The control of neutrophil infiltration into inflamed tissue.

The role of μ-calpain in neutrophil shape change.

---

by

Kimberley J Lewis

Neutrophil signalling group

Cardiff University School of Medicine

Cardiff

September 2011

Thesis submitted to Cardiff University for the degree of Doctor of Philosophy

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

DECLARATION

This work has not previously been accepted in substance for any degree and is not concurrently submitted in candidature for any degree.

Signed..... (candidate) Date..25/01/12..

STATEMENT 1

This thesis is being submitted in partial fulfilment of the requirements for the degree of PhD.

Signed..... (candidate) Date..25/01/12..

STATEMENT 2

This thesis is the result of my own independent work/investigation, except where otherwise stated. Other sources are acknowledged by explicit references.

Signed..... (candidate) Date..25/01/12..

STATEMENT 3

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organisations.

Signed..... (candidate) Date..25/01/12

STATEMENT 4 – BAR ON ACCESS APPROVED

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loans **after expiry of a bar on access** approved by the Graduate Development Committee

Signed..... (candidate) Date.....

Acknowledgments

Foremost I would like to express my sincerest gratitude to my supervisor Professor Maurice Hallett for firstly giving me this opportunity and then supporting me throughout my PhD research. I would also like to offer my greatest thanks to my co-supervisor Dr Sharon Dewitt, for endless amounts of great advice, technical tips and suggestions. I would also like to thank the remaining members of the Neutrophil Signalling Group, Dr Christian Parr, for guiding me through the world of molecular biology, Robert Francis for his help with apoptosis experiments and data regarding calpain in apoptotic neutrophils and Iraj Laffafian.

I would also like to thank the following people who have allowed me to work in their laboratories through the course of this research: Dr Phil Taylor and Dr Jacqueline McDonald for introducing me to and allowing me to utilise their immortalised mouse neutrophil progenitor cell line, Dr Richard Darley and Lorna Pearn for teaching me how to work with cord blood derived myeloid stem cells, and finally Dr Paul Brennan and Laurence Pearce for their endless help and discussions about lentiviral transfection.

I have been lucky to be surrounded by an amazing group of fellow students in my daily work throughout this project. I would like to thank Siobhan Webb, Geetha Elumalai, Dr Sivan Bokobza, Dr Astrid Escudero Esparza, Reezal Ishak and Ariel Sun for many helpful discussions and advice and for their friendship and support over the last three years.

I have to thank my family for all the support and encouragement they gave me throughout my childhood without which I would not be the person that I am today. And finally I owe thanks my boyfriend Owen Cox simply for being there to listen at the end of the day.

This work was supported by a grant from The Medical Research Council in conjunction with The British Lung Foundation.

Summary

Neutrophil spreading is an essential process in neutrophil extravasation and is a key step in the process of inflammation. Inhibition of neutrophil spreading is therefore a potential therapeutic target for the treatment of inflammatory diseases. Resting neutrophils have wrinkles in their plasma membrane which are held in place by cytoskeletal elements which act as a 'molecular velcro'. These proteins may be cleaved by cytosolic proteases such as the Ca^{2+}-activated protease μ calpain which contains a 'C2 like' domain. Calpain activation is also implicated in the expansion of the membrane during phagocytosis.

The primary challenge over the course of this project was expressing of fluorescent proteins in myeloid cells. In this thesis I show that lentiviral transduction was the most efficient method for the stable transfection of myeloid cell lines. Fluorescent-C2 domain translocates to the plasma membrane during experimental Ca^{2+} influx and during phagocytosis. A similar mechanism may occur in human neutrophils as μ-calpain is located at the plasma membrane in activated human neutrophils. I also found that neutrophils may have a mechanism of retrieving excess membrane from the phagosome after phagocytosis.

The work presented here has shown that by virtue of its 'C2 like' domain μ-calpain could translocate to the plasma membrane during the calcium influx which accompanies neutrophil spreading. μ calpain was shown to be present in the right place and at the right time in primary human neutrophils during neutrophil shape change and thus may play a key role in allowing the unwrinkling of the neutrophil membrane. Inhibition of calpain activity inhibits neutrophil spreading and therefore calpain poses a potential target for the treatment of inflammatory diseases.

Contents

Publications

In vivo functional analysis and genetic modification of in vitro-derived mouse neutrophils

McDonald JU, Cortini A, Rosas M, Fossati-Jimack L, Ling GS, **Lewis KJ**, Dewitt S, Liddiard K, Brown GD, Jones SA, Hallett MB, Botto M, Taylor PR.

FASEB J. 2011 Jun;25(6):1972-82. Epub 2011 Mar 2.

Localised and global Ca2+ influx and C2 domain translocation during phagocytosis

Lewis KJ, Dewitt S, Gadella TD, Hallett MB (2011)

(In preparation)

Membrane retrieval during phagocytosis by human neutrophils

Lewis KJ, Dewitt S, Hallett MB (2011)

(In preparation)

The role of phosphatidylserine in signalling shutdown in apoptotic neutrophils

Francis RJ, **Lewis KJ,** Kotecha S, Hallett MB (2011)

(In preparation)

Ca2+ dependent release of ezrin from the membrane during phagocytosis

Elumalai G, Dewitt S, **Lewis KJ,** Waddington R, Hallett MB (2011)

(In preparation)

Abstracts and conference presentations

The role of calpain translocation in neutrophil spreading

April 2011 **K J Lewis,** S Dewitt and M Hallett

European Phagocyte Workshop, ESCI Conference, Crete, Greece

The role of calpain translocation in neutrophil spreading

November 2010 **K J Lewis,** S Dewitt and M Hallett

Postgraduate Research Day, Cardiff University School of Medicine, Cardiff

Control of neutrophil infiltration into inflamed tissue

July 2010 **K J Lewis**

I3 IRG Annual Meeting, Atlantic College, Wales

The therapeutic potential of novel calpain inhibitors for reducing inflammatory neutrophil trafficking

July 2010 M B Hallett, S Dewitt, **K J Lewis**, G Elumalai, R Ishak, I Laffafian, C Parr

I3 IRG Annual Meeting, Atlantic College, Wales

Abbreviations

7TM	7 Trans Membrane
ADP	Adenosine Diphosphate
AM	Acetotoxymethyl Ester
ATRA	All Trans Retinoic Acid
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
BSS	Balanced Salt Solution
C3	Complement component 3
C3bi	Complement component 3bi
C5a	Complement component 5a
CAPN1	Calpain 1 (μ-calpain)
CAPN2	Calpain 2 (m-calpain)
CAPND3	Calpain 1 domain III
CAPNS1	Calpain 4 (calpain small subunit)
cDNA	complementary deoxyribonucleic acid
CFU-G	Colony Forming Unit - Granulocyte
CFU-GEMM	Colony Forming Unit – Granulocyte, Erythroid, Monocyte, Megakaryocyte
CFU-GM	Colony Forming Unit – Granulocyte, Macrophage
CGD	Chronic Granulomatous Disease

CIF	Calcium Influx Factor
CLSM	Confocal Laser Scanning Microscope
CNP	Circulating Neutrophil Pool
COPD	Chronic Obstructive Pulmonary Disease
CSF	Colony Stimulating Factor
CXCR1	CXC chemokine Receptor 1
CXCR2	CXC chemokine Receptor 2
DAG	Diacylglycerol
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced Green Fluorescent Protein
EGTA	Ethylene Glycol Tetraacetic Acid
ER	Endoplasmic Reticulum
ERM	Ezrin, Radixin, Moesin
FCS	Foetal Calf Serum
FERM	4.1 protein, Ezrin, Radixin and Moesin
FITC	Fluorescein isothiocyanate
FLAP	Fluorescence Loss After Photo bleaching
fMLP	*N*-Formylmethionyl-leucyl-phenylalanine

FPR	Formyl Peptide Receptor
FRAP	Fluorescence Recovery After Photo bleaching
G-CSF	Granulocyte – Colony Stimulating Factor
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte Monocyte – Colony Stimulating factor
HeBs	Hepes buffered saline
HRP	Horseradish Peroxidase
ICAM-1	Inter-Cellular Adhesion Molecule 1
ICAM-2	Inter-Cellular Adhesion Molecule 2
IFNα	Interferon alpha
IFNγ	Interferon gamma
IgG	Immunoglobulin G
IL-1	Interleukin 1
IL-3	Interleukin 3
IL-8	Interleukin 8
IP_3	inositol 1,4,5-trisphosphate
JAM A.B,C	Junctional Adhesion Molecule A,B, C
kDa	kilodaltons
LB	Lysogeny broth
LFA1	Lymphocyte function-associated antigen 1
MACS	Magnetic-activated cell sorting
MAE	Methyl-Amino-Ethanol

MNP	Marginated Neutrophil Pool
NADPH	Nicotinamide adenine dinucleotide phosphate
NPP	Neutrophil Progenitor/Proliferative Pool
NSP	Neutrophil Storage Pool
PAF	Platelet Activating Factor
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PECAM1	Platelet endothelial cell adhesion molecule 1
PI	Propidium Iodide
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
PKCβ	Protein Kinase C beta
PKCγ	Protein Kinase C gamma
PLA2	Phospholipase A2
PLCβ	Phospholipase C beta
PLCγ2	Phospholipase C gamma 2
PS	Phosphatidyl Serine
PSGL1	P-Selectin Glycoprotein Ligand 1
RFP	Red Fluorescent Protein
RNA	Ribonucleic Acid
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium

SCF	Stem Cell Factor
SERCa	sarco/endoplasmic reticulum Ca^{2+}-ATPase
SOC	Store Operated Channel
STIM1	Stromal interaction molecule 1
STS	Staurosporine
TBE	Tris/Borate/EDTA
TBS	Tris Buffered Saline
TNFα	Tumour Necrosis Factor alpha
TRIS	Tris(hydroxymethyl)aminomethane
TRPC	Transient Receptor Potential Cation channels
TRPM	Melastatin-related Transient Receptor Potential cation channels
UV	Ultra Violet
WT	Wild Type
YFP	Yellow Fluorescent Protein

List of figures

List of tables

CHAPTER 1

Introduction

1.1 The Immune System

1.1.1 Introduction

The human immune system has evolved a complex system of processes in order to protect the body from disease. Most infections with invading pathogens do not result in disease as the immune system deals with the infection efficiently. Disease normally only occurs when there are unusually high levels of pathogens, the pathogen is highly virulent or the host is in some way immuno-compromised. The body has several 'barriers to infection' in order to prevent invading pathogens from entering the body. These are anatomical, chemical and biological barriers. The anatomical barriers include skin, tears, saliva and mucus which act to prevent pathogens from entering the body. Chemical barriers such as lysozyme found in tears and the low pH conditions of sweat create a harsh environment for pathogens to live in. Finally the biological barrier is the body's own normal flora which colonise the skin and the digestive tract and compete with invading pathogens for space and nutrients. If a pathogen manages to pass the initial 'barriers to infection' then the next fundamental role of the immune system is the ability to distinguish these invading pathogens from the body's own cells in order to be able to specifically kill the pathogen without damaging any of the body's own tissue. This is known as self/non-self discrimination.

1.1.2 Innate and Acquired Immunity

There are 2 specific subdivisions of the immune system; innate and acquired (or adaptive) immunity. These subdivisions are made up of a diverse variety of cells which all perform specific functions (table 1.1.2.1). Innate immunity refers to the subset of cells, which an individual is born with, that are constitutively present in the blood and are able to act immediately upon infection. They are non specific and can react to any invading pathogen.

Acquired or adaptive immunity takes longer to respond to an infection but the response is much stronger than the innate response. It uses 'immunological memory' which is triggered when antibodies are produced after the initial infection with a specific pathogen which produces immunity to that pathogen. Immunological memory means that the next time the body comes into contact with that particular pathogen the immune system can respond immediately.

Cell type	**Primary Role**
Leukocytes	
-Neutrophils	Phagocytosis and microbial killing
-Basophils	Release histamine and other chemicals involved in inflammation
-Eosinophils	Destroy multicellular parasites
-Monocytes	Move to site of infection and differentiate into macrophages
-Lymphocytes	
-B cells	Make antibodies against antigens and develop into memory cells
-T cells	
-Cytotoxic T cells	Bind antigens on target cells/pathogens and destroy them
-Helper T cells	Release cytokines (to attract macrophages)
-Supressor T cells	Suppress immune responses
-NK cells	Kill virus infected and tumour cells
Plasma cells	Produce large quantities of antibodies
Macrophages	Phagocytosis and antigen presentation
Mast cells	Degranulate upon activation and release inflammatory mediators

Table 1.1.2.1 The roles of the different cells of the immune system (Widmaier, 2006)

1.1.3 The Inflammatory Response

When a tissue is damaged either by an injury or an invading pathogen the cells release chemical signals which trigger the first response of the immune system; the inflammatory response. Invading microbes, damaged cells and the cells surrounding them release a variety of inflammatory mediators which act to trigger the inflammatory response. Inflammatory mediators include: bradykinin; complement components including C3 and C5a; plasmin; thrombin; lysozyme granules; histamine; a variety of cytokines including Interferon γ (IFNγ), Tumour Necrosis Factor α (TNFα), Interleukin 1 (IL1) and Interleukin 8 (IL8); as well as the eicosanoids, leukotriene B4 and prostaglandins (*Griffin et al., 2003*).
These chemicals act together to produce the series of events known as the inflammatory response. They:

- Act on the smooth muscle in the blood vessels in the area of damage to cause vasodilation which leads to an increase in blood flow to that area. This is responsible for the reddening of the area seen in cases of inflammation.
- Cause an increase in vascular permeability in the immediate area which causes plasma to leak into the surrounding tissue leading to swelling and oedema.
- Cause an increase in expression of adhesion molecules on blood vessels that they act on. This results in the rolling and adhesion of phagocytes.
- Trigger extravasation of phagocytes which have slowed sufficiently on the blood vessel wall, whereby the phagocyte moves through the blood vessel wall into the surrounding tissue.
- Create a chemotactic gradient to attract the phagocytes to the area of damage/infection (*Widmaier, 2006*).

The main phagocytic cell involved in the inflammatory response is the neutrophil. Its main role is to remove damaged cells or microbes through phagocytosis. If the inflammatory response does not eliminate the infection then macrophages present antibodies which stimulate the acquired immune

response. If the body has already encountered the invading pathogen before then the acquired immune response will be triggered earlier on in the immune response and the infections will be more quickly eradicated.

1.2 Neutrophils

1.2.1 The Neutrophil

Neutrophils are the most predominant type of white blood cell in the body with about $7x10^9$ neutrophils being found in every litre of blood (Lewis et al., 2006). They are small (approx 10µm diameter) terminally differentiated phagocytic cells with a lifespan of only 3-4 days. Neutrophils are the first leukocytes to enter an area of inflammation, where their primary purpose is to internalise microorganisms and kill them by generating toxic free radicals. Neutrophils are essential for survival. For example the genetic defect chronic granulomatous disease (CGD) which affects one neutrophil specific protein and therefore impairs their function is fatal (*Segal, 1987, Segal, 1996*). However inappropriate activation of neutrophils in inflammatory diseases such as chronic obstructive pulmonary disease (COPD) can also be damaging to the body.

The different types of white blood cells were first described by Paul Ehrlich in 1898 (Ehrlich P et al, 1898) when he noted that there were cells which appeared to have more than one nuclei but in fact had one nucleus with many lobes (figure 1.2.1.1). He termed these cells 'cells with polymorphous nuclei'. Later they were renamed polymorphonuclear leukocytes (*Metchnikoff, 1905*), a term which is still used today. Metchnikoff also further classified the three types of polymorphonuclear leukocytes based on the staining properties of their granules. Basophils are stained with a basic stain, eosinophils stain with an eosin stain and neutrophils stain best with neutral dyes.

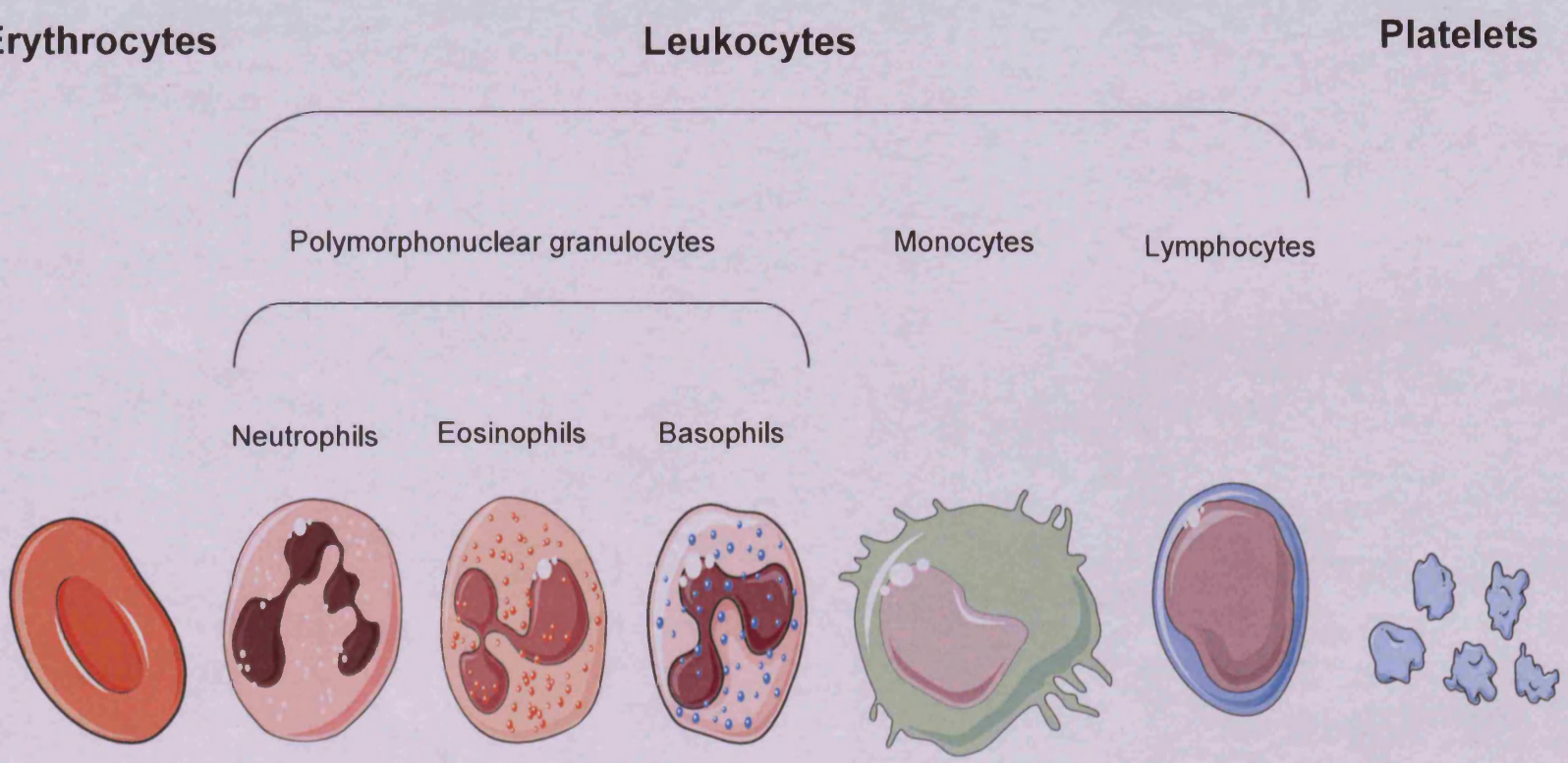


Figure 1.2.1.1: Cellular constituents of blood. Blood is made up of three main classes of cells; red blood cells (erythrocytes), white blood cells (leukocytes) and platelets. Leukocytes can be further divided into two groups; the polymorphonuclear granulocytes whose nuclei are made up of many lobes (neutrophils, eosinophils and basophils) and the mononuclear leukocytes (monocytes and lymphocytes). *Figure produced using Servier Medical Art.*

Establishing the role of the neutrophil proved controversial initially. As they were present in large numbers in pus and at the sites of infection it was first believed that they were generated at the infection site. However Waller was the first to show in 1846 that neutrophils in fact originated from the blood stream and that they left the blood vessels through a process known as diapedesis (*Cohnheim, 1867, Cohnheim, 1967, Waller, 1846*). It was also thought that they acted as agents of infection and ingested the bacteria in order to ferry them to the infection site and around the body. It was the 'thorn in starfish' experiments carried out by Metchnikoff in 1893 which first proposed the role of the neutrophil that we know today. He was able to show that when a transparent starfish embryo was pierced with a thorn that neutrophils moved towards the damaged area. This experiment he believed confirmed his theory that neutrophils were in fact beneficial to the body and that upon infection they moved towards the damaged area to phagocytose and kill the invading microbes (*Metchnikoff, 1905, Metchnikoff, 1893, Metchnikoff, 1901*).

1.2.2 Production – Myelopoiesis

As neutrophils circulating in the blood stream have a short half life of only 4-10 hours it is necessary for neutrophils to be constantly produced throughout a person's life. This production is called myelopoiesis and occurs in the bone marrow. Pluripotent stem cells in the bone marrow undergo a series of mitotic cell divisions to give rise to the stem cells responsible for all of the different blood cell lineages (figure 1.2.2.1). Neutrophils as well as all other granulocytes, monocytes, platelets and erythrocytes are derived from the myeloid stem cell lineage. Within the myeloid progenitor pool there are several subtypes of proliferative pools all containing cells that are at different stages of maturation, some cells are already committed to a certain fate whereas others are not. As the cells mature they become more committed to their fate.

Neutrophil production starts with the myeloid stem cell. The myeloid stem cell, the CFU-GEMM (colony forming unit – granulocyte, erythroid, monocyte, megakaryocyte) no longer has the ability to self replicate and differentiates into the CFU-GM (colony forming unit – granulocyte, macrophage) which then differentiates into the CFU-G (colony forming unit – granulocyte). These cells make up the neutrophil progenitor pool (NPP). As they differentiate these cells become progressively more committed (figure 1.2.2.2). However, the CFU-G is still not a fully committed cell and still has the potential to become any type of granulocyte. The first stages of committed neutrophil differentiation are the myeloblast, promyelocyte and the neutrophilic myelocyte. These cells are formed by mitotic divisions which take approximately one week and they make up the neutrophil proliferative pool (NPP). Following this stage of differentiation post mitotic maturation takes place. This leads to the formation of metakaryocytes, band cells and finally neutrophils. These constitute the neutrophil storage pool (NSP). There are several factors regulating the maturation of neutrophils including Granulocyte Macrophage – Colony Stimulating Factor (GM-CSF), Colony Stimulating Factor (CSF) and Interleukin 3 (IL-3) (*Okuda et al., 1992*).

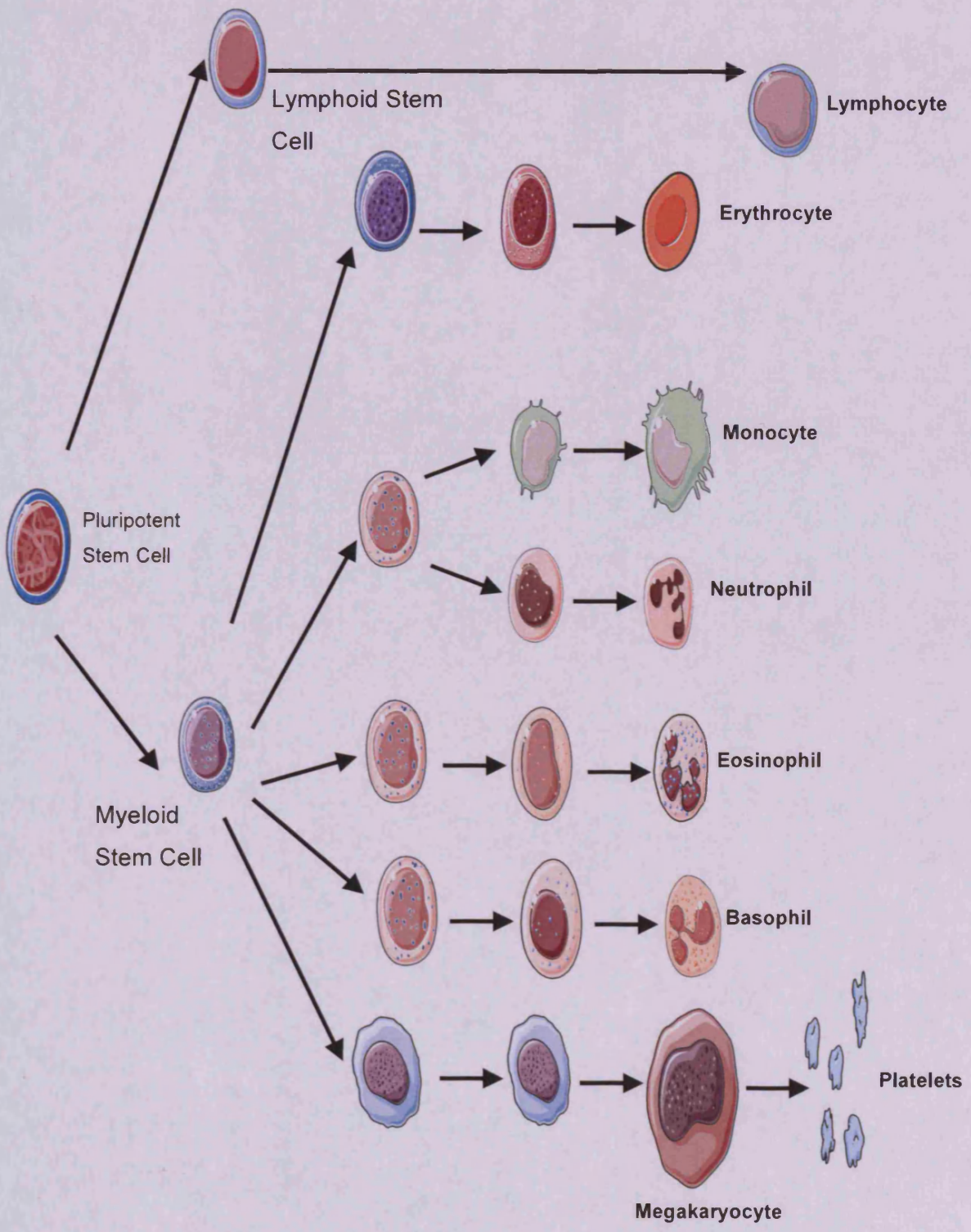


Figure 1.2.2.1: Production of the cellular constituents of blood. All blood cells are formed from a single pluripotent stem cell which undergoes a series of mitotic divisions to produce the myeloid and the lymphoid stem cells which are committed to that cell type. Neutrophils are derived from the myeloid lineage. *Figure produced using Servier Medical Art.*

As the neutrophils mature they become more deformable, they gain new receptors on their plasma membrane and finally they become mobile. This enables them to migrate through the endothelial pores in the bone marrow and into the blood stream. Here they become the circulating neutrophil pool (CNP). There are two pools of neutrophils in the peripheral blood stream. These are the circulating neutrophil pool (CNP) which are circulating freely in the blood stream and the marginated neutrophil pool (MNP) which are adhered to blood vessel walls via selectins on the endothelial cells. They roll along the blood vessel walls until receptors (β2 integrin) on the neutrophil surface encounters their corresponding ligands (Intercellular Adhesion Molecule 1 (ICAM1)) on the blood vessel endothelium. ICAM1 expression on blood vessel endothelium is enhanced on activated endothelial cells. This occurs at sites of infection or damage and this is the trigger for the neutrophil to extravasate from the blood vessel into the surrounding tissue.

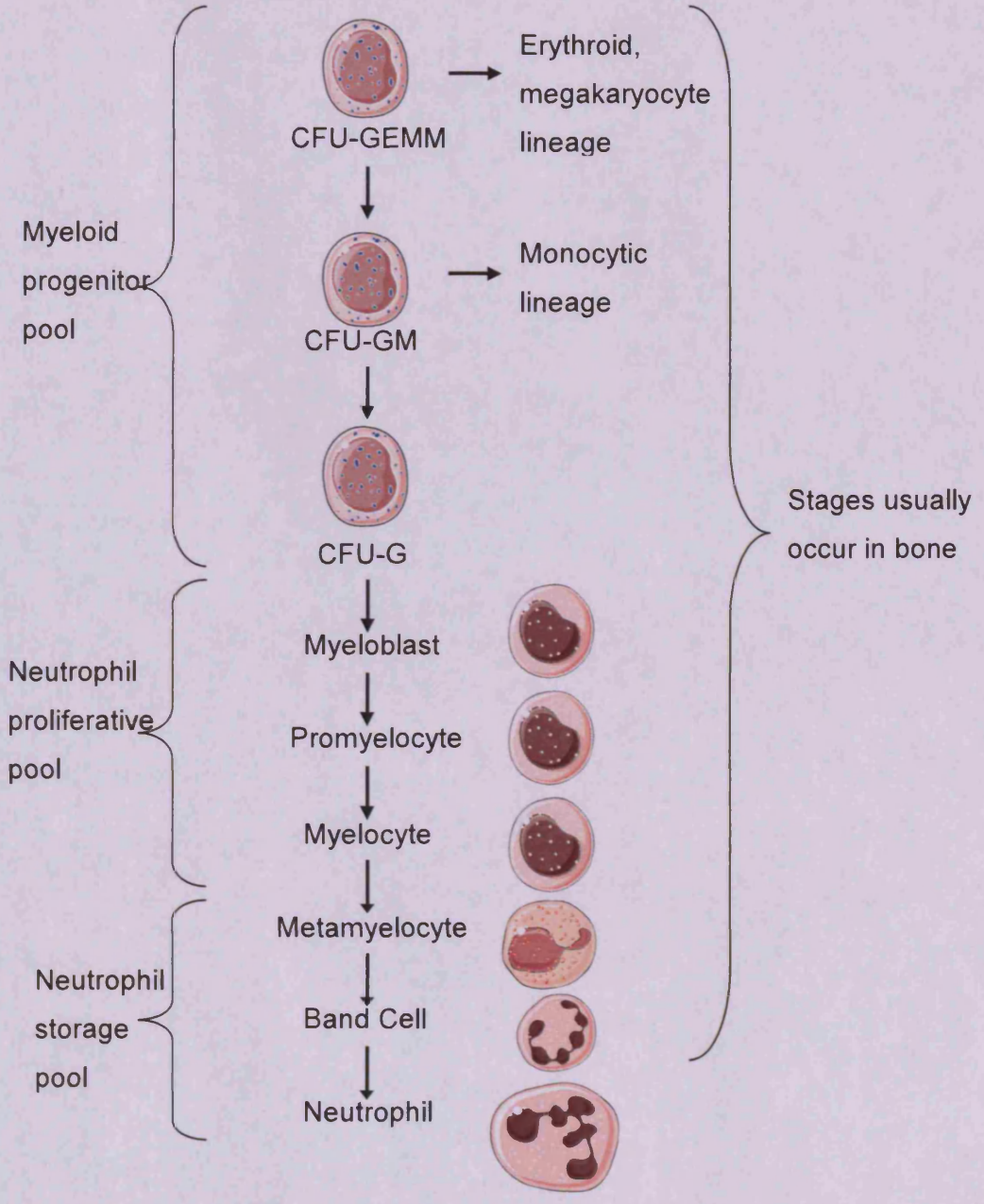


Figure 1.2.2.2: Neutrophil production. Neutrophils are derived from the myeloid lineage, starting with the myeloid stem cell, CFU-GEMM. The CFU-GEMM has the potential to become several different cell types. As it divides it becomes gradually more committed to a certain fate. The myeloblast is the first cell type committed to become a neutrophil. Several more mitotic divisions occur in the bone marrow before final maturation takes place. *Figure produced using Servier Medical Art.*

1.2.3 Neutrophil Structure

Neutrophils are small round cells, only approximately 10μm in diameter. They have a large multilobed nucleus which takes up 20% of the space in the cytosol of the cell (*Schmid-Schonbein et al., 1980*). The rest of the cytosol contains mostly granules which neutrophils have in very large numbers. Their function is to store pre-synthesised inflammatory mediators, enzymes and chemicals which would be harmful to the cell if allowed to diffuse freely in the cytosol. There are three primary types of granules which differ based on their contents. These are azurophilic granules, specific or small storage granules and gelatinase granules. The targeting of proteins to these granules is determined by the timing of protein synthesis during maturation and not by granule specific sorting and so not all granules are completely distinct; some have a mixture of contents. The contents of these granules can be harmful to the cell and so they are only secreted upon neutrophil activation. There is also a fourth form of granule known as secretory vesicles which are formed from endocytosis. There are very few mitochondria in neutrophils compared to other cells. Mitochondria only make up 0.2% of the organelle volume of a neutrophil compared with 22% of the volume in liver cells (*Hallett and Lloyds, 1997*). This is because neutrophils are not very metabolically active cells. Once mature, very little, if any protein synthesis occurs as they are terminally differentiated cells and once activated they are removed via apoptosis. Neutrophils have 84% more plasma membrane than is needed to enclose the contents of the cell (*Hogg, 1987*). This is specific to neutrophils as it contributes to their motility and ability to spread and phagocytose. This will be further discussed in section 1.5. Another factor that contributes to a neutrophils ability to be motile is the protein actin. Polymerised globular g-actin forms a layer of filamentous f-actin underneath the plasma membrane known as the cortical cytoskeleton. Actin polymerisation is continuous in a neutrophil but upon neutrophil activation there is a 4 fold increase in the proportion of f-actin in the cell (*Stossel, 1989*). There are many different receptors on the neutrophil which sense the environment around them and bind receptors enabling neutrophils to sense a chemotactic gradient as low as 0.1% over the cells length. Internal signalling molecules then allow for signal

transduction from the receptors on the cells plasma membrane throughout the cell.

1.2.4 Neutrophil Function

The neutrophils primary function is to kill microbes that could potentially cause damage to the body. However to do this they must be able to move from the blood stream into the tissues.

Neutrophils circulating in the blood are able to move freely between the circulating neutrophil pool (CNP) and the marginated neutrophil pool (MNP). Ligands on the surface of circulating neutrophils can bind selectins on the blood vessel endothelium. This causes the neutrophils to become loosely attached to the blood vessel endothelium so that they can roll along the blood vessel wall at a slower rate than those that are freely circulating in the blood. They can however still be washed off by the shear stress of the blood flowing past them. Microbes and damaged tissues stimulate tissue macrophages to release inflammatory mediators such as TNFα, which stimulates increased expression of ICAM-1 and other molecules such as Platelet Activating Factor (PAF) on the blood vessel endothelium (*Lorant et al., 1991*). Neutrophils constitutively express integrins on their surface. Neutrophils that are in the MNP and are loosely bound to the blood vessel endothelium via selectins are moving slowly enough for the integrins on the neutrophil to bind the ICAM-1 that is expressed on the blood vessel endothelium (*Lawrence and Springer, 1991*). This causes a firm adhesion of the neutrophil to the blood vessel wall and signals the neutrophil to flatten and pass through the endothelium into the tissue through a process known as diapesis or extravasation. (figure 1.2.4.1). The high levels of TNFα in the local environment around the area where the neutrophil extravasates "primes" the neutrophil for an increased oxidative response when it is finally activated at the site of infection (*Hallett and Lloyds, 1995, Lloyds et al., 1995*).

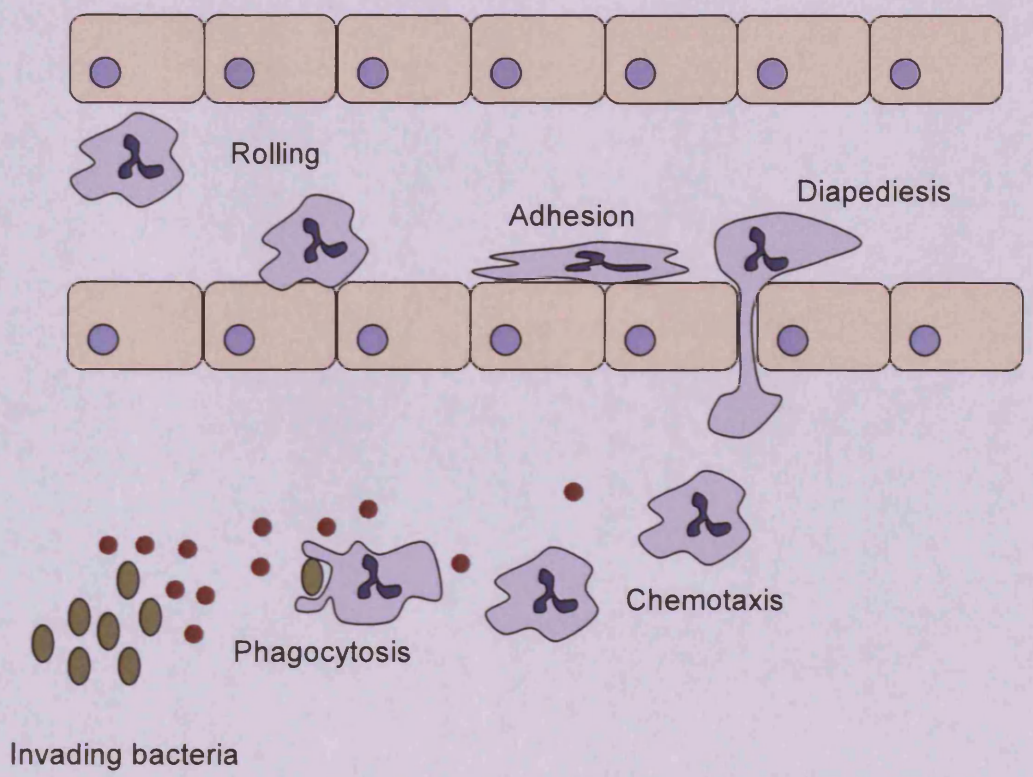


Figure 1.2.4.1: Neutrophil Function. Invading bacteria release inflammatory mediators which cause an increase in the expression of ICAM1 on the blood vessel endothelium. Neutrophils constitutively express integrins on their surface which enables them to bind to the ICAM1 on the endothelium and adhere and spread out before passing through the endothelium into the surrounding tissues in a process called diapedesis. Neutrophils then move along a chemotactic gradient created by chemicals released by invading bacteria and the damaged cells to the source of the infection where they phagocytose and kill any invading bacteria.

Formylated peptides released by the invading bacteria, the damaged cells and complement components and chemokines (IL-8, C5a and leukotrine B4) act as chemoattractants and attract the neutrophils that have left the blood stream to the area of damage. It is also thought that they can increase the lifespan of a neutrophil to up to 1-2 days, compared to 4-10 hours for a neutrophil circulating in the blood stream.

When a neutrophil encounters an invading microorganism opsonised with either immunoglobulin or complement (C3bi) it phagocytoses and destroys the microbe. Fc receptors on the neutrophil surface recognise and bind to immunoglobulins on foreign particles and β2 integrins (CD11b/CD18) recognise and bind complement (C3bi) opsonised particles (Lee et al., 2003). Activation of these receptors initiates a complex signalling cascade, discussed in section 1.3.5 which culminates in a global calcium signal, actin polymerisation and extensive membrane remodelling. The cell extends pseudopodia around the particle and encloses it within the phagosome taking it inside the cell where the phagosome fuses with the neutrophil granules containing degradative enzymes which destroy the microorganism. The global calcium signal is the trigger for granule fusion (Jaconi et al., 1990) and oxidase activation (Dewitt et al., 2003) with different granules having different calcium thresholds (Sengelov et al., 1993). Many of the enzymes are activated only after degranulation occurs when the pH of the granule which has fused with the phagosome activates the enzymes to destroy the microbe (*Hirsch and Cohn, 1960, Pryzwansky et al., 1979*). Respiratory/oxidase mediated killing occurs when a nicotinamide adenine dinucleotide phosphate (NADPH) enzyme on the membrane of the granules is activated releasing toxic oxygen species into the granule which destroy the microorganism. The degranulation and oxidation responses act together to ensure an extremely powerful and efficient method for the removal and killing of bacteria.

Once the neutrophil has destroyed as many of the invading microbes as possible it undergoes programmed cell death (apoptosis). It is then ingested by macrophages or other phagocytic cells which ensures that the harmful contents of the neutrophil granules are disposed of safely without any damage to surrounding tissues.

1.3 Calcium Signalling

1.3.1 Cytosolic free calcium measurement and visualisation.

The most common method of measuring cytosolic free calcium in neutrophils is using fluorescent calcium chelator indicators bound to acetoxymethyl esters (AM). Binding of the probe to an AM confers lipid solubility and masks the calcium binding site of the probe. When the probe is added to the cells it either precipitates in the medium or dissolves in the cell membrane. Once the probe has diffused across the cell membrane it comes into contact with non-specific esterases in the cytosol of the neutrophil which cleave the acetotoxymethyl ester, unmasking the calcium binding site of the probe and creating a hydrophilic molecule which is then trapped in the cytoplasm of the cell (*Hallett and Lloyds, 1997*). The most commonly used of these probes is Fura-2-AM: it has an affinity for Ca^{2+} of about 200nM which is in the mid-range of physiological cytosolic Ca^{2+} and thus sensitive to change in Ca^{2+} in this range; a high quantum yield which confers a high level of fluorescence in both the bound and unbound form of the probe so that less of the probe needs to be used to get a signal (less potential for calcium buffering from the probe); and the excitation wavelength is different for the calcium bound and calcium free form of the probe enabling the use of ratiometric imaging so that changes in fluorescence can be confidently interpreted as a consequence of a change in calcium concentration (*Tsein and Poenie, 1986*). However one of the excitation wavelengths is in the UV spectrum which is not ideal as many molecules found in neutrophils are excited by UV light. Therefore probes which are excited by longer wavelengths of light can sometimes be more useful. One such probe is

fluo4. It is highly fluorescent when excited at 488nm. At this longer wavelength the levels of auto fluorescence are generally much lower. It also has a high (100 fold) increase in fluorescence when bound to calcium, and a Kd near 345nM (Gee et al., 2000) making it sensitive to changes in cytosolic Ca^{2+}. However as there is no shift in excitation or emission wavelength, Ca^{2+} changes cannot be measured ratiometrically and confocal techniques must be employed for optical slicing (*Hallett and Lloyds, 1997*).

1.3.2 Cytosolic free calcium homeostasis

The calcium ion is a ubiquitous intracellular messenger that controls a wide range of processes in many different types of cells. Cytosolic Ca^{2+} levels are important for the control of most neutrophil behaviour including phagocytosis, spreading and possibly chemotaxis. Intracellular Ca^{2+} in neutrophils is actively buffered or sequestered into calcium storage organelles. This, along with calcium pumps in the plasma membrane and the low permeability of the plasma membrane in resting neutrophils, maintains a low resting free cytosolic calcium concentration. The free cytosolic calcium concentration ($[Ca^{2+}]$) in a resting neutrophil is 100nM (*Hallett and Lloyds, 1997*). Since the extracellular calcium concentration is up to 1mM, this creates a gradient of 1:10000 across the plasma membrane.

During neutrophil activation, intracellular calcium concentrations can reach up to 1μM due to the release of calcium from stores and the opening of calcium channels in the plasma membrane (*Demaurex et al., 1992)*. More recently, it has been suggested that Ca^{2+} concentrations can even reach concentrations of up to 30μM in the membrane wrinkles when the neutrophil is activated (Brasen et al., 2010)

1.3.3 Calcium stores in neutrophils

Neutrophils have a high capacity to buffer calcium. In order to do this the neutrophil has many intracellular calcium stores. Calcium can be taken up from the cytoplasm into these stores when calcium concentration in the cytoplasm is too high, and calcium that has been sequestered into these stores can be released into the cytoplasm when cytosolic calcium concentration is too low. There are two distinct calcium stores in neutrophils: a sub plasma membrane site and a juxtanuclear site. Stimulation of the neutrophil with *N*-Formylmethionyl-leucyl-phenylalanine (fMLP) triggers calcium release from the juxtanuclear site and CD11b/CD18 (integrin) cross linking stimulates calcium release from the sub plasma membrane site (Pettit and Hallett, 1998b). The juxtanuclear calcium storage site is likely to be either the Endoplasmic Reticulum (ER) or golgi (Davies et al., 1991). There is also evidence to suggest that there is a further inositol 1,4,5-trisphosphate (IP3) sensitive calcium storage site known as the 'calciosome'. The calcium sequestering protein calsequestin has been shown to co purify with the discrete IP3 sensitive organelle known as the 'calciosome' (Krause et al., 1989, Volpe et al., 1988). These calcium storage organelles have been shown to become redistributed around the phagosome during phagocytosis (Favre et al., 1996, Stendahl et al., 1994). Sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA)2b calcium ATPase pumps are also found on these IP3 sensitive calcium storage sites and act to pump calcium into the stores. These can be inhibited by a number of different inhibitors to prevent calcium being pumped into the calcium stores (Favre et al., 1996)

1.3.4 Calcium signalling by seven transmembrane receptors (G protein coupled receptors)

Chemoattractants such as fMLP act on seven transmembrane (7TM) receptors in the neutrophils plasma membrane to cause a cytosolic calcium signal in the neutrophil. Upon stimulation the cytosolic calcium concentration in the

neutrophil can rise from 0.1μM to 1μM. This signal is required for most neutrophil responses (Hallett and Campbell, 1984). The calcium signal caused by activation of 7TM receptors results from the release of calcium from intracellular stores such as the juxtanuclear calcium storage site (Davies et al., 1991) followed by an influx of calcium from channels in the plasma membrane (Pettit and Hallett, 1995). Activation of the 7TM receptor activates a G protein on the inner surface of the plasma membrane which leads to activation of Phospholipase C β (PLCβ) (Cockcroft and Gomperts, 1985), this cleaves Phosphatidylinositol 4,5-bisphosphate (PIP2) to liberate IP3. This results in the release of calcium from IP3 sensitive calcium stores. The release of calcium from IP3 sensitive intracellular stores is sufficient to activate Melastatin-related Transient Receptor Potential cation channels 2 (TRPM2) channels in the plasma membrane (Du et al., 2009) and may account for the Ca^{2+} influx phase which follows store release in neutrophils (figure 1.3.5.1). There is a delay between activation of the 7TM receptors and the increase in cytosolic calcium (Hallett et al., 1990). This delay can be explained by the time it takes for the signalling molecules to diffuse from the plasma membrane to the intracellular calcium stores (Hallett and Lloyds, 1997).

1.3.5 Calcium signalling via cross-linking of receptors (Integrin engagement)

A calcium signal can also be caused in neutrophils by cross-linking of IgG receptors (Fc (CD16/CD32) receptors) (Roberts et al., 1997) or integrin (CD11b/CD18) receptors on the neutrophils surface (Hellberg et al., 1996). This calcium signal is generated via a different mechanism to that generated by the activation of 7TM receptors.

Cross-linking of stimuli still generates release of calcium from intracellular stores followed by a global calcium signal due to calcium influx through channels in the plasma membrane (Davies and Hallett, 1995). However the

release of calcium from the intracellular stores is not sufficient to trigger the calcium influx (Pettit and Hallett, 1997). The site of calcium store release is also different to that triggered by 7TM receptors, being peripheral rather than central (Davies and Hallett, 1995), localised to the site of receptor cross linking (Pettit and Hallett, 1996). There is evidence from electron microscope studies supporting the presence of motile calcium storage sites close to the plasma membrane (Hoffstein, 1979). As with calcium signalling via 7TM receptors there is a delay between activation of the receptor and generation of the calcium signal. This can be explained by the time taken for the lateral diffusion of the receptors in the cell membrane to form aggregates capable of generating a calcium signal (Roberts et al., 1997). The signal can be inhibited by tyrosine phosphorylation inhibitors (Morgan et al., 1993) and inhibitors of the actin bundling protein L-plastin (Rosales et al., 1994), suggesting that the actin cytoskeleton is essential for transduction of the signal from the plasma membrane. Integrin engagement triggers tyrosine kinase dependent calcium mobilisation, Phospholipase C γ2 (PLCγ2) phosphorylation and an increase in levels of IP3 generation (Hellberg et al., 1996) which may suggest that the mechanism of calcium signalling via cross linking of stimuli is more similar to calcium signalling via 7TM receptors than was first thought. A possible mechanism is illustrated in figure 1.3.5.1. The exact mechanism however is yet to be resolved.

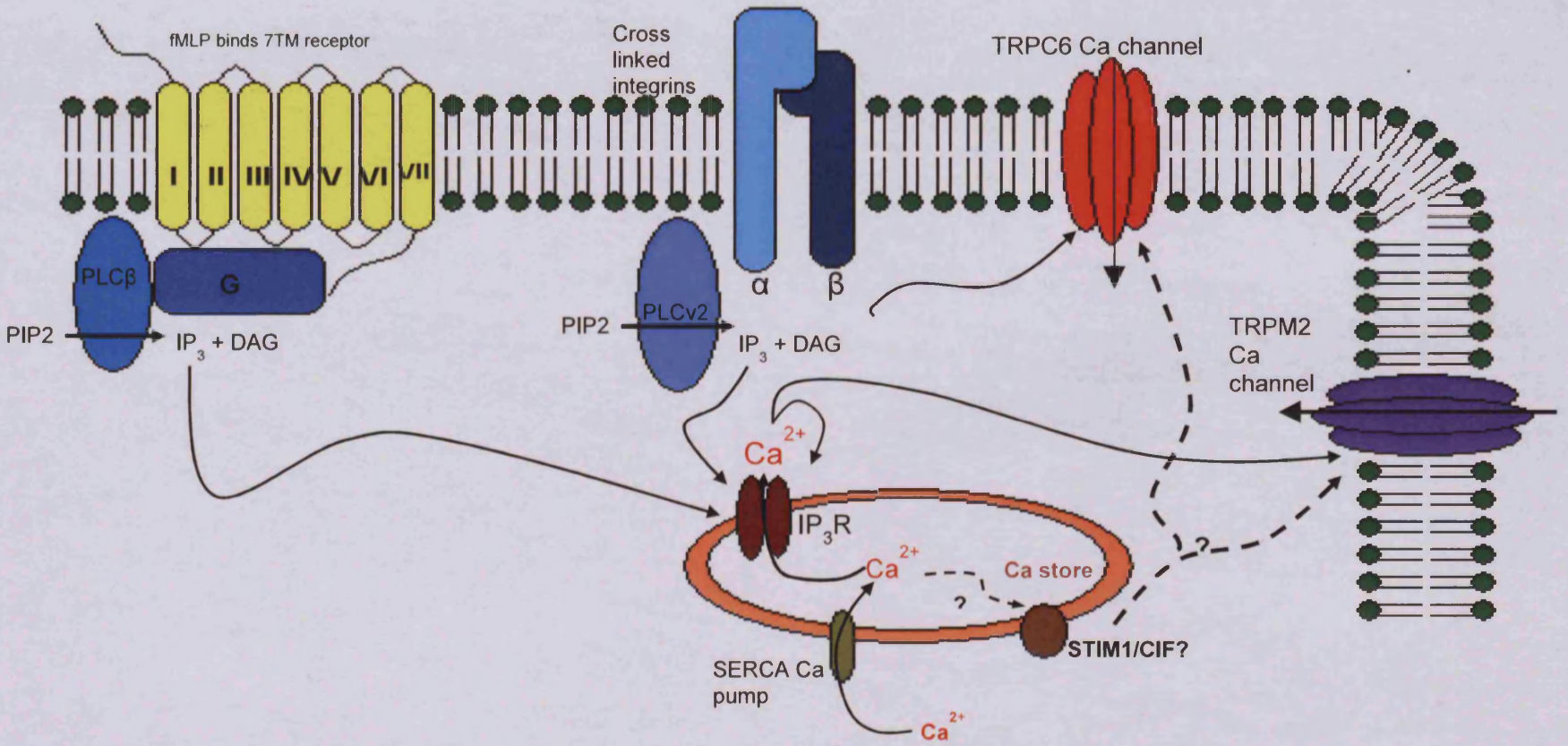


Figure 1.3.5.1: Schematic diagram of some of the possible routes for calcium signalling in the neutrophil. When a 7TM receptor is activated by a chemoattractant such as fMLP, its G protein activates PLCβ which converts PIP2 into IP3 and Diacylglycerol (DAG) and IP3 goes on to activate IP3 receptors on the intracellular calcium stores to trigger the release of calcium into the cytosol. Cross linking of integrins in the neutrophil plasma membrane results in the phosphorylation and activation of PLCγ2 and consequently the production of IP3 and DAG, leading to release of calcium from intracellular stores. Calcium release from stores is followed by calcium influx into the cytosol through plasma membrane calcium channels. Several possible mechanisms for this exist which are shown in the diagram, direct activation of calcium channels by DAG, activation of calcium channels by calcium released from the intracellular stores or a diffusible second messenger known as calcium influx factor (CIF). However, the exact mechanism by which calcium influx through the plasma membrane calcium channels is so far unknown.

1.3.6 Calcium influx

Calcium signalling via both 7TM receptors and via cross-linking of receptors involves calcium influx through channels in the plasma membrane. In both cases the calcium influx occurs after the release of calcium from intracellular stores, although the exact mechanism that triggers calcium influx remains unclear. Blocking this calcium influx inhibits most neutrophil responses (Hallett and Campbell, 1984, Marks and Maxfield, 1990). There is a delay between the addition of the stimulus to the neutrophil and the global calcium influx suggesting a series of stochastic events such as diffusion of small molecules must take place in order to trigger calcium influx (Hallett and Pettit, 1997, Pettit and Hallett, 1995). Although the sequence of events is similar for the generation of the calcium signal through the two different types of receptors the mechanisms appear to be quite distinct.

Inhibiting release of calcium from intracellular stores in neutrophils stimulated with the 7TM receptor agonist fMLP is difficult but micro-injection of the IP3-receptor blocker heparin, prevents the calcium influx from the plasma membrane channels (Davies-Cox et al., 2001). Also anucleated cytoplasts, where the juxtanuclear calcium store is removed along with the nucleus, are unable to produce a calcium signal when stimulated with C5a, a neutrophil chemoattractant (Gennaro et al., 1984). However inhibition of IP3 independent calcium stores in neutrophils stimulated with IgG immune complex had no effect on the calcium influx (Davies and Hallett, 1995). The timing of the calcium signal differs in both cases also. In neutrophils stimulated with fMLP calcium influx is seen at approximately 500ms after calcium is released from the intracellular stores (Pettit and Hallett, 1995). The delay is longer when the signal is triggered by integrin cross linking, with channel opening occurring between 10s and 100s after store release (Pettit and Hallett, 1996) . It was suggested that the two distinct mechanisms of calcium signalling may be due to the initial release of calcium being from two separate intracellular calcium stores.

The mechanism of calcium influx in both cases though is yet to be fully defined. Inhibition of SERCa pumps on the endoplasmic reticulum using agents such as thapsigargin causes calcium to leak from the calcium stores into the cytoplasm, this is followed by the opening of plasma membrane channels. It has been suggested that the channels open in response to a 'need' to replenish the calcium stores (Smyth et al., 2006). This is discussed further in relation to calcium channel activation in section 1.3.7. It has recently been shown that an increase in cytosolic calcium is sufficient to open TRPM2 channels in the plasma membrane and that IP3 receptor activation induced calcium release can activate these channels potentially causing calcium influx (Du et al., 2009). It may therefore be possible that in the case of 7TM stimulated calcium signalling, the release of calcium from the IP3 sensitive juxtanuclear calcium store is sufficient to trigger the calcium influx.

1.3.7 Calcium channels in the plasma membrane

Influx of calcium into the cytoplasm through calcium channels in the plasma membrane is a key step in the neutrophils cytosolic calcium signal. However the exact channels through which this influx occurs and how they are activated is still unknown. The calcium influx still occurs even in the presence of inhibitors of voltage sensitive calcium channels (Demaurex et al., 1994) suggesting that the channels in this case are not voltage gated channels. The channels are not directly activated by receptor occupancy but channel activity is linked to the emptying of the intracellular calcium stores (von Tscharner et al., 1986).

Three mechanisms have been proposed linking calcium being released from intracellular stores and the activation of calcium channels in the plasma membrane. Firstly is has been proposed that the IP3 receptor on the calcium store dynamically interacts with the calcium channel in the plasma membrane in order to activate it (Irvine, 1990). Secondly it has been suggested that there is

a requirement for a thus far unidentified diffusible second messenger known as the calcium influx factor (CIF) which acts to activate the calcium channels in the plasma membrane upon calcium release from internal stores. Stromal interaction molecule 1 (STIM1) is a 1 transmembrane calcium binding protein found in the plasma membrane which has been proposed to function as a calcium sensor and provide a trigger for the activation of calcium channels in the plasma membrane (Spassova et al., 2006). Finally, a secretory like mechanism involving close but reversible interactions between the plasma membrane and the endoplasmic reticulum has been proposed (Patterson et al., 1999).

The most studied candidates for potential calcium channels involved in the neutrophil calcium signal are the human homologues of the drosophila Transient Receptor Potential Cation (TRP) channels. Human neutrophils express TRPC1, TRPC3, TRPC4 and TRPC6 (Brechard and Tschirhart, 2008) although the activity of each channel is still uncertain. It has been shown that the TRPC channels are essential for the calcium influx phase of the calcium signal as cytoskeletal reorganisation that results in the internalisation of the TRPC channels inhibits calcium entry into the neutrophil (Itagaki et al., 2004). TRPC1 has been shown to be involved in calcium entry into platelets with the proposed mechanism involving coupling of the IP3 receptor with the TRPC1 channel (Rosado et al., 2002). TRPC3 and TRPC6 are both activated by diacylglycerol (DAG) (Hofmann et al., 1999). Activation of 7TM receptors results in the breakdown of PIP2 into IP3 and DAG, the DAG can then go on to activate TRPC6 calcium channels and cause calcium influx. TRPC6 is also activated by membrane phosphatidylinositol (3,4,5)-triphosphate (PIP3). Another well studied calcium channel in the neutrophil is the TRPM2 channel, which is activated adenosine diphosphate (ADP) ribose (Perraud et al., 2001). It has been reported that higher cytosolic calcium concentrations lower the ADP ribose concentration required to activate the channel but that ADP ribose is still essential for the channels activation (Heiner et al., 2005). However more

recently it has been shown that TRPM2 channels can be activated by an increase in cytosolic calcium alone (Du et al., 2009) making them the perfect candidate for the store operated calcium channel responsible for the calcium influx stage of the neutrophil cytosolic calcium signal.

1.3.8 Role of calcium signalling in neutrophil adhesion.

Neutrophil spreading is directly linked to an increase in cytosolic calcium. Multiple cytosolic Ca^{2+} signals have been observed during neutrophil spreading (Jaconi et al., 1991), in particular during CD11b/CD18 mediated adhesion (Pettit and Hallett, 1997). It has been shown that the neutrophil initially attaches to the substrate with no change in cytosolic calcium, this is followed by the global cytosolic calcium signal and finally neutrophil spreading (Kruskal et al., 1986), suggesting that the cytosolic calcium signal is the trigger for neutrophil spreading. This suggestion is further backed up by evidence that the release of caged cytosolic calcium in human neutrophils provides a calcium signal large enough to trigger neutrophil spreading when neutrophils are in contact with an integrin engaging surface (Pettit and Hallett, 1998a).

The calcium increase and, as a result, neutrophil spreading can be inhibited by incubating neutrophils with antibodies to CD11b/CD18 (Jaconi et al., 1991, Pettit and Hallett, 1998a). This illustrates that integrin engagement is the trigger for the calcium signal that causes neutrophil spreading. Cross linking antibodies bound to CD11b/CD18 integrins can trigger a local calcium signal (Petersen et al., 1993) as a result of calcium release from the sub plasma membrane storage site (Pettit and Hallett, 1998b). The exact mechanism by which integrin engagement may lead to a cytosolic calcium signal was discussed in section 1.3.5 but briefly, it has been shown that integrin engagement in neutrophils triggers the phosphorylation of PLCγ2 and the formation of IP3 (Hellberg et al., 1996). The calcium concentration has been shown to increase locally at the integrin engagement sites as the neutrophil

initially adheres, followed by a global cytosolic calcium signal which accompanies neutrophil spreading (Pettit and Hallett, 1996).

The cytosolic calcium signal is also required for the rapid shape change observed during integrin-mediated phagocytosis (Dewitt and Hallett, 2002). The mobilisation of intracellular calcium promotes β2 integrin mediated adhesion in neutrophils (Leitinger et al., 2000) and increases the amount of Lymphocyte function-associated antigen 1 (LFA1) integrin clustering in lymphocytes (Stewart et al., 1998). This all confirms that the global cytosolic calcium signal is the key signal for triggering neutrophil spreading and neutrophil shape change.

1.4 Neutrophil extravasation

1.4.1 Triggers and chemoattractants

Neutrophil extravasation is triggered by the inflammatory response, discussed in section 1.1.3. Invading microbes, damaged cells and the cells surrounding them release a variety of inflammatory mediators which act to trigger the inflammatory response. Inflammatory mediators involved in neutrophil extravasation include: TNFα C5a; fMLP; IL1, IL8 and leukotriene B4 (*Griffin et al., 2003*). These have different effects on neutrophils and the blood vessel endothelium which act to trigger neutrophil extravasation. TNFα is a cytokine released by monocytes and macrophages in response to lipopolysacharides and IL-1 (other molecules involved in the inflammatory process) (Gallin and Snyderman, 1999). Similar to IL-1 it acts to increase the expression of adhesion molecules on the blood vessel endothelium. It also acts to 'prime' the neutrophils ready for transmigration and activation. Complement component C5a acts as a chemoattractant and fMLP is a formylated tripeptide produced by bacteria. Both act as neutrophil chemoattractants and lead to an accumulation of neutrophils at the site of inflammation once the neutrophils have migrated

across the endothelium. IL-1 is a cytokine produced by monocytes and macrophages at the site of inflammation and acts on nearly all cell types (Gallin and Snyderman, 1999). It acts to increase the expression of adhesion molecules on the blood vessel endothelium in order to increase the slow rolling of neutrophils in the blood vessel (Dinarello, 1996). IL-8 is a chemokine produced by macrophages and neutrophils in areas of inflammation. It acts as a chemoattractant, triggering a calcium signal in the neutrophils by binding the G-protein coupled receptors CXC chemokine receptor 1 (CXCR1) and CXC chemokine receptor 2 (CXCR2) (Gallin and Snyderman, 1999) . The mechanism behind the generation of the calcium signal from G protein coupled receptor activation is discussed in section 1.3.4. Leukotriene B4 is another chemokine produced by macrophages which acts as a potent neutrophil chemoattractant (Gorska et al., 2010).

1.4.2 Rolling and adhesion

Before neutrophils leave the blood stream they first roll along the blood vessel endothelium binding transiently via selectins to facilitate rolling and then integrins on the neutrophil bind ICAM-1 on the blood vessel endothelium where they adhere and spread out before they extravasate from the blood vessel into the tissue

- P selectin glycoprotein ligand 1 (PSGL1)

P selectin glycoprotein ligand 1 (PSGL1) is found on the microvilli of neutrophils and other leukocytes (McEver and Cummings, 1997). It binds P-selectin on endothelial cells. This binding is strong enough to slow the neutrophils to a slow roll along the endothelium so that they do not get washed away by shear force but is not strong enough to cause firm adhesion to the endothelium (McEver and Cummings, 1997). This rolling is inhibited by antibody blockade of PSGL1 (Norman et al., 1995) and PSGL1 knock out cells derived from mouse bone marrow show no L-selectin dependent rolling (Sperandio et al., 2003). PSGL1 interacts with the cytoskeleton via Ezrin Radoxin Moesin (ERM) domain

proteins (Zarbock and Ley, 2008) and the binding of PSGL1 initiates signalling events which induce the activation of the neutrophils and the β2 integrins which initiate firm adhesion (Simon et al., 2000).

- L selectin

L-selectin is expressed by all neutrophils and is a type 1 transmembrane glycoprotein which interacts with sialylated ligands expressed on the endothelial surface of blood vessels (Zarbock and Ley, 2008). It is involved in the rolling and activation of neutrophils. The cytoplasmic domain of L-selectin interacts with α actinin and forms complexes with other cytoskeletal proteins such as viniculin (Pavalko et al., 1995) and the ERM proteins ezrin and moesin (Ivetic et al., 2002). Disruption of this interaction by deletion or mutation of the cytoplasmic tail of L-selectin impaired or eliminated neutrophil rolling in vitro and in vivo (Dwir et al., 2001, Kansas et al., 1993). This suggests that the cytoskeletal interaction of L-selectin is important for stabilising the neutrophil tether. Cross linking of the L-selectins leads to β2 integrin activation which is the trigger for the firm adhesion of the neutrophil to the endothelium (Simon et al., 1995).

- Integrins

Integrins are type 1 transmembrane cell adhesion molecules consisting of two non covalently associated subunits, α and β (Zarbock and Ley, 2008). Uniquely, β2 integrins are only expressed in immune cells. The predominant integrins expressed on neutrophils are $\alpha_L\beta_2$ (LFA1) and $\alpha_M\beta_2$ (Mac1) but they also express $\alpha_x\beta_2$ and $\alpha_4\beta_2$ at lower levels (Zarbock and Ley, 2008). Integrins are involved in slow rolling, adhesion, post adhesion strengthening, migration, respiratory burst, phagocytosis and polarisation of neutrophils. Binding of integrins results in a signalling cascade leading to a cytosolic calcium signal (discussed in section 1.3.5), which triggers neutrophil shape change. Once neutrophils are bound to selectins and rolling slowly along the endothelium they are moving slow enough for integrins to bind their ligand ICAM1 which triggers firm adhesion and spreading before extravasation (Lawrence and Springer,

1991). β2 integrins can regulate adhesion by altering their conformation and clustering in the plasma membrane in response to external stimuli. Knocking out of LFA1 integrins in mice abolished rolling and decreased adhesion efficiency posing a role for LFA1 in controlling the rolling velocity of neutrophils (Dunne et al., 2002) LFA1 knockout mice also display a decrease in the firm attachment of neutrophils and a decrease in neutrophil influx to areas of infection which also suggests a role for LFA1 in adhesion and migration of neutrophils (Ding et al., 1999). Further evidence for the role of LFA1 in neutrophil migration comes from experiments showing that LFA1 is evenly distributed on the membrane at neutrophil arrest but redistributes to the neutrophil - endothelial cell junction and co localises with its ligand ICAM1 during transmigration and is further redistributed to the uropod as transmigration is completed (Shaw et al., 2004). Mac1 integrins are stored in granules in the cytoplasm in resting neutrophils which are incorporated into the plasma membrane upon neutrophil activation (Borregaard et al., 1994). Once incorporated into the membrane they play a role in regulating rolling velocity and migration (Dunne et al., 2002). Once integrins have been activated and the neutrophil is adhered to the endothelium and spread then extravasation into the surrounding tissue can occur.

1.4.3 Transmigration

After selectin mediated slow rolling has lead to integrin engagement, firm adhesion and neutrophil spreading, the next step in neutrophil extravasation is transmigration through the endothelial cell wall of the blood vessel into the surrounding tissue. This is a multistep and complex process and the exact mechanism by which this occurs, and even the route that the neutrophil takes is still not fully understood. The paracellular route of transmigration, during which the neutrophil migrates through the junctions between the endothelial cells, is generally agreed to be the most likely route of neutrophil transmigration through the endothelium (figure 1.4.3.1). There is however some evidence that neutrophils may also migrate through the endothelial cell itself, this is known as

the transcellular route (figure 1.4.3.2). Both of these mechanisms will be discussed in this section.

- *Paracellular route*

The paracellular route of neutrophil transmigration involves the migration of the neutrophil through the junctions between the endothelial cells without disturbing the integrity of the cells of the blood vessel wall. This route is thought to be regulated in part by several key molecules found in the intercellular junctions of the endothelium. A schematic view of this mechanism of this is shown in figure 1.4.3.1.

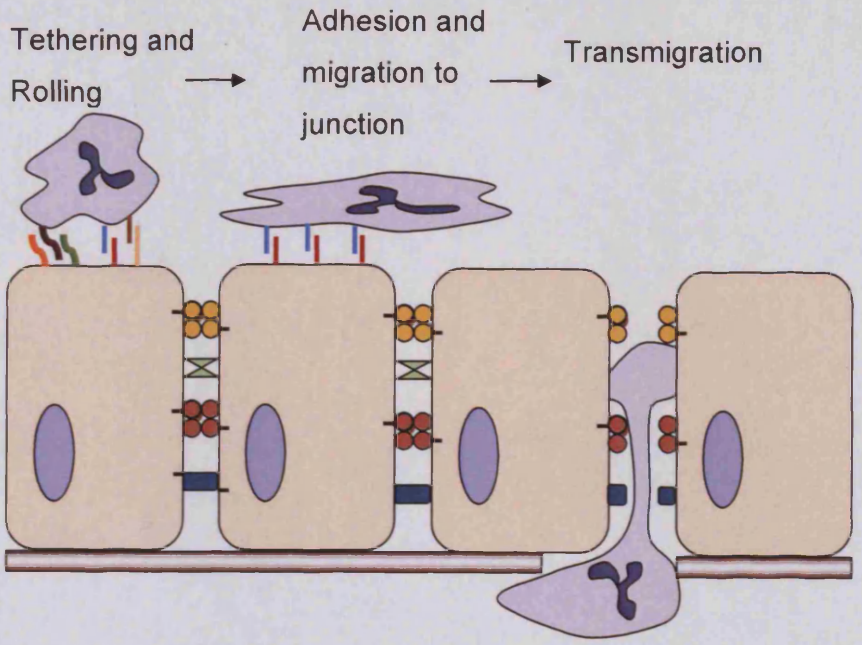


Figure 1.4.3.1: The paracellular route of neutrophil transmigration. Neutrophils roll along the endothelium via selectins and integrins before transmigrating between the endothelial cells. (Garrido-Urbani et al., 2008)

Platelet-endothelial cell adhesion molecule 1 (PECAM1) is a member of the immunoglobulin super family and is expressed on endothelial cells, leukocytes and platelets (Garrido-Urbani et al., 2008). Antibody blockade of this molecule blocks the migration of neutrophils into the inflamed peritoneal cavity of mice in vivo which suggests that it plays a role in neutrophil extravasation (Bogen et al., 1994). It later became apparent that PECAM1 may in fact play two distinct roles in leukocyte transmigration as two different antibodies to different parts of the molecule led to arrest of neutrophils at different stages of the transmigration process, one before the cells had moved through the endothelial cell junctions and one after the cells had moved through the cell junctions but before they passed through the basement membrane (Nakada et al., 2000, Wakelin et al., 1996). PECAM1 is involved but is not essential for neutrophil transmigration. Knocking out ICAM2 in mice led to a decrease in neutrophil accumulation at sites of inflammation suggesting that ICAM2 may play a role in PECAM1 independent transmigration (Huang et al., 2006).

Vascular endothelial cadherin (VE-cadherin) is expressed in the plasma membrane of endothelial cells at the adherens junctions of the endothelium and is linked to the actin cytoskeleton via catenin complexes (Garrido-Urbani et al., 2008). It is displaced from the plasma membrane of the endothelial cells during neutrophil transmigration but is replaced after the neutrophil has passed through the endothelial layer (Shaw et al., 2001). Antibody mediated inhibition of VE-cadherin increases vascular permeability and enhances the entry of neutrophils into inflamed peritoneum in mice. This suggests that VE-cadherin is essential for the maintenance of the endothelial barrier and that the opening of this barrier is essential for neutrophil transmigration (Gotsch et al., 1997). Engagement of ICAM1 leads to phosphorylation of VE-cadherin, inhibition of which decreases neutrophil transmigration suggesting that this is an essential step (Allingham et al., 2007).

The junctional adhesion molecule super family consists of 3 classical members; JAM A, JAM B and JAM C. JAM A is expressed by endothelial cells (Bradfield et al., 2007) and leukocytes but there have been contradictory reports as to its role in transmigration (Garrido-Urbani et al., 2008). JAM B is expressed at the intercellular junctions of high endothelial venules (Palmeri et al., 2000). JAM C is expressed by endothelial cells (Bradfield et al., 2007), inhibition of JAM C decreases neutrophil migration (Aurrand-Lions et al., 2005, Chavakis et al., 2004) whereas over expression increases recruitment of leukocytes to areas of inflammation in mice (Aurrand-Lions et al., 2005) suggesting this JAM plays the most important role in neutrophil transmigration.

- *Transcellular route*

Some experiments have also shown evidence for a transcellular route of neutrophil transmigration where neutrophils transmigrate through the endothelial cell itself. A schematic diagram of how this may work is shown in figure 1.4.3.2.

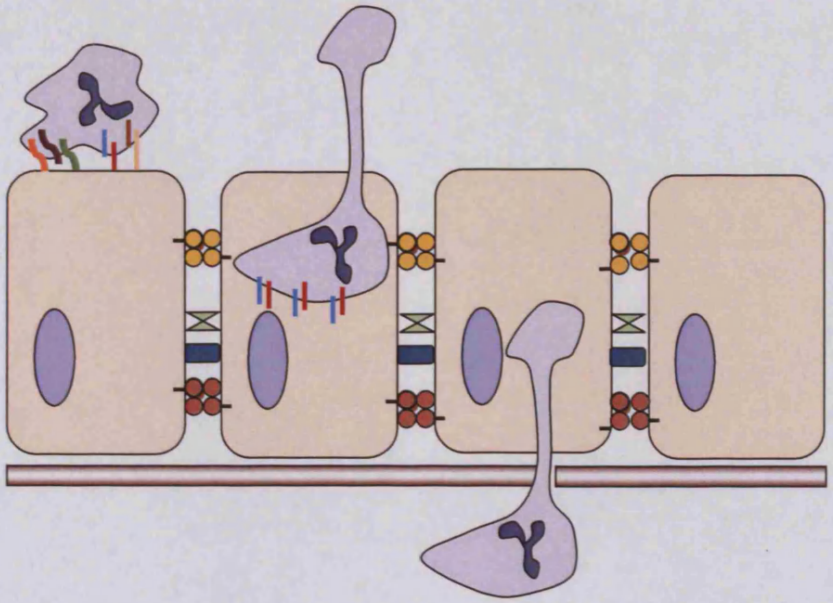


Figure 1.4.3.2: The transcellular route of neutrophil transmigration. After neutrophils firmly adhere to the endothelium via integrins and ICAM1, the ICAM1 is internalised into the endothelial cell creating a channel for the neutrophil to travel through. (Garrido-Urbani et al., 2008)

Electron microscopy studies provide evidence of neutrophil transmigration through endothelial cells in response to fMLP stimulation (Feng et al., 1998). The proposed mechanism for this involves clustering of ICAM1 on the endothelial cell. As it has been shown that ICAM1 translocates to caveolin rich domains in the plasma membrane at the end of actin stress fibres (Millan et al., 2006), the proposed mechanism of neutrophil migration involved the internalisation of the ICAM1 which is then trancytosed to the basal plasma membrane through caveolae. This creates a route through which the neutrophil can follow (Millan et al., 2006). Despite evidence for both the transcellular and the paracellular route of neutrophil transmigration the exact mechanism by which this takes place is yet to be conclusively identified.

1.5 Neutrophil spreading

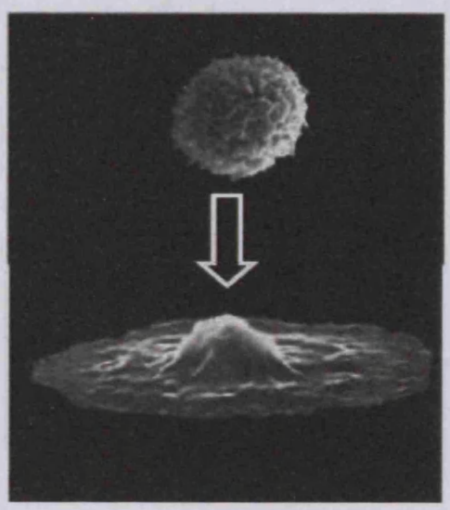
1.5.1 The surface area problem

When a neutrophil spreads on the blood vessel endothelium the surface area of the cell increases by more than two fold (Dewitt and Hallett, 2007). It is not possible for the plasma membrane to simply stretch to accommodate this increase in surface area as the biochemical nature of the lipid bilayer that makes up the plasma membrane means that it can only stretch approximately 4% before rupturing (Hamill and Martinac, 2001). It is also unlikely that the neutrophil gains this extra membrane through exocytosis of intracellular granules. Neutrophils have approximately 1300 primary granules and approximately 4600 secondary granules. If all these granules were to fuse with the plasma membrane, it would increase the surface area of the plasma membrane by 540μm^2 (Nusse and Lindau, 1988). Although this would be sufficient to increase in surface area for the neutrophil to spread, fusion of all of the neutrophil granules to the plasma membrane would release all of the degradative enzymes contained in neutrophil granules into the surrounding tissue causing massive damage. Not to mention that these calculations are

only theoretical and that it is difficult to release more than a third of neutrophil granules experimentally (Dewitt and Hallett, 2007). Cytoskeletal expansion or swelling has also been ruled out as a mechanism of membrane expansion as a computer simulation showed that the object to be internalised would be initially pushed out before being pulled back into the cell, whereas this is not seen experimentally. Also the measurements of the forces involved in phagocytosis experimentally differ to those predicted by the computer model suggesting that membrane expansion is an active rather than a passive process (Herant et al., 2006)

1.5.2 Plasma membrane wrinkles

The final possibility is that the reservoir of extra membrane required for neutrophil spreading comes from the 'un-wrinkling' of the neutrophil membrane as it spreads out (figure 1.5.2). Surface folds on macrophages have been shown to disappear rapidly after phagocytosis (Petty et al., 1981) suggesting a similar mechanism in other phagocytic cells. Neutrophil membrane wrinkles are approximately 300nm in length (Bruehl et al., 1996, Shao et al., 1998), cover approximately 30-40% of the neutrophil plasma membrane (Bruehl et al., 1996) and can be stretched when enough force is applied (Shao et al., 1998).



Un-activated 'spherical' neutrophil with membrane wrinkles still in place.

Activated adherant neutrophil with no membrane wrinkles

Figure 1.5.2: Scanning electron microscope images of a resting 'spherical' neutrophil and an activated adherent neutrophil. Image adapted from (Bessis, 1973).

Biophysical experiments comparing membrane expansion during phagocytosis to forcibly stretched areas of membrane using a micropipette have shown that tension remained low during phagocytosis until 80% of the membrane had expanded compared to only 30% expansion when the membrane was forcibly expanded, suggesting that there is only a limited amount of slack in the wrinkles in the neutrophil membrane (Herant et al., 2005). It is known that neutrophil spreading is directly linked to an increase in cytosolic calcium concentration as a large rise in cytosolic calcium accompanies neutrophil spreading and un-caging cytosolic Ca^{2+} or IP_3 provides an increase in Ca^{2+} large enough to trigger neutrophil spreading (Pettit and Hallett, 1998a). This raises the possibility that an enzyme or second messenger activated by the cytosolic calcium signal which triggers neutrophil spreading and phagocytosis is actively increasing the amount of membrane available. L-selectin is found on the apical surface of neutrophil membrane wrinkles (Bruehl et al., 1996, Erlandsen et al., 1993) and β2 integrins are found on the cell body between the membrane wrinkles (Erlandsen et al., 1993). This would be a logical position if the neutrophil did unwrinkle its membrane during spreading as during extravasation neutrophils first roll along the blood vessel endothelium binding transiently via selectins before they spread and make firm adhesions via β2 integrins. L-selectin is bound to the actin cytoskeleton by the linker molecule ezrin (Ivetic et al., 2002) and β2 integrin is anchored between the plasma membrane and the cytoskeleton via talin (Sampath et al., 1998), both members of the ERM family of proteins. It is possible that these molecules hold the membrane wrinkles in place in resting neutrophils like a 'molecular velcro'. Cleavage of these molecules would release the membrane when the neutrophil is activated.

1.6 Talin

1.6.1 Structure

Talin is a member of the ERM family of proteins. It is a 270kD protein consisting of a 47kD head at the N terminal and a 220kD flexible rod domain at the C terminal. The head domain is made up of a FERM (4.1 ezrin, radixin, moesin) domain which consists of three sub domains, F1-3 (Moser et al., 2009). The F3 domain is responsible for binding the NPxY domain in the cytoplasmic tail of integrin, one of talins main functions (Cheung et al., 2009). There is an additional F0 domain which bears no homology to any known protein domains (Moser et al., 2009). The rod domain comprises a series of helical bundles with several binding sites for the actin binding protein viniculin (Moser et al., 2009). Talin, as a whole contains 3 actin binding sites each of which is adjacent to one of the viniculin binding sites (Hemmings et al., 1996). The rod domain also contains a second integrin binding domain. Both integrin binding sites are required for high affinity binding of talin to integrin (Gingras et al., 2009). At the C terminus talin contains a THATCH (talin/H1P1R/Sla2P actin tethering C terminal homology) domain. This domain is crucial for the primary linkage between talin and f-actin (Moser et al., 2009). The key structural elements of talin are shown in figure 1.6.1.1.

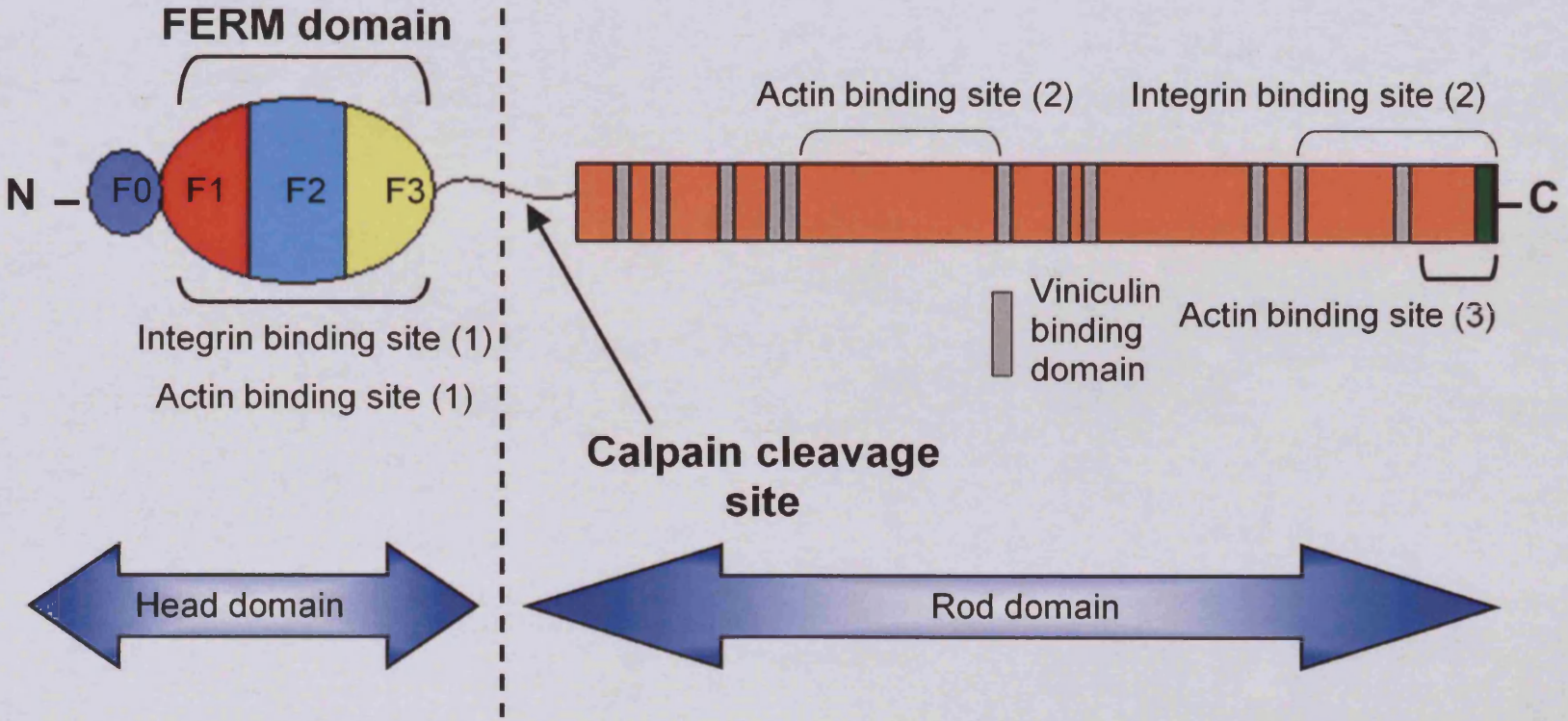


Figure 1.6.1.1: Structure of talin. Talin is made up of a head domain and a rod domain. The head domain contains the FERM domain where integrin and actin bind talin. The rod domain contains further integrin and actin binding sites as well as multiple viniculin binding sites. The calpain cleavage site is between the head domain and the rod domain

1.6.2 Function

The primary role of talin is to connect integrin to the cytoskeleton. Talin has been shown to bind β1,2 and 3 integrins (Gingras et al., 2009, Sampath et al., 1998). There has also been some evidence to show that talin plays a role in the activation of integrins. Mice deficient for talin show integrin mediated cell adhesion defects. However, talin alone is not sufficient to activate integrins. The protease calpain cleaves talin between the FERM domain in the head and the rod domain (Critchley and Gingras, 2008) and this is thought to be a key step in focal adhesion complex turnover and control of the actin cytoskeleton. Talin co localises with calpain in focal adhesion complexes (Lebart and Benyamin, 2006), where it can be cleaved by calpain. A decrease in talin proteolysis due to a down regulation of m-calpain has been shown to make focal adhesion complexes last longer in the cell due to a decrease in focal adhesion complex turnover, suggesting that talin proteolysis is a key mechanism in the disassembly of focal adhesion complexes (Franco et al., 2004). The binding of talin to integrin may explain this. It has been reported that the head domain of talin has a higher affinity for the cytoplasmic domain of β2 (Ivetic and Ridley, 2004) and β3 integrins (Yan et al., 2001) than intact talin within focal adhesion complexes. It was shown that calpain cleavage of the talin head group from the whole molecule increases the affinity of the head group for integrins (Yan et al., 2001) and as a result increases integrin clustering (Ivetic and Ridley, 2004). This would lead to a more stable focal adhesion which may contribute to the effect observed when talin proteolysis was decreased (Franco et al., 2004). Talin has also been shown to be involved in the final stages of cell spreading. When talin is knocked out completely there is no β integrin activation and the final stages of cell spreading do not occur, re-expression of the head group leads to spreading only at the cell edges and re-expression of the whole molecule rescues the defect and the cells are able to spread (Zhang et al., 2008). However, talin deletion had no effect on the early integrin independent stages of cell spreading (Zhang et al., 2008). Talin's exact role in neutrophils is still unknown. However, it has been shown that intact talin co localises with the β2 integrin subunit in un-activated neutrophils (Sampath et

al., 1998). A cleaved 190kD talin subunit is found in activated neutrophils and this subunit no longer associates with β2 integrin (Sampath et al., 1998). This shows that talin is cleaved in activated neutrophils although the reason for this cleavage is as yet unknown, as discussed in section 1.5.2 talin may provide the tether between β2 integrins and the actin cytoskeleton which holds the neutrophils plasma membrane wrinkles in place in the un-activated cell.

1.7 μ-Calpain

1.7.1 The calpain family

The calpains are cytosolic calcium activated proteases found in all vertebrate cells. The family consists of two main members m-calpain (calpain 2) and μ-calpain (calpain 1) as well as other less well characterised "calpains" and calpain- like molecules (Goll et al., 2003). However, μ-calpain is the predominant calpain found in neutrophils and other blood components such as platelets and erythrocytes. Both μ-calpain and m-calpain are made up of a large subunit and small subunit which together form a heterodimer. The small subunit is common to both calpains and is encoded on a single gene on chromosome 19 in humans (Goll et al., 2003). The large subunit of m and μ calpain are different gene products and are encoded on chromosome 1 and chromosome 2 respectively (Goll et al., 2003). m-calpain is made up of an 80kD large subunit (Aoki et al., 1986, Imajoh et al., 1988) and the 28kD common small subunit. The calcium requirement for half maximal activation of m-calpain is very high, 400-800μM calcium and the optimum pH for activation is 7.2-8.2 (Goll et al., 2003). μ-calpain is made up of an 80kD large subunit, slightly larger than the m-calpain large subunit (Aoki et al., 1986, Imajoh et al., 1988), and the 28kD common small subunit. The calcium requirement for half maximal activation of μ-calpain is considerably lower, 3-50μM calcium and the optimum pH for activation is 7.2-8.2 (Goll et al., 2003). There is only one known endogenous protein inhibitor specific for calpain, calpastatin. Binding of

calpastatin to calpain requires calcium to also be bound to calpain and the inhibition is reversible (Otsuka and Goll, 1987)

1.7.2 μ-calpain structure

Calpain 1 or μ-calpain is the predominant calpain found in neutrophils (*Goll et al., 2003*) and as previously stated, is made up of a large proteolytic subunit (80kDa) and the small regulatory subunit (28kDa). Each subunit is then made up of several different domains. The large subunit has a clear 4 domain structure made up of domains I – IV (Aoki et al., 1986). Domains V and VI make up the small subunit (*Goll et al., 2003*). Figure 1.7.2.1 shows the important features and the domains of μ-calpain.

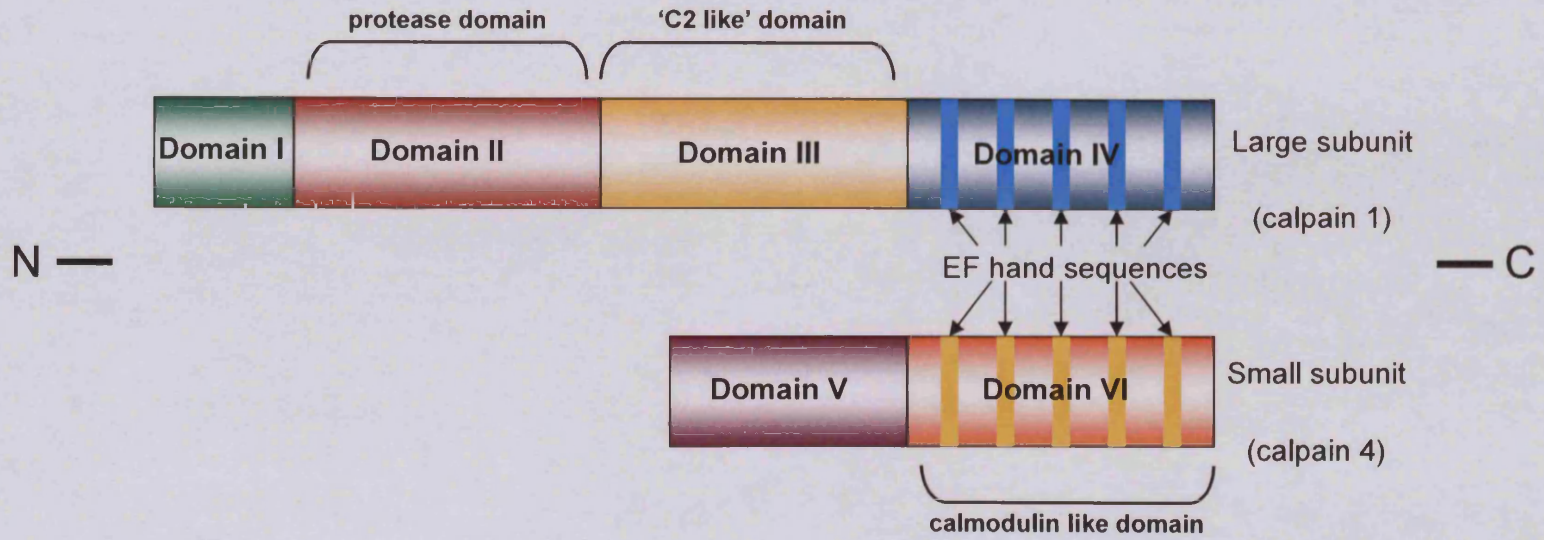


Figure 1.7.2.1 Structure of μ-calpain. μ-calpain is made up of a large 80kD subunit (also known as calpain 1) and a small 28kD subunit (also known calpain 4) which come together to form a heterodimer. The large subunit is made up of four domains and contains the protease domain and the C2 like domain. There are also several EF hand calcium binding domains found in domain IV and domain VI. The small subunit is made up of 2 domains with domains VI forming the calmodulin like domain.

Domain VI in the small subunit contains a calmodulin like domain which contains 4 EF hand calcium binding sequences (Ohno et al., 1986). X ray crystallography studies on common small subunit have shown that there is also potentially a 5th EF hand domain (Blanchard et al., 1997). Crystallographic structures are not yet available for the large subunit of μ-calpain.

Domain I at the N terminal of the large subunit of μ-calpain bears no amino acid sequence homology to any other proteins. Domain II of the large subunit is also known as the protease domain as it contains amino acid residues which form a catalytic triad similar to that found in cysteine proteinases (Goll et al., 2003). Domain III contains a C2 like domain which could enable the protein to translocate to the plasma membrane and bind phosphatidylserine in areas of high calcium (Tompa et al., 2001). This domain may also have a regulatory function. Domain IV is marginally homologous to calmodulin and contains 4 potential EF hand sequences which enable calcium binding (Goll et al., 2003).

1.7.3 μ-calpain activation

The catalytic triad is not fully assembled in unactivated calpain, suggesting that calcium binding must induce a conformational change and reorientate the protease domain into an active state (Hosfield et al., 1999). μ Calpain (calpain 1) requires unusually high cytosolic calcium concentrations to be activated, around 30 μM (*Michetti et al., 1997*). This is much higher than the physiological calcium concentration found in the bulk cytoplasm where μ-calpain is located in resting cells. There has been much debate as to whether autolysis of the calpains occurs in order to decrease the calcium concentration required for their activation. Both m and μ calpain can undergo autolysis when incubated with calcium (Cong et al., 1993), however the calcium concentrations required to induce autolysis are around the levels required for calpain activation therefore they would be higher than encountered in living cells and μ-calpain which had not undergone autolysis is still active as a proteinase (Cong et al., 1993). Also

oxidation of μ-calpain inhibits its proteolytic activity but not autolysis (Guttmann et al., 1997). This suggests that proteolysis and autolysis are two separate events and that autolysis of calpain is not required for its activity. This leaves the question of how calpain activity is regulated in the cell. There have been suggestions of the existence of a calpain activator protein in human erythrocytes which associates with the plasma membrane and activates calpain at physiological levels (Salamino et al., 1993) but there is yet to be any evidence of this in other cell types. During calcium influx in human neutrophils there are areas of calcium concentrations high enough to activate calpain just below the plasma membrane, especially within the membrane wrinkles (Davies and Hallett, 1998, Brasen et al., 2010). μ-calpain has been shown to translocate from the cytosol to the plasma membrane (Gil-Parrado et al., 2003). Calpain's C2 like domain may enable it to translocate from the cytoplasm and bind to the phosphatidylserine on the membrane where calcium is highest. This may be a potential mechanism by which calpain would encounter calcium concentrations high enough for proteolysis to occur.

1.7.4 μ-calpain substrates

Calpain cleaves the ERM proteins talin and ezrin but not moesin (Ivetic and Ridley, 2004, Shcherbina et al., 1999). These proteins associate with the cytoskeleton and are found at the plasma membrane. It is therefore probable that calpain is involved in cytoskeletal regulation in processes such as cell spreading and focal adhesion formation. There have been several reports of calpains involvement in focal adhesion formation and maintenance (Franco et al., 2004, Lebart and Benyamin, 2006, Undyala et al., 2008). Cleavage of talin by calpain appears to be the crucial step in calpains role in focal adhesion turnover, calpain co-localises with talin in focal adhesion complexes and a deficiency of calpain results in a decrease in focal adhesions formed (Lebart and Benyamin, 2006). There are also many reports of calpains involvement in cell spreading, motility and adhesion of other cell types which likely involves calpain mediated cleavage of cytoskeletal proteins (Croce et al., 1999, Dourdin

et al., 2001, Rosenberger et al., 2005, Stewart et al., 1998, Wiemer et al., 2009).

1.7.5 Potential role of μ-calpain in neutrophil spreading

There is evidence that inhibition of calpain can lead to a decrease in inflammation in animal models (Chatterjee et al., 2005, Cuzzocrea et al., 2000, Marzocco et al., 2004, Yoshifuji et al., 2005). The exact mechanism by which calpain inhibition leads to a decrease in inflammation is so far unknown. It has been demonstrated that inhibition of calpain impairs neutrophil spreading and phagocytosis (Dewitt and Hallett, 2002, Wiemer et al., 2009). This leads to the possibility that the mechanism by which calpain inhibition decreases inflammation involves the impairment of neutrophil function, namely neutrophil spreading. If neutrophil spreading was inhibited this would lead to a decrease in neutrophils leaving the bloodstream at areas of inflammation as neutrophil spreading is a key step in neutrophil transmigration, as discussed in section 1.4. As discussed in section 1.5 in order to spread onto the blood vessel endothelium before extravasation the neutrophil needs to unwrinkle its plasma membrane. The wrinkles in the neutrophil plasma membrane are held in place by cytoskeletal proteins among which are the calpain substrates talin and ezrin (Ivetic et al., 2002, Sampath et al., 1998). Should calpain cleave these proteins this would lead to unwrinkling of the neutrophil plasma membrane allowing the neutrophil to spread out and transmigrate through the blood vessel endothelium. There are two key features of calpain that make this possible. Firstly, calpain is activated by high calcium concentrations of around 30μM (Michetti et al., 1997). Under physiological conditions calcium concentrations this high are found specifically in the neutrophil membrane wrinkles, but not the bulk cytosol, during calcium influx, which occurs before neutrophil spreading (Brasen et al., 2010). Secondly by virtue of its 'C2 like' domain, calpain would be able to translocate from the cytosol where it is located under resting conditions to the plasma membrane where the calcium concentration would be high enough for proteolysis to occur when the neutrophil is activated and

calcium influx occurs. It has been observed that unactivated calpain is located in the cytoplasm and upon ionomycin induced calcium influx it translocates to the plasma membrane (Gil-Parrado et al., 2003). These two features of calpain would ensure that the protease is activated specifically at the cell membrane upon neutrophil activation where it would be able to cleave the cytoskeletal proteins talin and/or ezrin and release the neutrophil membrane wrinkles allowing neutrophil spreading to occur.

1.8 Clinical application – Chronic Obstructive Pulmonary Disease

1.8.1 Causes and symptoms

Chronic obstructive pulmonary disease (COPD) is term used to describe a number of conditions involving the long term inflammation of the lungs including chronic bronchitis and emphysema. It is one of the most common forms of lung disease and affects more than 900,000 people in the UK. COPD primarily affects people over the age of 40. The primary cause of COPD is smoking although it can also be caused by coal dust and other work related and environmental pollutants and can also be caused by inherited α1 anti trypsin deficiency. During COPD the bronchioles become inflamed and narrowed and this leads to damage to the alveoli. Symptoms include a persistent cough, high amounts of phlegm and shortness of breath. These are usually only present when undertaking physical activity in patients with mild forms of COPD but in severe cases these symptoms can persist throughout everyday life.

1.8.2 Current treatment

There is currently no cure for COPD. Current NICE guidelines for the treatment of COPD include the cessation of smoking and management of diet as well as

the use of bronchodilators such as β2 agonists and anticholinergics, and nebulisers to treat the everyday symptoms. Antibiotics are used to treat further lung infections and oxygen therapy can be used when not enough oxygen is getting into the blood stream. In cases where the disease is exacerbated temporarily for example by an infection inhaled corticosteroids are prescribed on a short term basis. In severe disease where exacerbations are regular occurrence non invasive ventilation where air is forced into the lungs through a sealed face mask can be used to ensure the patient receives adequate oxygen. Only as a last resort in very severe cases is surgery and a lung transplant considered.

1.8.3 Potential role of calpain inhibitors

As COPD is primarily caused by the inflammation of the bronchioles then a therapy that would decrease the inflammation may provide long term relief. Neutrophils are the most abundant white blood cells found at areas of inflammation and are responsible for releasing degradative enzymes into the tissue and causing damage. Inhibition of neutrophil trafficking to the lung by inhibiting a key step in the process of neutrophil spreading such as calpain activation would prevent the neutrophils from leaving the blood stream and entering the area of inflammation. Although calpain inhibition may not completely obliterate the number of neutrophils leaving the blood stream and entering the lung space, it would reduce the numbers and may therefore be of benefit to patients suffering from COPD.

1.9 Aims of the thesis

The aims of this thesis are to establish that calpain translocation and activation plays a role in neutrophil shape change and that membrane is actively recovered after neutrophil shape change.

These aims will be achieved by demonstrating:

(i) The route of calcium influx during neutrophil shape change (phagocytosis)

(ii) The role of μ-calpains 'C2 like' domain in calpain translocation

(iii) That calpain translocation occurs during neutrophil morphological change (including spreading and phagocytosis): and that calpain translocation and activation has a role in the control of neutrophil morphology change

(iv) A potential route for the retrieval of excess plasma membrane after neutrophil shape change

A key route to achieving these aims will be the establishment of an efficient method for stably expressing foreign proteins in myeloid cell lines.

CHAPTER 2

Materials and Methods

2.1 Cell line maintenance

The HL60, PLB-985 and NB4 cell lines are myeloid cell lines isolated from blood or bone marrow samples from patients with myeloid leukaemia (Collins et al., 1977, Lanotte et al., 1991). They are immature myeloid cells that have been immortalised so that they may be grown continuously in culture. HL60 and PLB-985 cells are slightly more mature than NB4 cells.

HL60, PLB-985 and NB4 cells were cultured in RPMI medium supplemented with 10% heat inactivated Foetal Calf Serum (FCS), 5mM glutamine, 100µg/ml streptomycin and 100µg/ml penicillin at 37°C in 5% CO_2.

The MyPH8B6 cell line was immortalised from bone marrow cells isolated from the femurs of Black6 mice by J.Mcdonald according to the method described by Wang et al (Wang et al., 2006). They showed that enforced production of HoxB8 blocks the differentiation of stem cell factor (SCF) or granulocyte-macrophage colony stimulating factor (GM-CSF) dependent myeloid progenitors. Myeloid progenitors isolated from mouse bone marrow were therefore transfected with oestrogen dependent HoxB8 such that when the cells are grown in the presence of oestrogen they express HoxB8 and are held in an immortal state, but when the oestrogen is removed from the cell medium the cells cease to express HoxB8 and are able to continue their maturation.

MyPH8B6 cells were cultured in OptiMem medium (Invitrogen) supplemented with 10% heat inactivated FCS, 5mM glutamine, 100µg/ml streptomycin, 100µg/ml penicillin, 30µM β-mercaptoethanol, 1µM β-estradiol and 10ng/ml stem cell factor at 37°C in 5% CO_2.

3T3 NIH cells are a mouse fibroblast cell line which is easily transfectable. 3T3 cells were cultured in DMEM medium supplemented with 10% heat inactivated FCS, 5mM glutamine, 100µg/ml streptomycin and 100µg/ml penicillin at 37°C in 5% CO_2.

RAW 264 cells are a mouse monocyte-macrophage cell line. They are relatively easy to transfect and are capable of phagocytosing opsonised

zymosan particles. RAW cells were cultured in DMEM medium supplemented with 10% heat inactivated FCS, 5mM glutamine, 100µg/ml streptomycin and 100µg/ml penicillin at 37°C in 5% CO_2.

For long term storage cells were frozen in either; 10% DMSO, 90% medium supplemented with FCS (HL60, NB4 3T3 and RAW cells) or FCS with 10% DMSO (MyPH8B6 cells). $5x10^6$ (1 confluent flask) cells were frozen per cryovial.

A stock solution of 20% DMSO in medium was prepared. Cells were centrifuged at 1600rpm for 4 minutes and re-suspended in 500µl medium and added to a cryovial along with 500µl stock medium+DMSO solution so that final concentration of DMSO was 10% and cells were exposed to DMSO for as little time as possible. Cryovials were then frozen down slowly (1°C per minute) in the -80°C freezer overnight then moved to the liquid nitrogen for long term storage.

To defrost, cells were thawed quickly in a water bath and transferred into 15mls pre-warmed RPMI/DMEM to dilute the DMSO. Cells were allowed to recover for approx 20 minutes in 37°/5% CO2 incubator, then centrifuged at 1600rpm for 4 minutes and then re-suspended in 5mls warm RPMI/DMEM and put into flasks.

2.2 Isolation of human neutrophils from peripheral blood.

2.2.1 White blood cell isolation from human blood.

Neutrophils were isolated from blood donated by healthy volunteers as previously described (Davies et al., 1991). 2.5mls of 6% dextran (Mr 70,000) was added to 10ml heparinised blood which was allowed to sediment for approx 30 minutes. Once the blood had separated the middle 'buffy coat' layer (figure 2.4.1.1) which contains the white blood cells was removed and centrifuged at 2000rpm for 0.7 minutes.

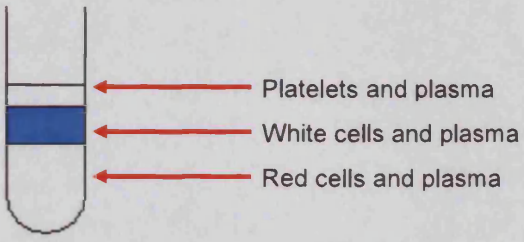
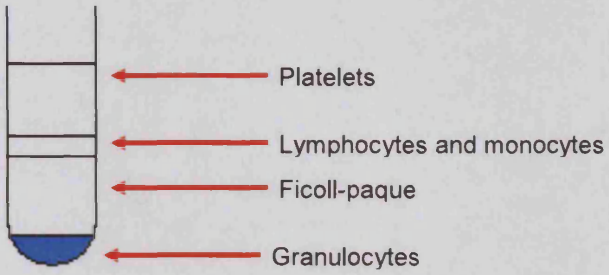


Figure 2.2.1.1: Sedimentation of whole blood. Whole blood is sedimented with 6% dextran at room temperature for 45 minutes. Red blood cells being the heaviest sediment to the bottom of the tube and platelets being the lightest remain in the top layer with the neutrophils being found in the 'buffy coat' layer between the red blood cells and the platelets.

After the supernatant was removed the pellet was re-suspended in 1ml water to lyse the red blood cells followed by 20ml BSS to restore osmolarity. Cells were centrifuged at 2000rpm for 0.5 minutes and the pellet was re-suspended in 5ml Krebs-BSA. The typical proportion of neutrophils in the preparation is high, and these cells are used for single cell experiments, as less preparation means less risk of activation, and less time taken, so cells are 'fresher'

2.2.2 Neutrophil isolation from a white blood cell preparation

This type of preparation was used when a pure neutrophil preparation was required. 5ml ficoll-paque solution was added underneath the cell suspension and the suspension was centrifuged at 1000rpm for 25 minutes. The supernatant was then decanted to leave the pellet which contains the neutrophils (figure 2.2.2.1)



2.2.2.1: Separation of granulocytes from other white blood cells. Granulocytes are separated from other white blood cells using ficoll-paque density gradient centrifugation.

2.3 Purification of plasmid DNA

Plasmids pEYFP-C1-calpain 4 (calpain 4-YFP) (Gil-Parrado et al., 2003), pMX-Mu-calpain-EGFP (calpain 1-GFP) (Lokuta et al., 2003), C2-gamma-YFP and C2-beta-RFP (Adjobo-Hermans et al., 2008) were all kind gifts from other laboratories.

Plasmids were transformed into Invitrogen oneshot® top10 *Eshcericia coli* (*E.coli)* cells according to the manufacturers protocol. Briefly, plasmid is transformed into chemically competent *E.coli* (OneShot® TOP10 cells) by adding the plasmid to the cells and then incubating the cells on ice and then heat shocking to permeabalise the cell membrane to allow the plasmid to enter. Nutrient rich SOC medium is added to increase the efficiency of the transformation. Bacteria were then spread onto Ampicillin selective LB agar plates at different concentrations in order to grow one plate containing evenly spaced colonies. Plates were incubated overnight at 37°C.

Plasmids were then purified using Machery-Nagel Nucleobond® Extra Maxi kit (Abgene) according to the manufacturer's protocol. Briefly, the bacterial cells are lysed by adding SDS and NaOH which breaks the phospholipid bilayer by denaturing the proteins involved in holding it together. Plasmid DNA is separated from other nucleic acids and proteins through anion exchange. The solution is applied to an anion exchange column made from anion exchange resin which consists of hydrophilic, pourus silica beads with a methyl-amino-ethanol (MAE) group. This functional group has an overall positive charge which means that under acidic conditions the negatively charged phosphate backbone of the DNA binds to the resin.

Large double stranded chromosomal DNA is denatured by the addition of an acidic solution to neutralise the column. In its single stranded form

chromosomal DNA will be washed from the column at a lower pH than the double stranded plasmid DNA. Increasing concentrations of salt buffer are added to the column to wash off any protein, RNA and chromosomal DNA. The more interactions that molecule can make with the resin the higher salt concentration is required to wash it off. Small nucleic acids and single stranded DNA make fewer interactions than the double stranded plasmid DNA and so are washed off at a lower pH. A high salt concentration elution buffer is then applied to the column finally to wash off the plasmid DNA. The plasmid DNA is then recovered from the supernatant by ethanol precipitation. This precipitate can then be re-suspended in a buffer of ddH20. Plasmids were digested to check plasmid integrity and cut plasmid was run on a 0.8% agarose gel at 100 volts for approx 45 minutes. Plasmids were quantified using a spectrophotometer to analyse absorbance at 260nm

Where plasmid concentration was too low, plasmids were precipitated using sodium acetate precipitation. 10% of the sample volume 3M sodium acetate (pH 5.2) was added to the plasmid along with double the sample volume ice cold 100% ethanol. Sample was vortexed and centrifuged for 15 minutes at maximum speed at 4°C. Supernatant was removed carefully without disturbing the pellet and replaced with 1ml ice cold 70% ethanol. Sample was centrifuged for 10 minutes at maximum speed at 4°C. The supernatant was removed and the pellet was air dried at 55°C for 5 minutes and re-suspended in 30µl PCR quality H_2O.

2.4 Plasmid Sequencing

All plasmids for which there were known primer sites before, after or within the insert were sequenced. The resulting sequences were then entered into a Basic Local Alignment Search Tool (BLAST) search to confirm that the plasmids contained the correct insert (Appendix IV).

2.5 Differentiation of cell lines into phagocytosis-competent cells

2.5.1 Differentiation of the NB4 cell line

All trans-retinoic acid (ATRA) was purchased from Sigma-Aldrich® in powder form which was diluted in DMSO to desired concentration in an argon atmosphere under dimmed light to prevent decomposition. ATRA is a vitamin A derivative that acts via the retinoic acid nuclear receptor to cause differentiation in the NB4 cell line (Idres et al., 2001).

Sterile cover slips were placed in a 6 well plate and $2x10^5$ NB4 cells in 2mls RPMI with varying concentrations of All Trans Retinoic Acid (ATRA) (1, 0.75, 0.5, 0.25, 0.1 or 0.01μM) was placed in each well. Controls were cells treated with DMSO or received no treatment. Cells were monitored daily.

To see if it was possible to achieve a high transfection frequency and a high level of differentiation $2x10^6$ NB4 cells were cultured in a flask in the presence of 0.01μM ATRA for 3 days prior to transfection with pmaxGFP plasmid using the Amaxa nucleofector device according to the manufacturer's protocol. Cells were recovered in RPMI supplemented with 0.01μM ATRA and were placed in 2mls RPMI supplemented with 0.01μM ATRA on cover slips in a 6 well plate and allowed to differentiate for a further 2 days before checking if the transfection had been successful.

2.5.2 Differentiation of the HL60 cell line

Treatment with DMSO causes the HL60 cell line to differentiate into neutrophil like cells. The exact molecular mechanism that causes this differentiation is still unknown.

Sterile cover slips were placed in a 6 well plate and $1x10^5$ HL60 cells in 2mls RPMI with varying concentrations of DMSO (1.3%, 1.7% and 1.9%) were placed in each well. Control cells received no treatment. Cells were checked

daily to determine the optimum length of time that they needed to be treated with DMSO for maximum levels of differentiation.

For calpain translocation experiments $2x10^6$ HL60 cells were cultured in a flask in the presence of 1.3% DMSO for 5-7 days prior to transfection with the pEYFP-C1+30cDNA (calpain 4) plasmid using nucleoporation according to the manufacturer's instructions. Cells were recovered in RPMI supplemented with 1.3% DMSO and were placed in 1ml RPMI supplemented with 1.3% DMSO on cover slips in a 6 well plate and left overnight to adhere to the cover slips before observing them on the CLSM.

2.5.3 Differentiation of the MyPH8B6 cell line

$7x10^5$ cells were centrifuged at 350xg for 5 minutes and washed 3 times in PBS. Cells were seeded at $7x10^4$ cells/ml in 10ml OptiMem medium (Invitrogen) supplemented with 10% heat inactivated FCS, 1% glutamine, 100µg/ml streptomycin, 100µg/ml penicillin, 30µM β-mercaptoethanol, SCF (20ng/ml) and G-CSF (20ng/ml). Cells were maintained in this medium for 4 days until differentiation was complete. Additional medium was added daily to renew the growth factors.

2.6 Cytosolic calcium measurement

2.6.1 Cytosolic calcium measurement using Fura Red Calcium indicator

Fura Red was loaded into the cells in an AM form using the same method described in section 1.3.2 for Fura2 (figure 2.6.1.1). Fura red is a non ratiometric calcium indicator which is excited at the same wavelength in both the calcium free and calcium bound forms. Calcium free fura red is excited by 488nm light and emits light at 650nm. Calcium bound fura red is excited at 488nm light and emits light at 650nm but at a lower intensity. (figure 2.6.1.2) Changes in cytosolic calcium concentration can be detected by the decrease in

fluorescence intensity of the indicator. The calcium concentration in the cell at any given time can be calculated from the fluorescence intensity if the indicator using the following equation:

$[Ca^{2+}]_i = Kd \times (Fmax \div F) - 1$

Where, Fmax is the maximum fluorescence intensity of the indicator and F is the fluorescence of the indicator at any given point, relative to the minimum value of zero.

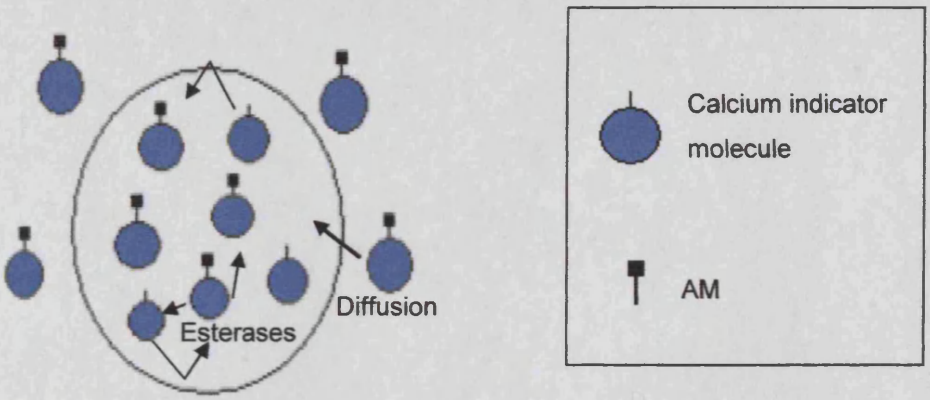


Figure 2.6.1.1: AM bound calcium indicator loading of cells. The acetotoxymethyl ester that is bound to the fura-red molecule makes the molecule lipid soluble. This allows the fura-red to pass through the plasma membrane of the cell. Once in the cytoplasm of the cell non-specific esterases within the cytoplasm digest the ester bond linking the acetotoxymethyl ester to the fura-red molecule. This leaves the hydrophilic form of fura-red trapped in the cytoplasm.

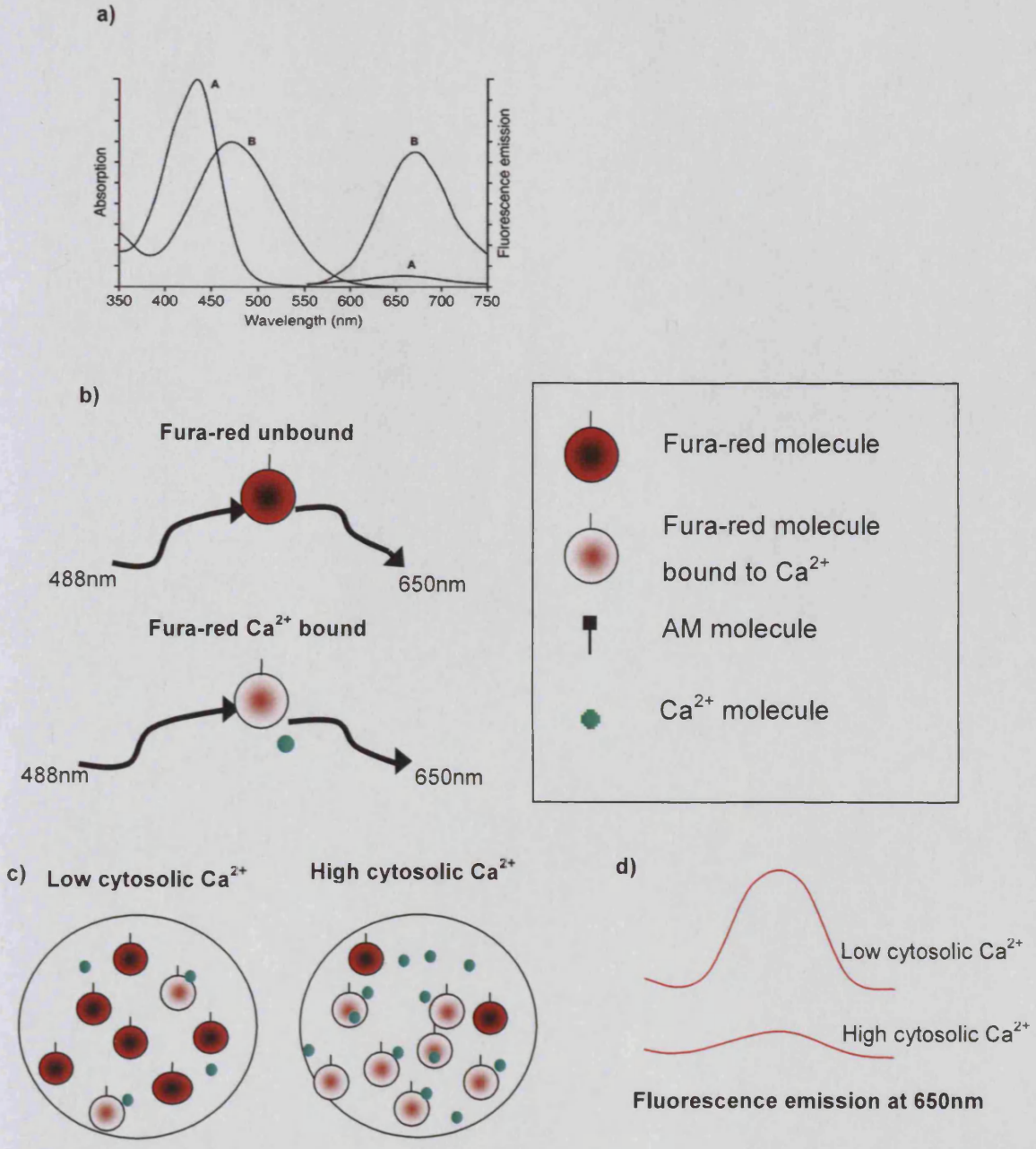


Figure 2.6.1.2: Measurement of calcium concentration using fura-red. (a) Fluorescence excitation (excited at 488nm) and emission (detected at 650nm) spectra of Ca^{2+}-saturated (A) and Ca^{2+}-free (B) fura-red in pH 7.2 buffer. (b) Unbound fura-red is excited by the 488nm wavelength emits high levels of fluorescence at the 650nm wavelength and calcium bound fura-red is excited by the same wavelength but emits less fluorescence than the unbound conformation. (c) Increasing the cytosolic calcium within the cell causes an increase in the amount of calcium bound fura-red and d) lead to a decrease in the overall fluorescence of the cell detected at 650nm

2.7 Ca^{2+} elevating experiments

Ionophore increases intracellular calcium by creating artificial calcium channels in the plasma membrane which allows calcium to enter the cell. Thapsigargin increases intracellular calcium by inhibiting the CaATPase on the sarcoplasmic and endoplasmic reticulum. This increases intracellular calcium by stopping the cell from pumping calcium from the cytoplasm into the intracellular calcium stores. Depletion of calcium from the intracellular stores then causes the plasma membrane calcium channels to open causing further calcium influx. fMLP causes a physiological calcium signal in neutrophils and in neutrophil like cells. fMLP binds to the formyl peptide receptor (FPR). This is a G protein coupled receptor and its fMLP binding leads to the activation of numerous second messenger proteins (Selvatici et al., 2006). These act to initiate the release of Ca^{2+} from intracellular stores. This produces the initial small increase in Ca^{2+} seen upon fMLP treatment. Depletion of Ca^{2+} from these stores results in the opening of Ca^{2+} channels in the plasma membrane, this leads to Ca^{2+} influx into the cytoplasm and results in a large increase in cytosolic Ca^{2+}.

Ca^{2+} elevating experiments were performed on all cell lines including cord blood derived neutrophils. Differentiated cells were placed on cover slips on a heated stage on the Confocal Laser Scanning Microscope (CLSM). Adhered cells were treated first with 1μM fMLP to try and produce a physiological calcium signal. Cells were then treated with 1μM or 10μM ionophore, 1μM or 5μM thapsigargin and finally 26mM $CaCl_2$ was added 50:50 to their medium so that the final concentration of the $CaCl_2$ in the medium was 13mM to further increase the intracellular calcium.

2.8 Confocal Microscopy

Confocal imaging of cells was carried out on a Leica SP2 or SP5 confocal microscope using a x64 oil immersion objective. Fura red calcium indicator and green and yellow fluorescent proteins were excited using the 488nm laser. Red fluorescent proteins were excited with the 543nm laser. Emission was detected at the appropriate wavelength for the fluorophore being imaged. Where more than one fluorophore was being imaged in the same sample sequential imaging techniques were employed. Sequential imaging allows for simultaneous imaging at two different wavelengths whilst minimising interference and 'crosstalk' between the two images by recording the image for the different wavelengths sequentially between frames.

CHAPTER 3

Translocation of C2 domain proteins

3.1 Introduction

Domain III of calpain contains a C2-like domain which is very important for its ability to act as a protease localised to the cell periphery. This C2 domain enables the protein to translocate to the plasma membrane where it binds phosphatidylserine at areas of high calcium (Tompa et al., 2001). In this chapter, data will be presented to show that that classical C2 domain constructs, as a surrogate model for translocation of C2 containing proteins such as calpain-1, can be used to map out Ca^{2+} influx sites and potential sites of calpain translocation during activation of myeloid cells.

3.1.1 C2 domains

C2 domains are Ca^{2+} dependent phospholipid binding domains found in a wide range of eukaryotic proteins (Nalefski and Falke, 1996). The most common C2 domains are around 130 amino acid residues in length with a 3D structure made up of an 8 stranded anti parallel β sandwich consisting of a pair of 4 stranded β pleated sheets (Nalefski and Falke, 1996). The C2 domain was first discovered in protein kinase C and is the 2^{nd} of 4 conserved domains in that protein. C2 domains were also found in the neuronal protein synaptotagmin. As both protein kinase C and syntaptotagmin bind phosphatidyl serine upon an increase in calcium concentration it was postulated that the C2 domain may be involved in calcium regulated phosphatidyl serine binding (Bazzi and Nelsestuen, 1987). Experiments showing that the C2 domain from phospholipase A2 translocated to membrane vesicles upon increased calcium concentration (Clark et al., 1991) and the C2 domain from synaptotagmin binding phospholipid vesicles upon calcium increase (Davletov and Sudhof, 1993) confirmed that the C2 domain was indeed a calcium dependent phospholipid binding domain. A fully saturated C2 domain can bind two calcium ions (Shao et al., 1996). It is still not known how the binding of calcium to the C2 domain mediates phospholipid binding but there have been two models proposed. The first is a ternary complex model in which the calcium is

bound simultaneously by the phosphatidyl serine and the C2 domain therefore mediating the binding of the lipid and the protein (Nalefski and Falke, 1996). This model is based on data from other molecules which are not homologous to the C2 domain but do bind phosphatidyl serine with the aid of calcium ions such as AnnexinV. Also the $[Ca^{2+}]_{1/2}$ of C2 domains from protein kinase C is reduced in the presence of membrane phospholipids suggesting that there is some sort of binding equilibrium taking place between the calcium and the membrane phospholipids (Kohout et al., 2002). The second proposed model is the conformational change model which suggests that a conformational change of the C2 domain upon binding calcium exposes necessary functional groups which in turn enable the C2 domain to bind to phosphatidyl serine (Davletov and Sudhof, 1994). This model is based upon observations that in certain C2 domains calcium binding induces a conformational change in the protein (Davletov and Sudhof, 1994), however as yet no direct evidence exists to suggest that this conformational change leads to phosphatidyl serine binding. As still very little is known about the mechanism of phosphatidyl serine binding by C2 domains both very different theories remain plausible. The existence of a ternary complex method of binding phosphatidyl serine via calcium by other non C2 domain proteins suggests that it is not unlikely that C2 domain proteins employ a similar method however experimental evidence of a conformational change in C2 domains upon calcium binding makes the second theory just as feasible. Until further direct experimental evidence is achieved the mechanism of phosphatidyl serine binding by C2 domains will remain unknown.

3.1.2 Calpains C2 like domain

Domain III of calpain is known as the 'C2 like' domain (figure 3.1.2.1). X-ray crystallography images of m-calpain confirm that domain III of m-calpain is a calcium regulated phospholipid binding domain (Tompa et al., 2001). As well as amino acid sequence homology to the classical C2 domains the 'C2 like' domain of m-calpain folds into an anti-parallel β sandwich consisting of a pair of 4 stranded β pleated sheets (Tompa et al., 2001). This is the same

conformation as the original classical C2 domains discovered in protein kinase C (PKC). m-calpains 'C2 like' domain can also bind to phosphatidyl serine in a calcium dependent manner (Tompa et al., 2001) further confirming its function as a C2 domain. As these studies were all done on domain III from m-calpain further investigation is needed to confirm that domain III from μ calpain is also a C2 domain. However the amino acid sequences of domain III from calpain 1 and calpain 2 are 62% identical and 82% similar (figure 3.1.2.2) so it is likely that the two domains would act in a similar manner. It has been shown that domain III is required for membrane targeting of μ calpain (Gil-Parrado et al., 2003) and it had been suggested that calpain activation may be caused by its association with the plasma membrane (Molinari and Carafoli, 1997). In any case, the C2 domain is clearly important for physiological calpain activation.

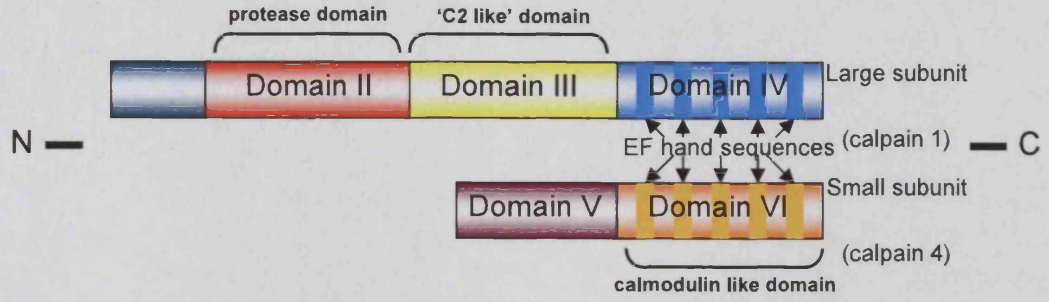


Figure 3.1.2.1 Structure of μ-calpain. μ-calpain is made up of a large 80kD subunit (also known as calpain 1) and a small 28kD subunit (also known calpain 4) which come together to form a heterodimer. Domain III is known as the 'C2 like' domain.

```
CAPN1 D3 PDALKSRTIRKWNTTLYEGTWRRGSTAGGCRNYPATFWVNPQFKIRLDETDDPDDYGDRE
CAPN2 D3 PDTLTSDTYKKWKLTKMDGNWRRGSTAGGCRNYPNTFWMNPQYLIKLEEEDEDEEDG--E
         **:*.* * :**: *  :*.************** ***:***: *:*:* *: :: *  *

CAPN1 D3 SGCSFVLALMQKHRRRERRFGRDMETIGFAVYEVPPELVGQPAVHLKRDFFLANASRARS
CAPN2 D3 SGCTFLVGLIQKHRRRQRKMGEDMHTIGFGIYEVPEELSGQTNIHLSKNFFLTNRARERS
         ***:*::.*:******:*::*.**.****.:**** ** **. :**.::***:* :* **

CAPN1 D3 EQFINLREVSTRFRLPPGEYVVVPSTFEPNKEGDFVLRFFSEKSAGTVELDD 172
CAPN2 D3 DTFINLREVLNRFKLPPGEYILVPSTFEPNKDGDFCIRVFSEKKADYQAVDD 170
         : ******* .**:******::**********:*** :*.****.*.   :**
```

Figure 3.1.2.2: Protein sequence alignment of domain III from calpain 1 and domain III from calpain 2. Sequences are 82% similar and 62% idenditcal. * denotes identical residues and : or . denotes homologous residues.

In the case of neutrophil spreading, it is hypothesised that the role of the C2 domain in calpain is to permit calpain to translocate to the plasma membrane upon cytosolic Ca^{2+} increase therefore ensuring that calpain is activated specifically at the plasma membrane where it can cleave its target molecule(s).

3.1.3 Calcium influx and C2 domain translocation

Neutrophil spreading and phagocytosis are directly linked to an increase in cytosolic calcium concentration (Dewitt and Hallett, 2002, Jaconi et al., 1991, Kruskal et al., 1986, Pettit and Hallett, 1998a, Pettit and Hallett, 1997). This increase in cytosolic calcium concentration is caused by calcium ion influx across the plasma membrane and blocking this influx inhibits most neutrophil responses (Hallett and Campbell, 1984, Marks and Maxfield, 1990). Despite substantial evidence pointing to the importance of the calcium influx and the cytosolic calcium signal to neutrophil activity the exact channels through which this calcium influx occurs are still unknown. There are several potential candidates which are discussed in more detail in section 1.3.7.

To further investigate potential triggers of calcium influx and the potential calcium channels involved in calcium influx it would be useful to know if the plasma membrane calcium channels opened globally, all over the plasma membrane during calcium influx or locally i.e. just at the position on the plasma membrane where the neutrophil is stimulated. Observation of C2 domain translocation during a global stimulation such as fMLP or a local stimulation such as attachment of a particle during phagocytosis can help to answer this question. As C2 domains are calcium sensitive phosphatidyl serine binding domains (Clark et al., 1991, Davletov and Sudhof, 1993) they will translocate from the cytosol to the plasma membrane and bind phosphatidyl serine only where calcium concentration is high (i.e. where the plasma membrane calcium channels are open). This translocation would occur immediately prior to a global increase in cytosolic calcium concentration which must "lag" behind Ca^{2+} influx itself. Therefore where the C2 domain binds the plasma membrane in the

first few ms after stimulation (either via fMLP or phagocytosis) will give an indication as to where on the plasma membrane the calcium channels are open when the neutrophil is activated.

C2 domains from protein kinase C are able to translocate to the plasma membrane in response to an increase in cytosolic calcium concentration in a variety of cell types (Adjobo-Hermans et al., 2008). C2 domain translocation has been used successfully to analyse different types of calcium signal in rat basophilic leukaemia cells (Teruel and Meyer, 2002) and oscillations in C2 domain membrane binding have been used to illustrate the calcium regulation of PLCβ (Adjobo-Hermans et al., 2008). Different types of C2 domains display different calcium binding properties. In the two studies highlighted above the C2 domain from protein kinase C γ was used. This particular C2 domain has a $[Ca^{2+}]_{1/2}$ of 0.7μM and as such has a very high calcium affinity when compared to another C2 domain from the same family, protein kinase C β, which has a $[Ca^{2+}]_{1/2}$ of 5μM (Kohout et al., 2002). As such the C2 domain from PKCγ (C2γ) will translocate and bind to the plasma membrane at much lower calcium concentrations than the C2 domain from PKCβ. As C2γ is highly sensitive to changes in calcium concentration, it may be possible to utilise C2γ plasma membrane binding to reveal the location of calcium influx during phagocytosis (i.e. global versus local influx)

3.1.3 Aims of the chapter.

The aims of this chapter are therefore to use classical C2 domain constructs as a surrogate model for translocation of C2 containing proteins such as calpain-1. In addition, translocation of C2 domain constructs will enable the mapping of Ca^{2+} influx sites during activation of myeloid cells.

In this chapter the specific objectives are:-

- To characterise the translocation of different C2 domains

- To use C2 domain translocation to investigate where in the membrane calcium channels open to cause calcium influx during phagocytosis
- To investigate the role of calpains C2 like domain

These objectives will be achieved with the use of fluorescently tagged classical C2 domains which will be expressed by different cell types. Their translocation in response to calcium influx will be investigated using pharmacological and physiological methods of raising calcium within the cells.

3.2 Materials and Methods

3.2.1 Transfection of cell lines

3.2.1aTransfection of the 3T3 cell line using nucleofection

$1x10^6$ cells were transfected with C2γ-YFP or C2β-RFP using the Amaxa nucleofector device according to the manufacturer's protocol. Cells were centrifuged at 2000rpm for 10mins and re-suspended in 100µl nucleofector solution (kit V). 2µg plasmid was added to the cells which were then transferred to a cuvette and electroporated using programme X-001 on the Amaxa nucleofector device. Cells were recovered in 500µl pre-warmed DMEM before being transferred to glass bottomed 'confocal dishes' containing 1ml pre-warmed DMEM per transfection. Cells were incubated at 37 °C in 5% CO_2 overnight before being used for calcium elevating experiments on the CLSM the next day.

3.2.1b Transfection of the RAW 264 cell line using nucleofection

$2x10^6$ cells were transfected with C2γ-YFP or C2β-RFP using the Amaxa nucleofector device according to the manufacturer's protocol. Cells were centrifuged at 750rpm for 10mins and re-suspended in 100µl nucleofector solution (kit V). 2µg plasmid was added to the cells which were then transferred to a cuvette and electroporated using programme D-035 on the Amaxa nucleofector device. Cells were recovered in 500µl pre-warmed DMEM before being transferred to glass bottomed 'confocal dishes' containing 1ml pre-warmed DMEM per transfection. Cells were incubated at 37°C in 5% CO_2 for 4 hrs before being used for calcium elevating experiments on the CLSM.

3.2.1c Transfection of partially differentiated PLB-985 cells using nucleofection.

$2x10^6$ cells were differentiated in 1.3%v/v DMSO for 4 days. These cells were then transfected with C2-gamma-YFP or C2-beta-YFP using the Amaxa nucleofector device according to the manufacturer's protocol. Cells were centrifuged at 2000rpm for 10mins and re-suspended in 100µl nucleofector solution (kit V). 2µg plasmid was added to the cells which were then transferred to a cuvette and electroporated using programme X-001 on the Amaxa nucleofector device. Cells were recovered in 500µl pre-warmed RPMI before being transferred to glass bottomed 'confocal dishes' containing 1ml pre-warmed RPMI per transfection. Cells were incubated at 37°C in 5% CO_2 for 3 hours before being used for calcium elevating experiments on the CLSM the next day.

3.2.2 Confocal Microscopy and calcium elevating experiments

Pharmacologically stimulated cytosolic calcium increase was achieved by treating the cells with a cocktail containing 10µM ionophore, 5µM thapsigargin 13mM $CaCl_2$ as described in section 2.7. Physiologically stimulated cytosolic calcium influx was achieved by treating the cells with mouse serum. Cytosolic calcium concentration was monitored using fura red calcium indicator as described in section 2.6. All experiments were imaged using a Leica SP2 confocal microscope as described in section 2.8.

3.2.3 Phagocytosis experiments

RAW 264 expressing C2-gamma YFP were presented with zymosan particles opsonised using mouse serum using a micropipette and manipulator. Cells were allowed to bind the particle before the micropipette was removed and phagocytosis was allowed the complete (see phase contrast image sequence in figure 3.3.2.4 for demonstration). C2 domain translocation and/or cytosolic calcium concentration was recorded continuously during phagocytosis.

3.2.4 Induction of apoptosis in RAW-264 cells

RAW 264 cells were transfected with YFP tagged C2 domain from protein kinase C as described in section 3.2.1b. As RAW 264 cells are immortalised they do not undergo spontaneous apoptosis as neutrophils do. Therefore Staurosporine (STS) was needed to induce apoptosis. It is thought that STS induces apoptosis by inhibiting various kinases in the cells (Yamaki et al., 2002) in a non-specific manner. To induce apoptosis, STS (4µM) was added to the transfected RAW 264 cells 3.5 hours before CLSM visualisation. C2 domain translocation was pharmacologically stimulated as described in section 3.2.2.

3.2.4.1 Visualisation of phosphatidylserine (PS) externalisation

Phosphatidylserine that had been externalised to outer leaflet of the plasma membrane was visualised using AnnexinV-FITC (1µl of AnV-FITC in 0.02% NaN_3, PBS and 1% BSA to 100µl of sample), excited at 488nm, with a maximum emission wavelength of 570nm.

3.2.1.2 Identification of necrotic cells with propidium iodide

Necrotic cells were identified by their uptake of propidium iodide nucleic acid stain which is excluded from viable cells.

3.2.5 Immunofluorescent staining for calpain1 in apoptotic neutrophils

White blood cells, including neutrophils were isolated from human blood as described in section 2.2.1. Half of the sample was allowed to age for 24 hours in order for apoptosis to occur and half of the sample was fixed immediately. Before fixing and permeabilisation AnV-FITC (1µl) and $CaCl_2$ (1µl) were added to 100µl aged and non-aged neutrophil suspension and incubated for 30 minutes at 37°C. This mixed suspension was allowed to settle onto glass slides

pre-coated with fibronectin (5µl of 0.1% fibronectin) for 20 minutes at 37°C. Cells were washed twice in Krebs-BSA before being fixed in 100µl 3.7% formaldehyde for 10 minutes at room temperature. Cells were washed 3 times in PBS and then permeabilised in 0.1% triton for 4 minutes after which they were washed a further 2 times in PBS. Non specific binding sites were blocked by incubating the cells in 4% horse serum for 1 hour before the addition of the primary antibody. Primary antibody was monoclonal anti-calpain 1 (6C-12) (Santa Cruz – sc32327). Primary antibody was diluted 1 in 100 (in 4% horse serum) before use and cells were incubated in primary antibody overnight at 4°C. The following morning cells were washed thoroughly in PBS before secondary antibody was added. Secondary antibody was goat anti-mouse IgG Rhodamine (Santa Cruz santa cruz – sc-2092). Cells were incubated in secondary antibody for 1 hour at room temperature in the dark. Secondary antibody was removed by washing thoroughly twice with PBS. Microscopic visualisation of the cells, once stained, was carried out using the CLSM.

3.3 Results

3.3.1 Classical C2 domains

3.3.1.1 C2 domain translocation in response to pharmacologically stimulated calcium influx.

As a preliminary to using the translocation of fluorescently tagged-C2 domains, it was necessary to ensure that the constructs expressed biologically active protein in myeloid cells. Both human (PLB-985 cells) and mouse (RAW 264 cells) myeloid cell lines were transfected, and investigated by utilising a strategy aimed to elevate cytosolic Ca^{2+} (and influx) pharmacologically. Cytosolic calcium levels were raised using a "cocktail" of ionomycin (10μM), thapsigargin (5μM) and 13mM $CaCl_2$ which was shown to be effective in increasing Ca^{2+} concentration and inducing C2 translocation using a model cell line 3T3 cells (figure 3.3.1.1). Using this strategy, C2 translocation was shown to be triggered in both PLB-985 cells (figure 3.3.1.2) and RAW 264 cells (figures 3.3.1.3 and 3.3.1.4). This translocation was reversed when Ca^{2+} influx was stopped/reduced by replacement of the cocktail with physiological medium (figures 3.3.1.3 and 3.3.1.4). Simultaneous measurement of cytosolic Ca^{2+} showed the dependency on cytosolic calcium influx being maintained at a high level of calcium influx (figure 3.3.1.5). It was concluded that active C2 construct was generated in myeloid cells and that its translocation was a monitor of Ca^{2+} influx.

3.3.1.2 C2 domain translocation in response to physiologically stimulated calcium influx.

In order to establish whether C2 translocation was sensitive enough to detect Ca^{2+} influx triggered physiologically, rather than pharmacologically, mouse serum (as a source of mouse C5a) was used. This physiological stimulus caused a transient increase in cytosolic Ca^{2+} in the mouse cell line RAW 264 which was accompanied by a clear translocation of C2 domain to the plasma

membrane (n=3). The C2 domain was released from the plasma membrane before cytosolic calcium concentration decreased to resting levels (figure 3.3.1.6)

3.3.2 C2 domain translocation during phagocytosis

The preceding results demonstrate the usefulness of C2 domain translocation as a surrogate measure for the translocation of C2 proteins, like calpain. Using RAW 264 cells transfected with C2 domain constructs, it was found that the Ca^{2+} influx accompanying phagocytosis is sufficient to stimulate C2 domain proteins to translocate from the cytoplasm to the plasma membrane (figure 3.3.2.1 and 3.3.2.2). This suggests that calpain may also translocate to the plasma membrane during phagocytosis (see chapter 4 section 4.3.2.4). However RAW 264 cells like neutrophils can still undergo phagocytosis without the accompanying Ca^{2+} influx (figure 3.3.2.4).

Although it is known that phagocytosis is accompanied by a physiological Ca^{2+} influx, the sites at which influx occurs are unknown. C2 translocation thus provides a useful means of mapping out sites of Ca^{2+} influx during phagocytosis. The duration of the Ca^{2+} influx and the location of the start of the Ca^{2+} influx varied between cells. Some cells exhibited a fast, short calcium influx where the C2 domain translocated immediately to the whole plasma membrane and was quickly released (figure 3.3.2.1), whereas other cells displayed a longer Ca^{2+} influx (figure 3.3.2.2). In this example C2 can be seen to initially translocate to the plasma membrane of the phagocytic cup in the first few seconds after particle binding, and before the global calcium signal (figure 3.3.2.3). Subsequently, the cytosolic calcium concentration increases and the C2 domain translocates to the plasma membrane throughout the cell (figure 3.3.2.2). These results suggest that although it is possible that Ca^{2+} influx channels may be opened initially at the particle contact site they are also opened at sites remote from the initial particle contact site.

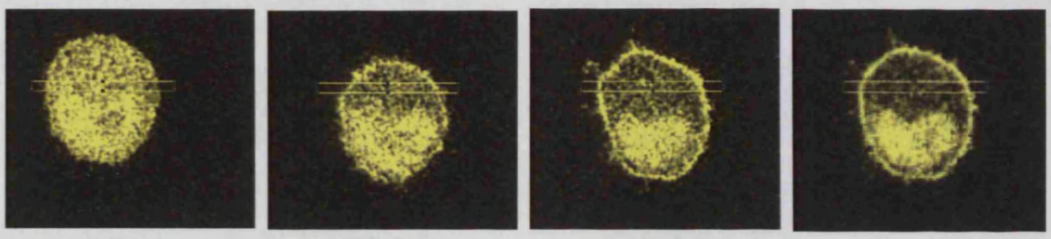
3.3.3 C2 domain translocation in apoptotic RAW cells

During apoptosis around 50% of the phosphatidyl serine on the inner leaflet of the plasma membrane which C2 domains bind to during calcium influx is externalised to the outer leaflet of the plasma membrane. Cells with externalised phosphatidyl serine were identified by staining positive with AnnexinV-FITC (figure 3.3.3.1). C2 domain proteins in cells with externalised phosphatidyl serine did not translocate as much as C2 domain proteins in normal cells when cytosolic calcium was increased using pharmacological stimuli as described in section 3.2.2. Translocation of YFP tagged C2 domain was significantly decreased in PS externalised cells (figures 3.3.3.2 and 3.3.3.3). There is no significant difference in C2 domain distribution between AnV+ve cells and AnV-ve cells before the addition of the pharmacological stimulus (P=0.9 at 3.5s) but once the stimulus has been added and the C2 domain has translocated C2 domain distribution is significantly different in AnV+ve and AnV-ve cells (P=0.02 at t=39s)(figure 3.3.3.2).

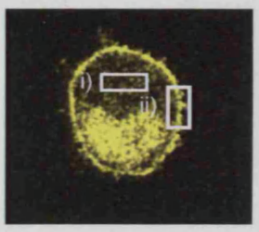
3.3.4 Location of calpain in apoptotic neutrophils with externalised phosphatidyl serine

Neutrophil adhesion and spreading has previously been shown to involve an increase in the cytosolic calcium concentration. Healthy human neutrophils fixed and stained for calpain after spreading on glass, show enriched calpain at the plasma membrane (figure 3.3.4.1). Stimulation of healthy (Annexin V negative), human neutrophils with Ca^{2+} elevating reagents (ionomycin and thapsigargin) also triggers and enrichment of calpain at the plasma membrane (figure 3.3.4.2). However, apoptotic neutrophils which have externalised phosphatidyl serine (Annexin V positive), fail to accumulate calpain at the plasma membrane when the cytosolic calcium concentration is increased, as illustrated by the difference between cytoplasmic fluorescence intensity and membrane fluorescence intensity (figure 3.3.4.2).

a)



b)



c)

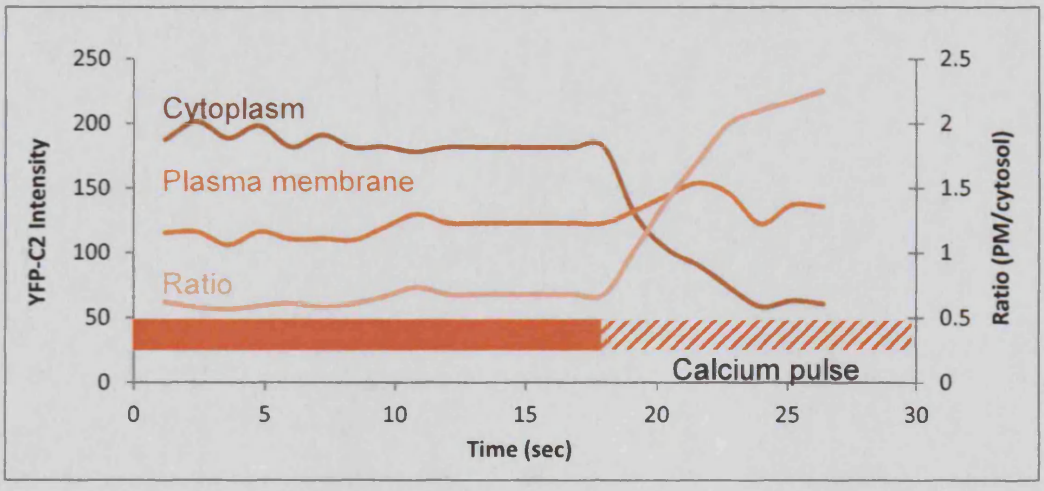
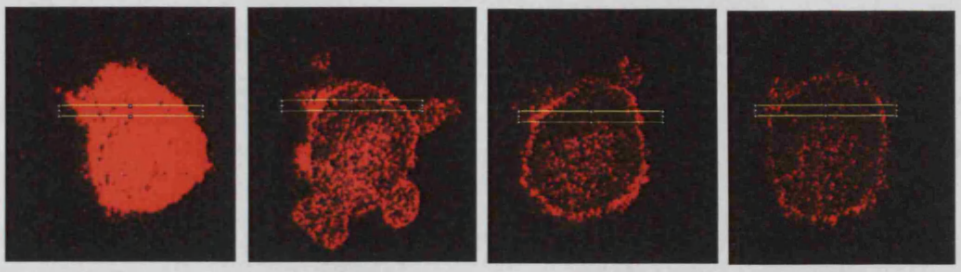


Figure 3.3.1.1: Typical C2γYFP translocation in 3T3 cells in response to pharmacologically stimulated calcium influx *(n=7)*. A typical experiment where 3T3 cells expressing C2γYFP were treated with 10μM ionomycin and 5μM thapsigargin in medium containing 13mM calcium and C2 domain translocation was observed. YFP fluorescence was imaged throughout the experiment. a) When the treatment is added to the cells to increase the cytosolic calcium the C2 domain translocates from the cytosol to the plasma membrane. b) Fluorescence intensity measurements were taken for the cytosol (i) and the plasma membrane (ii) for the entire time of the treatment and are displayed in c) along with the ratio of these intensities. The decrease in intensity of fluorescence in the cytosol is evident, as the plasma membrane increases in intensity as the C2 domain translocates.

a)



b)

c)

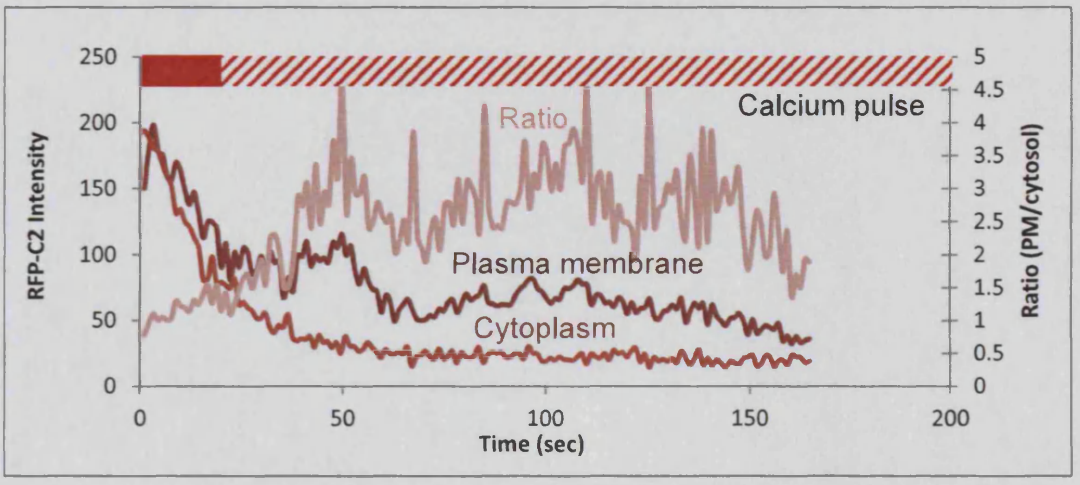
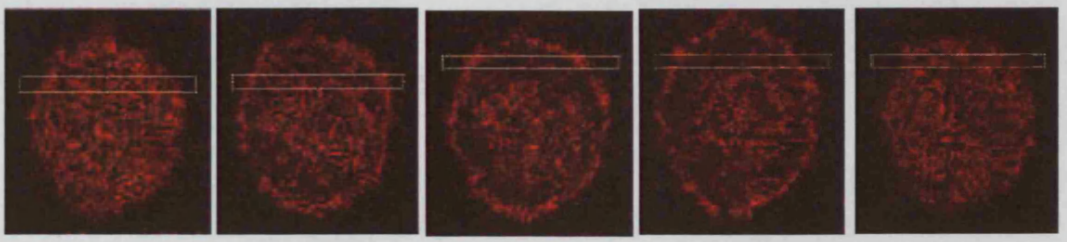


Figure 3.3.1.2: Typical C2βRFP translocation in PLB-985 cells in response to pharmacologically stimulated calcium influx *(n=2)*. A typical experiment in which PLB-985 cells expressing C2βRFP were treated with 10μM ionomycin and 5μM thapsigargin in medium containing 13mM calcium and C2 domain translocation was observed. RFP fluorescence was imaged throughout the experiment. a) When the treatment is added to the cells to increase the cytosolic calcium the C2 domain translocates from the cytosol to the plasma membrane. b) Fluorescence intensity measurements were taken for the cytosol (i) and the plasma membrane (ii) for the entire time of the treatment and are displayed in c) along with the ratio of these intensities. The decrease in intensity of fluorescence in the cytosol is evident, as the plasma membrane increases in intensity as the C2 domain translocates.

a)



b)

c)

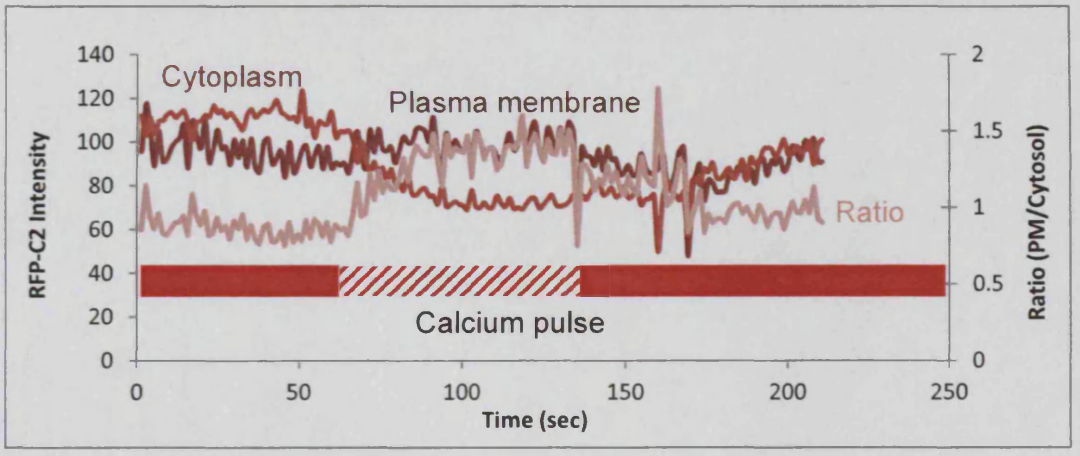
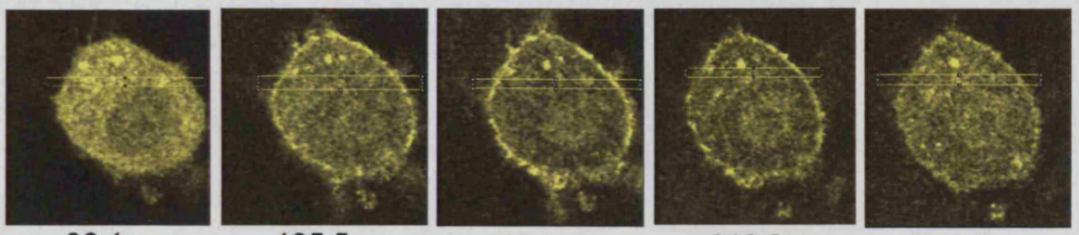
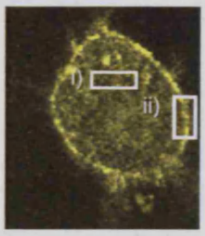


Figure 3.3.1.3: Typical C2βRFP translocation in RAW 264 cells in response to pharmacologically stimulated calcium influx *(n=2)*. A typical experiment, where RAW 264 cells expressing C2βRFP were treated with 10μM ionomycin and 5μM thapsigargin in medium containing 13mM calcium for 80 seconds to increase cytosolic calcium concentration before the medium was replaced with normal medium to decrease the cytosolic calcium to normal levels. RFP fluorescence was imaged throughout the experiment. a) When calcium is increased in the RAW cell expressing the C2 domain it translocates from the cytosol to the plasma membrane, the C2 domain is then released from the plasma membrane after the stimulus is removed and replaced with normal medium as the cytosolic calcium returns to resting levels. b) Fluorescence intensity measurements were taken for the cytosol (i) and the plasma membrane (ii) for the entire time of the treatment and are displayed in c) along with the ratio of these intensities. The decrease in intensity of fluorescence in the cytosol is evident, as the plasma membrane increases in intensity as the C2 domain translocates with the reverse being apparent when the pharmacological stimulus is removed and cytosolic calcium decreases.

a)



b)



c)

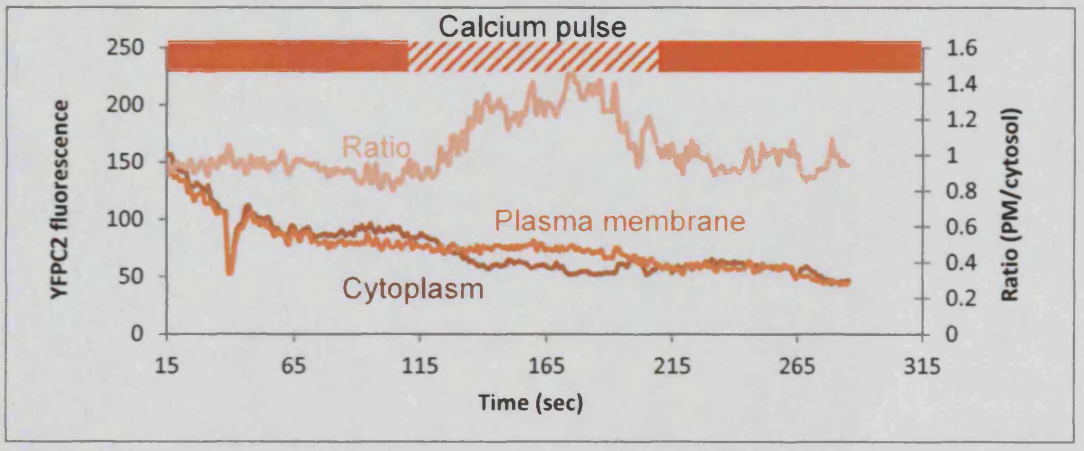
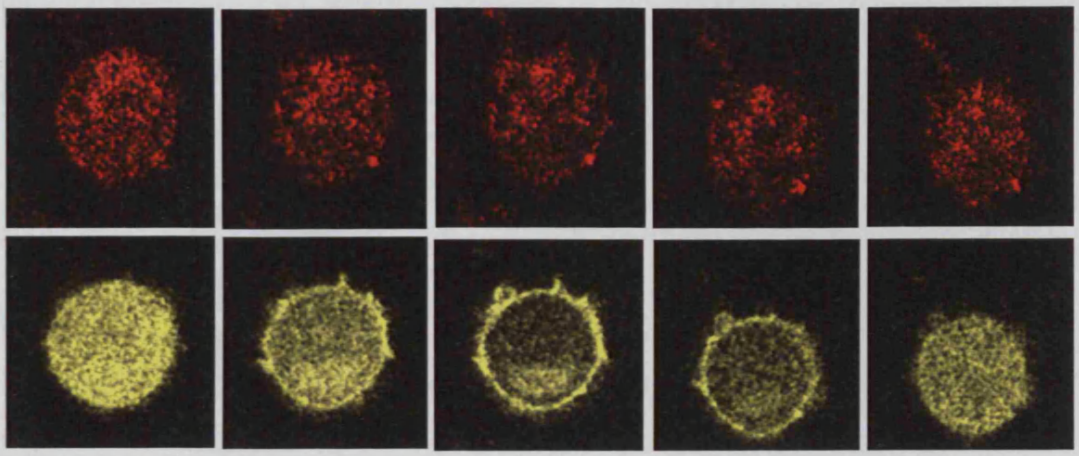
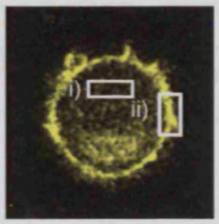


Figure 3.3.1.4: Typical C2γYFP translocation in RAW 264 cells in response to pharmacologically stimulated calcium influx *(n=3)*. A typical experiment, where RAW 264 cells expressing C2γYFP were treated with 10μM ionomycin and 5μM thapsigargin in medium containing 13mM calcium for 80 seconds to increase cytosolic calcium concentration before the medium was replaced with normal medium to decrease the cytosolic calcium to normal levels. YFP fluorescence was imaged throughout the experiment. a) When calcium is increased in the RAW cell expressing the C2 domain it translocates from the cytosol to the plasma membrane, the C2 domain is then released from the plasma membrane after the stimulus is removed and replaced with normal medium as the cytosolic calcium returns to resting levels. b) Fluorescence intensity measurements were taken for the cytosol (i) and the plasma membrane (ii) for the entire time of the treatment and are displayed in c) along with the ratio of these intensities. The decrease in intensity of fluorescence in the cytosol is evident, as the plasma membrane increases in intensity as the C2 domain translocates with the reverse being apparent when the pharmacological stimulus is removed and cytosolic calcium decreases.

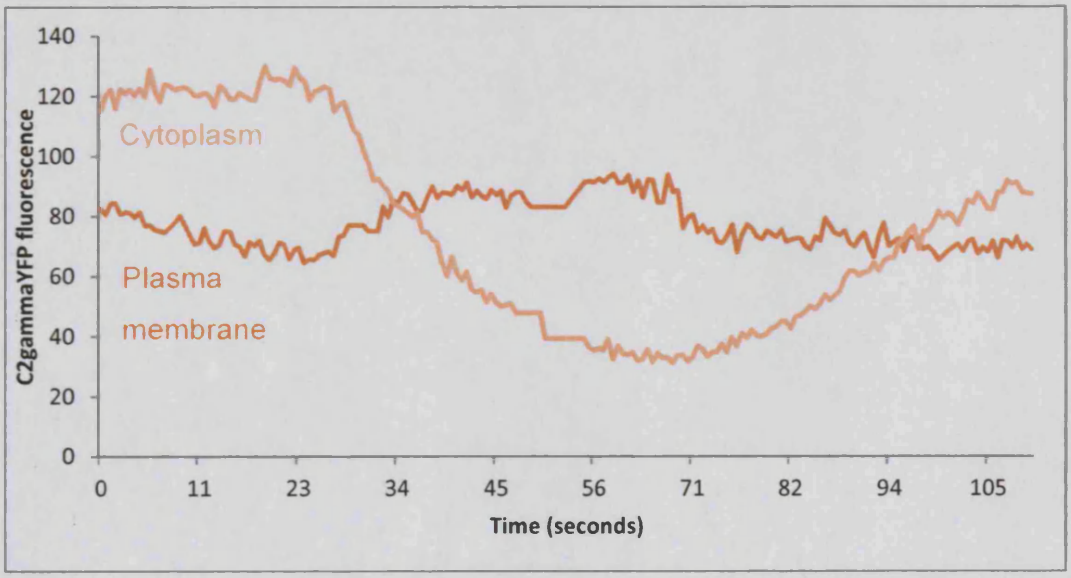
a)



b)


i)
ii)

c)


140
120
100
80
60
40
20
0
C2gammaYFP fluorescence
Cytoplasm
Plasma
membrane
0
11
23
34
45
56
71
82
94
105
Time (seconds)

d)

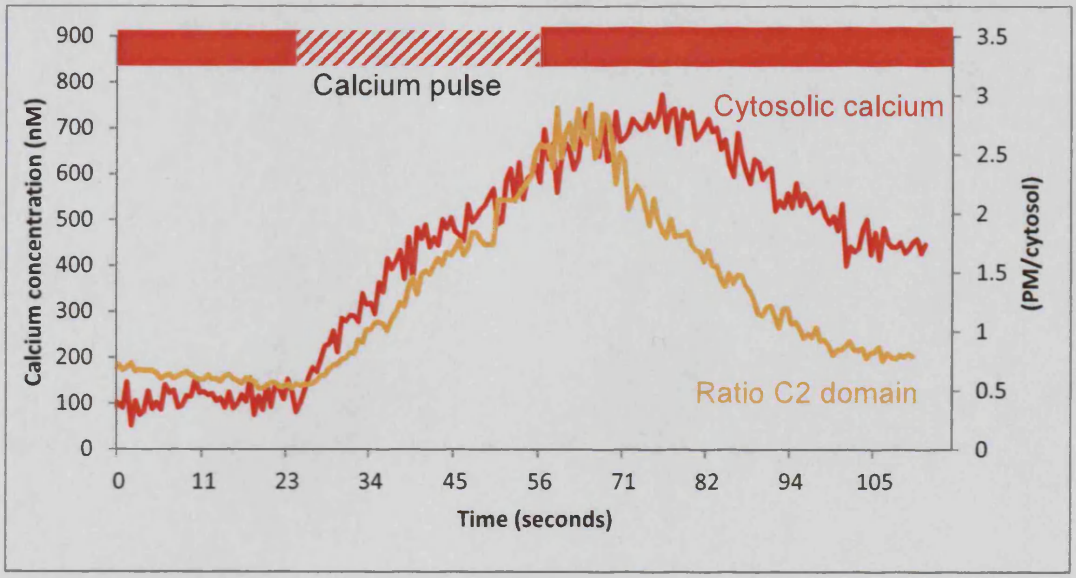
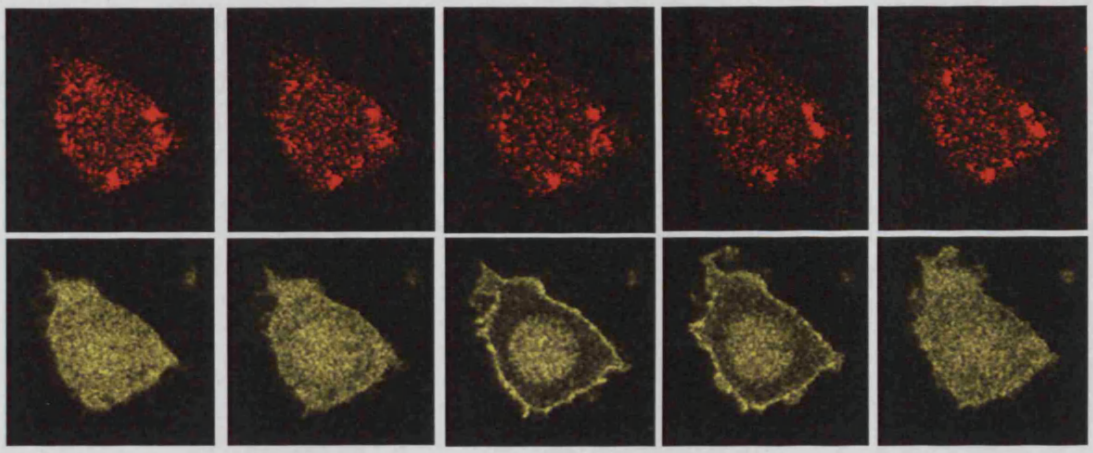
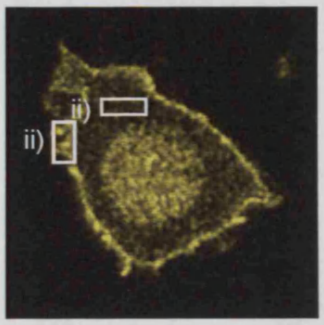


Figure 3.3.1.5: Typical C2 domain translocation in RAW 264 cells in response to pharmacologically stimulated calcium influx accompanied by cytosolic calcium measurment *(n=6)*. Typical experiment, in which RAW 264 cells expressing C2γYFP were loaded with fura red Ca^{2+} indicator before pharmacological stimulus was added. YFP and fura red fluorescence were imaged simultaneously throughout the experiment. Cells were treated with 25μM thapsigargin and 10μM ionomycin in medium containing 13mM calcium for 30 seconds to increase the cytosolic calcium concentration before the medium was replaced with normal medium to decrease the cytosolic calcium to normal levels. a) Treatment of RAW cells expressing C2 domain proteins with ionomycin and thapsigargin increased cytosolic calcium concentration; this was accompanied by translocation of the C2 domain to the plasma membrane. Removal of the stimulus sees cytosolica calcium concentration return to resting levels and the C2 domain is released from the plasma membrane back into the cytosol. b) Fluorescence intensity measurements were taken for the cytosol (i) and the plasma membrane (ii) for the entire time of the treatment and are displayed in c) The decrease in intensity of fluorescence in the cytosol is evident, as the plasma membrane increases in intensity as the C2 domain translocates with the reverse being apparent when the pharmacological stimulus is removed and the C2 domain is released back into the plasma membrane. Fura red fluorescence intensity was also imaged at the same time as the YFP fluorescence intensity d) Shows the quantification of cytosolic calcium concentration from the fura red fluorescence intensity along with the ratio of the YFP PM and cytosol fluorescence for the entire series. The C2 domain translocates as soon as the cytosolic calcium concentration increases and is released from the plasma membrane as soon as the stimulus is removed but the cytosolic calcium concentration is still high.

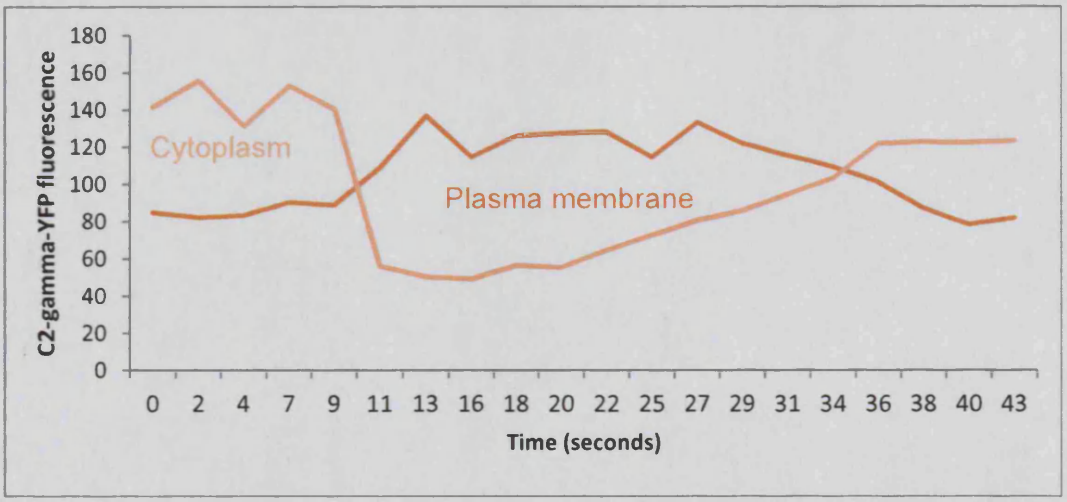
a)


9.0s
11.2s
13.5s
27.0s
38.2s

b)


ii)
ii)

c)


C2-gamma-YFP fluorescence
180
160
140
120
100
80
60
40
20
0
Cytoplasm
Plasma membrane
0 2 4 7 9 11 13 16 18 20 22 25 27 29 31 34 36 38 40 43
Time (seconds)

d) e)

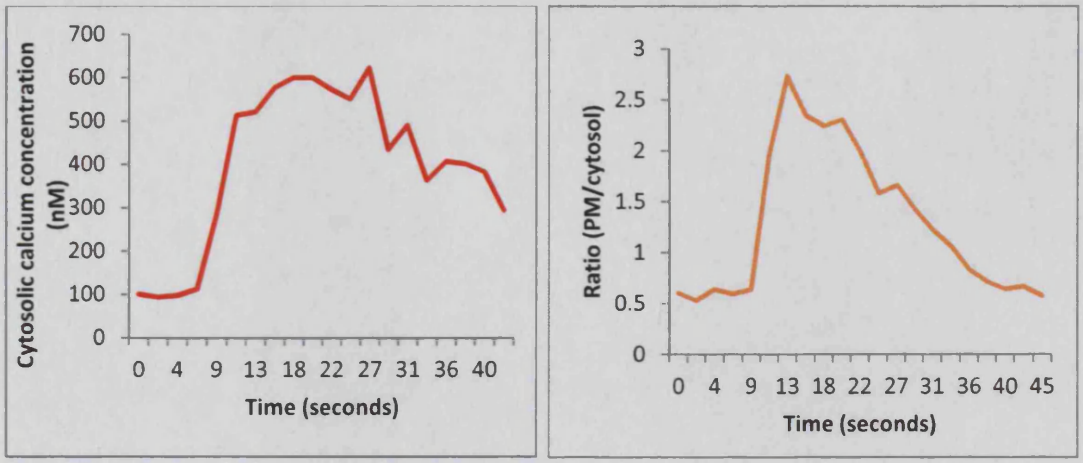
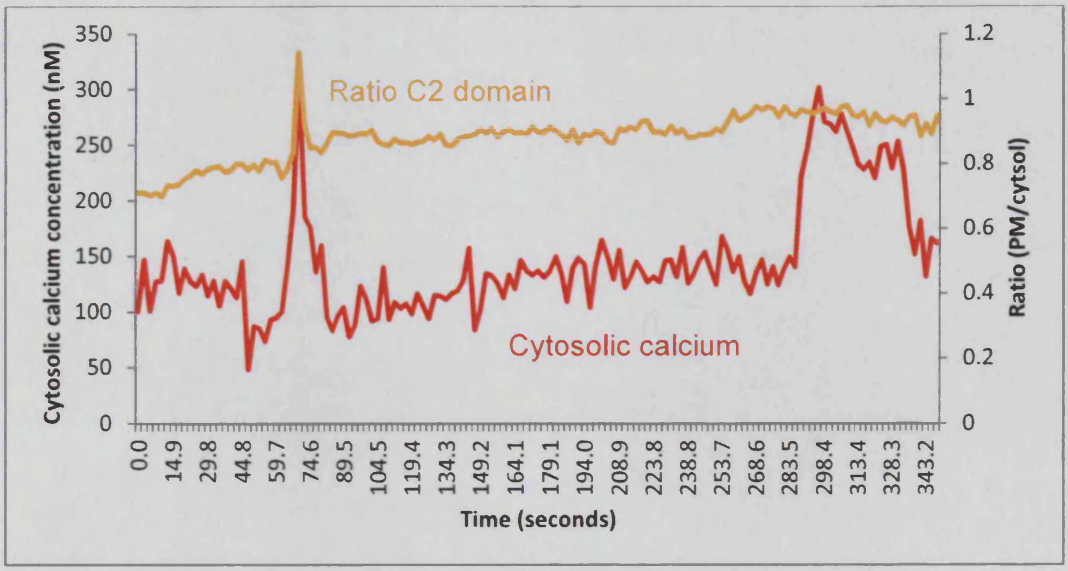
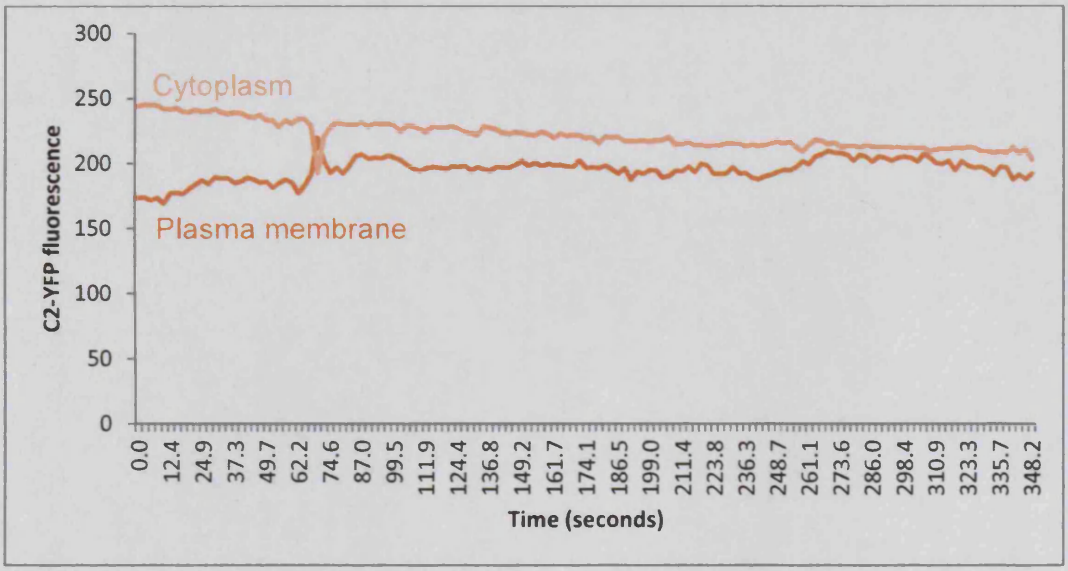


Figure 3.3.1.6: Typical C2 domain translocation in RAW 264 cells in response to physiologically stimulated calcium influx *(n=3)*. a) A typical experiment in which, RAW 264.7 cells expressing C2γYFP were loaded with the fura red Ca^{2+} indicator and a cytosolic calcium influx was stimulated with mouse serum. Fura red fluorescence and YFP were imaged simultaneously throughout the experiment and mouse serum was added at t=5s. a) The upper row (red) shows a transient decrease in fura red fluorescence intensity, corresponding to a transient increase in cytosolic Ca^{2+} (quantified in (d)) The bottom row (yellow) shows a corresponding translocation of C2γYFP from cytosol to plasma membrane, which is then released from the plasma membrane as the cytosolic calcium returns to resting levels. b) C2γYFP fluorescence intensity measurements were taken for the cytosol (i) and the plasma membrane (ii) for the entire time series and are displayed in (c). c) The decrease in intensity of fluorescence in the cytosol is evident, as the plasma membrane increases in intensity as the C2 domain translocates with the reverse being apparent when the C2 domain is released back into the cytosol. d) Fura red fluorescence intensity was also imaged at the same time as the YFP fluorescence intensity. This was used to calculate to cytosolic calciu concentration shown in the graph e) The ratio of C2YFP fluorescence at the plasma membrane and the cytosol showing C2 domain translocation. When graphs (d) and (e) are compared it can be seen that the C2 domain is released from the plasma membrane back into the cytosol whilst the bulk cytosolic calcium concentration is still high.

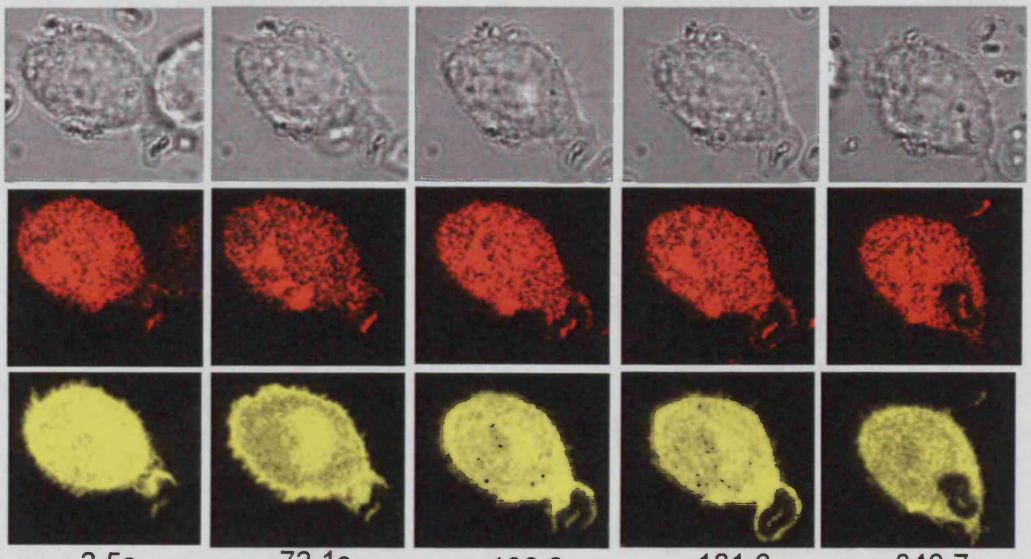
a)


Cytosolic calcium concentration (nM)
Ratio (PM/cytsol)
Ratio C2 domain
Cytosolic calcium
Time (seconds)
0
50
100
150
200
250
300
350
0.2
0.4
0.6
0.8
1
1.2
0.0
14.9
29.8
44.8
59.7
74.6
89.5
104.5
119.4
134.3
149.2
164.1
179.1
194.0
208.9
223.8
238.8
253.7
268.6
283.5
298.4
313.4
328.3
343.2

b)


C2-YFP fluorescence
Cytoplasm
Plasma membrane
Time (seconds)
0
50
100
150
200
250
300
0.0
12.4
24.9
37.3
49.7
62.2
74.6
87.0
99.5
111.9
124.4
136.8
149.2
161.7
174.1
186.5
199.0
211.4
223.8
236.3
248.7
261.1
273.6
286.0
298.4
310.9
323.3
335.7
348.2

c)



d)

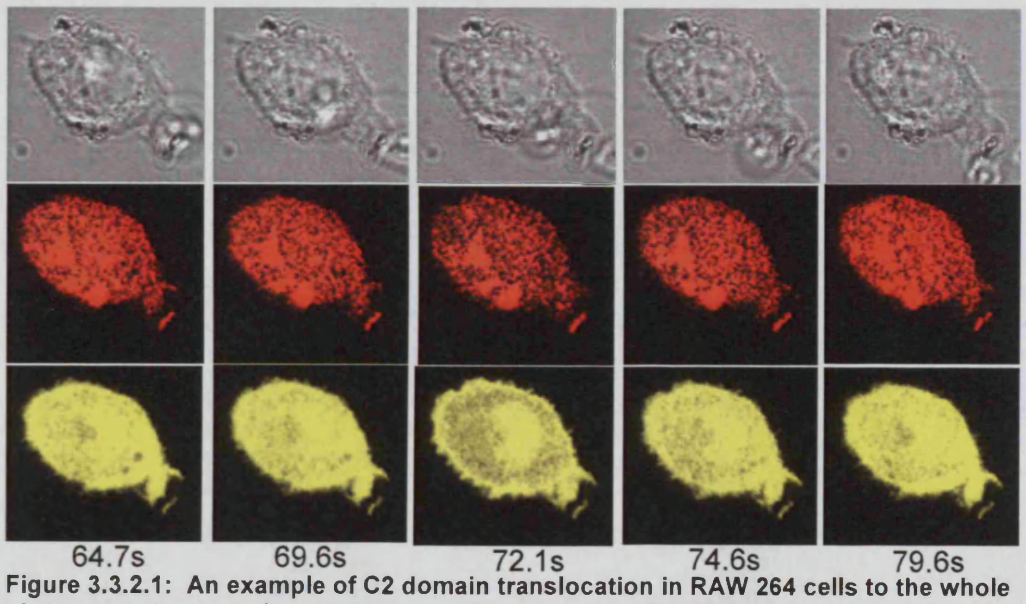
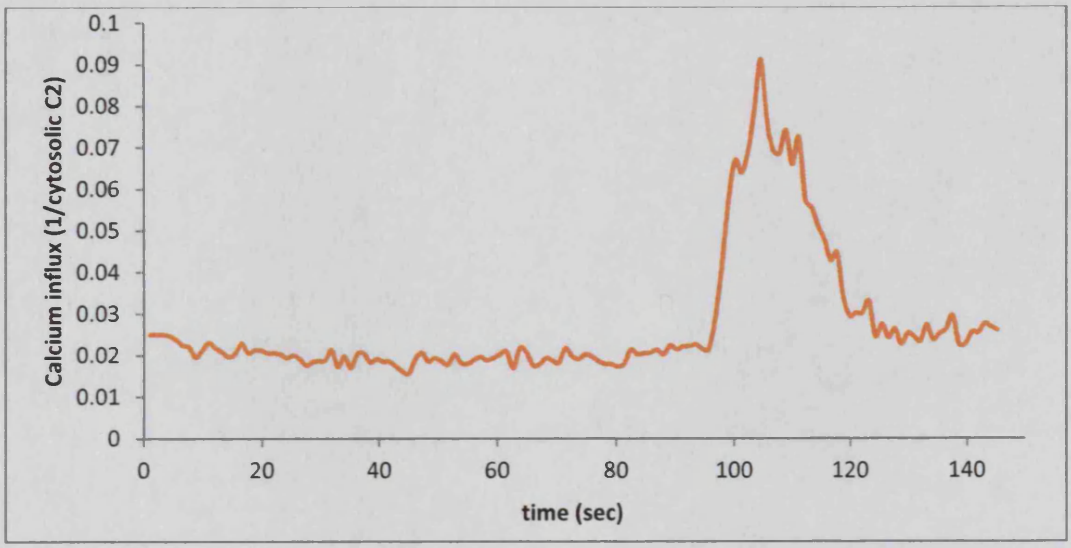
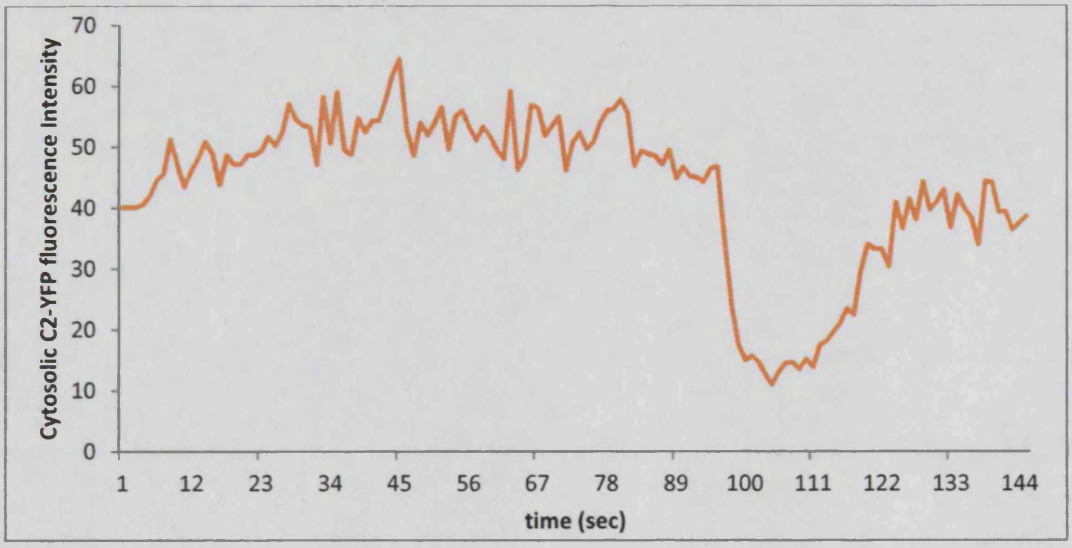


Figure 3.3.2.1: An example of C2 domain translocation in RAW 264 cells to the whole plasma membrane before phagosome closure *(n=2)*. RAW 264 cells expressing C2γYFP and loaded with fura red calcium indicator were allowed to phagocytose mouse serum opsonised zymosan particles in order to induce a physiological calcium increase. Fura red and YFP were imaged simultaneously throughout the experiment. a) Cytosolic calcium concentration was calculated from the fura red fluorescence intensity (red line). There is a very fast cytosolic calcium signal (t=72.1s) accompanied by a brief translocation of the C2 domain from the cytosol to the plasma membrane (shown by the ratio of plasma membrane fluorescence and cytosolic fluorescence (yellow line)). b) Shows an increase in the fluorescence intensity at the plasma membrane and a decrease in fluorescence in the cytosol as the C2 domain translocates. c) Shows the entire process of phagocytosis, which completes over 100 seconds after the cytosolic calcium signal. d) Shows the C2 domain translocation and the accompanying decrease in fura red fluorescence at 72.1 seconds.

a)


Calcium influx (1/cytosolic C2)
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0
20
40
60
80
100
120
140
time (sec)

b)


Cytosolic C2-YFP fluorescence Intensity
70
60
50
40
30
20
10
0
1
12
23
34
45
56
67
78
89
100
111
122
133
144
time (sec)

c)


i)

d)

Figure 3.3.2.2: An example of C2 domain translocation before phagosome closure in RAW cells *(n=1)*. RAW 264 cells expressing C2γYFP were allowed to phagocytose mouse serum opsonised zymosan particles in order to induce a physiological calcium increase. YFP fluorescence was imaged throughout the experiment. a) The decrease in cytosolic calcium concentration observed when the C2 domain translocates was used as a measure of the rate of calcium influx. b) Shows the decrease in cytosolic YFP fluorescence as the C2 domain translocates to the plasma membrane. c) Fluorescence intensity of the YFP was measured in the cytosol throughout the experiment. d) Illustration of C2 domain translocation before closure of the phagocytic cup. There is a sharp increase in cytosolic calcium concentration after the cell has bound the particle which is illustrated by the translocation of the C2 domain (a,d). This influx lasts ~25 seconds and the C2 domain is released from the plasma membrane before the cell continues to phagocytose the particle. The fluorescence intensity of the cytosol decreases sharply at ~95s as the cell binds the particle and the C2 domain translocates to the plasma membrane indicating the start of calcium influx, within ~25s the fluorescence intensity in the cytosol has returned to starting levels indicating the end of calcium influx (b,d)

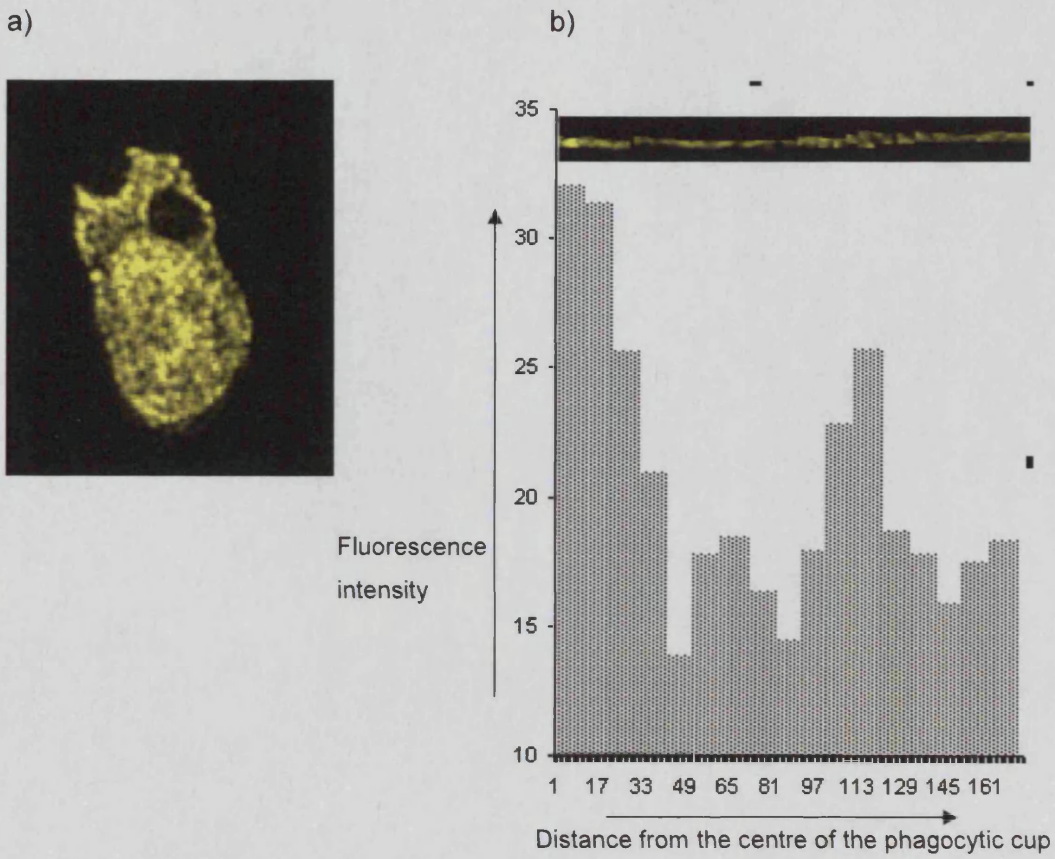


Figure 3.3.2.3: An example of C2 domain translocation to the plasma membrane around the phagosome in RAW 264 cells *(n=1)*. RAW cells expressing C2γYFP were allowed to phagocytose mouse serum opsonised zymosan particles in order to induce a physiological calcium increase. YFP fluorescence was imaged throughout the experiment. a) Image of the cell upon part. When the cell initially binds the particle the C2 domain can be seen to move initially to the plasma membrane around the phagocytic cup in the first few seconds after particle binding. b) An illustration of the fluorescence intensity of the plasma membrane if it were taken from the middle of the phagocytic cup around the outside if the cell to the middle of the opposite side of the cell. The units on the graph are arbitrary units, illustrating fluorescence intensity on the y axis and distance from the centre of the phagocytic cup on the x axis. This illustrates that the fluorescence intensity is high initially at the phagocytic cup and decreases gradually as you move away from the cup suggesting that the C2 domain is moving first to the phagocytic cup and then making its way around the rest of the plasma membrane.

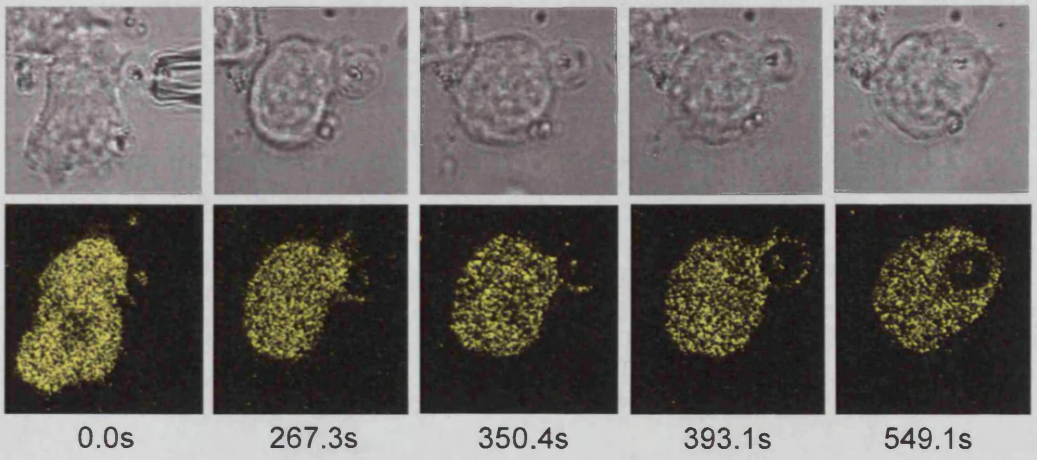


Figure 3.3.2.4: An example of phagocytosis in Raw 264 cells from particle binding to completion without an accompanying calcium influx *(n=3)*. The upper sequence shows phagocytosis of a serum opsonised particle presented to a RAW 264 cell expressing C2γYFP. The lower sequence shows the failure of the C2 domain to translocate in the absence of a Ca^{2+} signal during the process of phagocytosis.

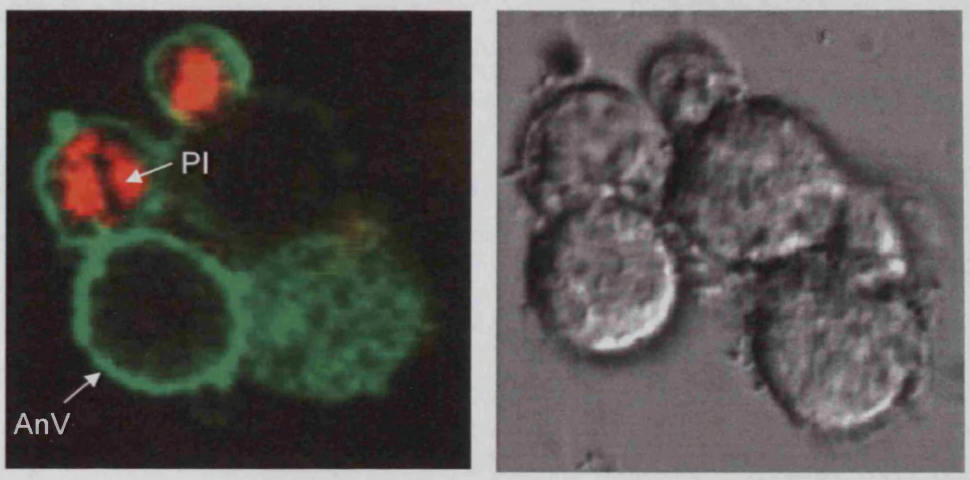


Figure 3.3.3.1: Example of how phosphatidyl serine externalised RAW 264 cells were identified by AnnexinV staining. PS externalised cells stain positive with AnnexinV at the cell membrane. Dead cells were identified byPropidium Iodide (PI) nuclear staining.

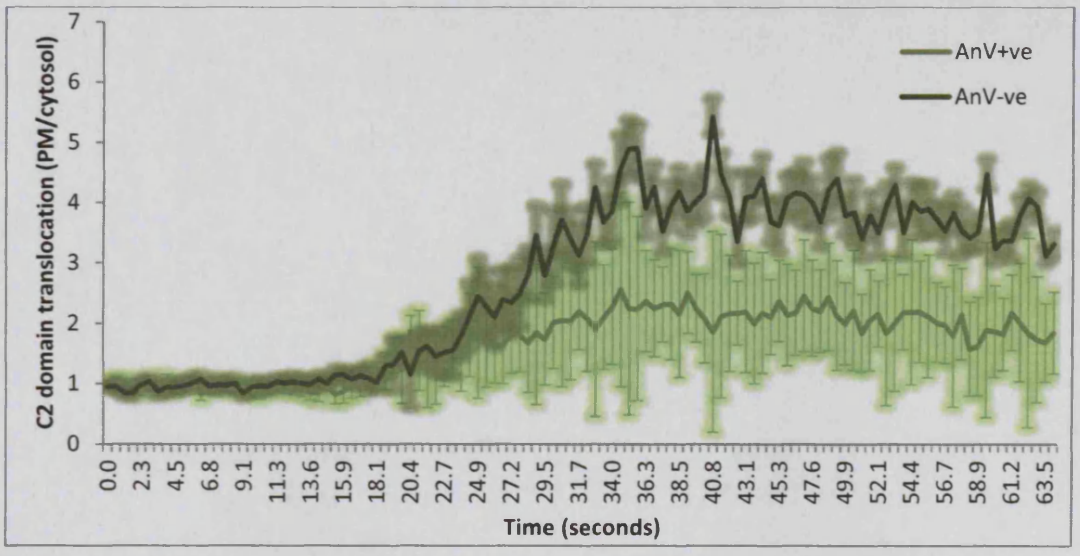


Figure3.3.3.2: C2 domain translocation in PS normal and PS externalised cells. C2 domain translocation was quantified as a ratio between the increase in plasma membrane fluorescence and the decrease in cytosolic fluorescence over time, this ratio is shown here. C2 domain translocation was reduced significantly in cells which displayed externalised phosphatidyl serine. Data shown represents the mean translocation from 9 separate experiments. The shaded area indicates the standard error of the mean.

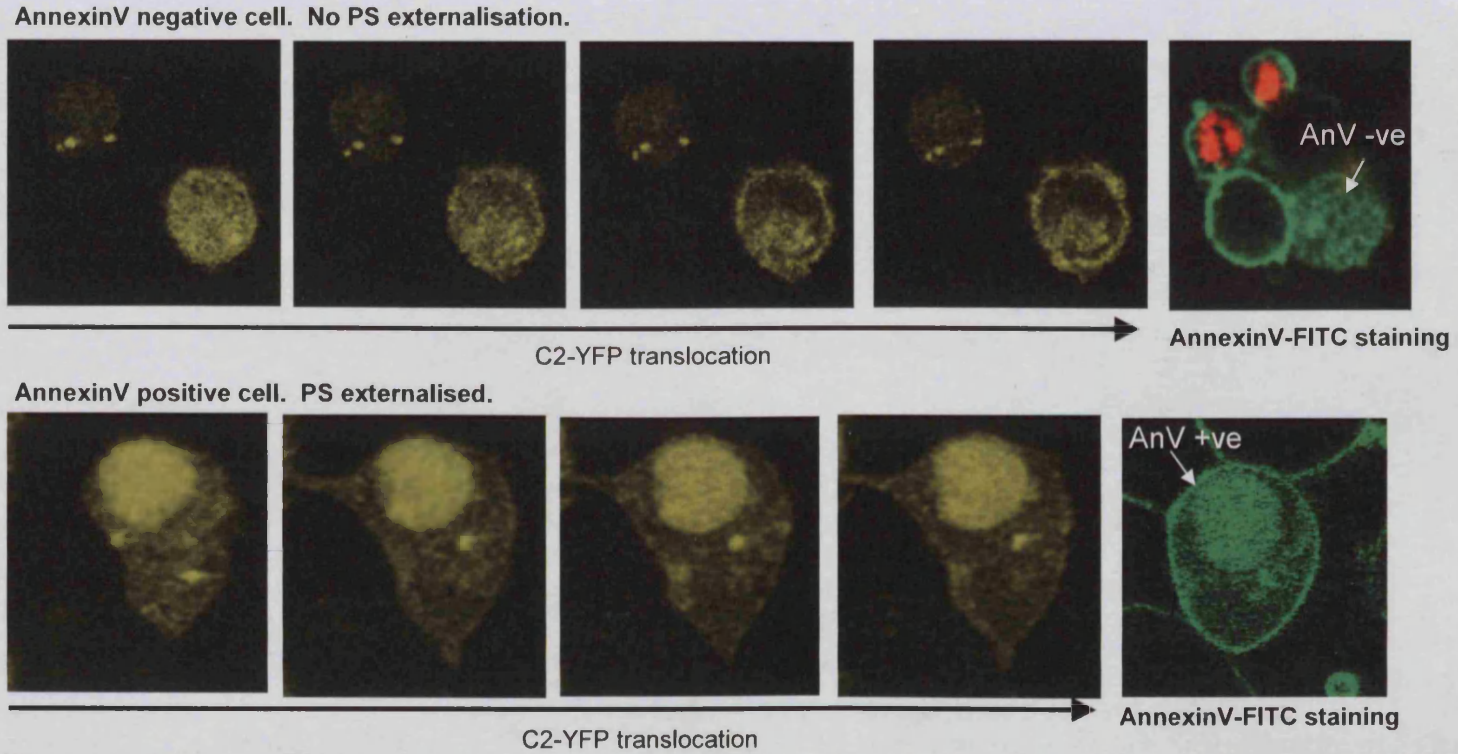


Figure 3.3.3.3: Typical C2 domain translocation in AnV negative RAW 264 cells with no Ps externalisation and in AnV positive RAW 264 cells with PS externalised. (AnV negative (n=5) and AnV positive (n=9)). Apoptosis was induced in RAW cells expressing C2YFP before 5μM ionomycin and 10μM thapsigargin was added to the cells to stimulate cytosolic calcium concentration increase, stimulus was then removed and replaced with fresh tissue culture medium to allow cytosolic calcium concentration to return to resting levels. YFP fluorescence was measured during the stimulation to monitor C2 domain translocation. After stimulation and removal of the stimulus AnnexinV was added to the cells and FITC fluorescence was monitored in order to identify which cells stained positive for AnnexinV at the membrane and where therefore AnnexinV positive apoptotic cells. Propidium Iodide was then added to the cells to identify any necrotic cells. TRITC fluorescence and FITC fluorescence were measured simultaneously for this part of the experiment. The top panel shows C2 domain translocation in an AnnexinV negative cell, followed by a still image of the AnnexinV and Propidum Iodide staining showing that the cell in which the C2 domain translocated was neither apoptotic nor nectrocic. One other cell in the field of view was apoptotic (although this cell was not expressing YFP) and that two of the cells in the field of view were necrotic (one of which was expressing YFP and the other was not). The bottom panel shows decreased C2 domaint translocation in an AnnexinV positive cell, followed by a still image of AnnexinV and Proidium Iodide staining showing that all cells in the field of view were indeed apoptotic and none where necrotic.

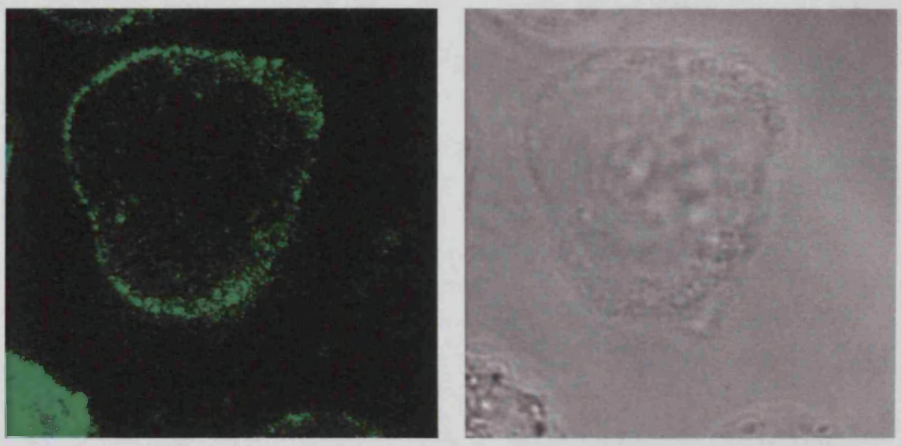


Figure 3.3.4.1: Location of calpain in spread human neutrophils. Healthy human neutrophil fixed and stained for calpain location after being allowed to spread onto a glass coverslips. Calpain staining is localised to the plasma membrane.

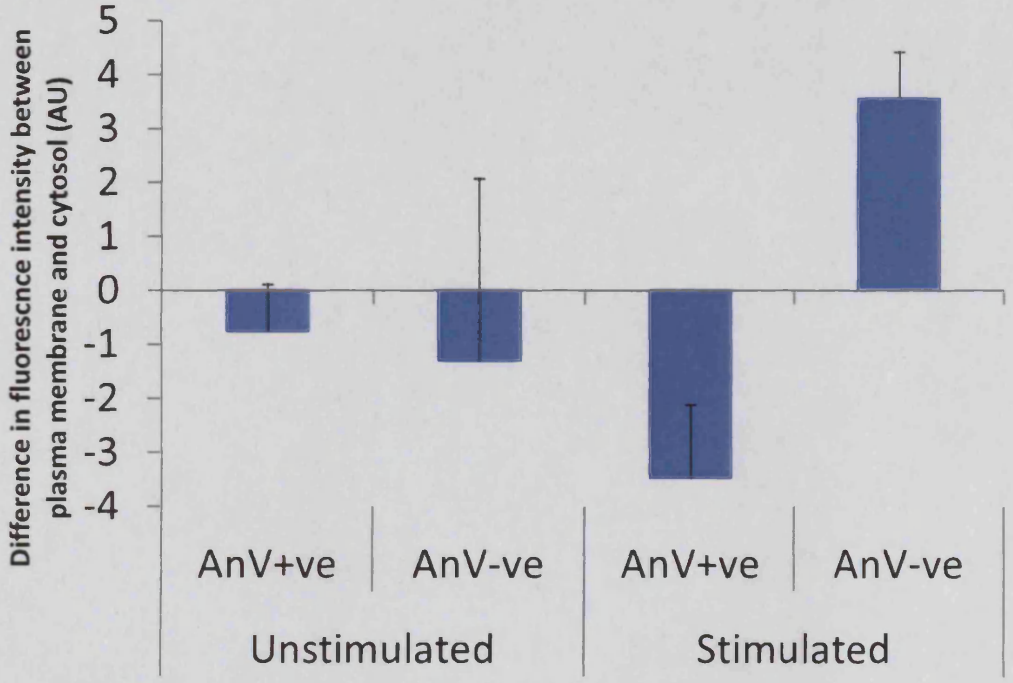


Figure 3.3.4.2: Difference in fluorescence intensity between plasma membrane and cytosol in apoptotic human neutrophils fixed and stained for calpain. Cells *(n=30)* staining positively and negatively for Annexin V as an indicator of phosphatidyl serine externalisation were stimulated with 5μM ionomycin and 5μM thapsigargin to initiate Ca^{2+} influx and increase the cytosolic Ca^{2+} concentration. The difference in fluorescence between the plasma membrane and the cytosol was used as a confirmation of the membrane location of the calpain staining (measured in arbitrary units). Cells with a high difference between plasma membrane fluorescence and cytoplasmic fluorescence were deemed to have calpain located at the plasma membrane and cells with no difference between the plasma membrane fluorescence and the cytosolic fluorescence were deemed to have cytoplasmic calpain staining. Calpain was found to be located at the plasma membrane in AnnexinV negative cells and in the cytsol in AnnexinV positive cells.

3.4 Discussion

The data here show that classical C2 domains are able to translocate in response to both physiological and pharmacological stimuli. Provided that calcium influx is triggered (monitored by a rise in Ca^{2+}concentration) C2 domain translocation to the plasma membrane occurs. Monitoring C2 domain translocation provides an indication of when the calcium channels in the plasma membrane are open during calcium influx.

3.4.2 C2 domain translocation during phagocytosis

The data presented here illustrate that the calcium influx triggered by phagocytosis is sufficient to trigger C2 domain translocation. C2 domain translocation occurred before phagosome closure suggesting that calcium influx starts before closure of the phagosome. Not all of the cells displayed C2 domain translocation during phagocytosis suggesting that calcium influx did not occur in all cells. Previous reports have shown that calcium influx is not necessary for particle internalisation but is for bacterial killing once the particle has been internalised (Dewitt et al., 2003, Jaconi et al., 1990). It is therefore not unusual that not all of the cells in this study displayed calcium influx during phagocytosis. The data presented in this chapter show some suggestion that C2 domain proteins translocate first to the membrane around the phagosome before the rest of the plasma membrane. This suggests that the initial source of calcium influx is from the calcium channels in the membrane around the phagosome. Previous studies have shown that after phagosome closure there is a decrease in calcium concentration within the phagosome suggesting that calcium is entering the cell from the phagosome through calcium channels in the phagosomal membrane (Lundqvist-Gustafsson et al., 2000). Local increase in cytosolic calcium concentrations around the phagosome have also been reported (Jaconi et al., 1990). The C2 domain used in these experiments has a very high calcium affinity (0.7μM) (Kohout et al., 2002) and as such will translocate readily to the membrane when calcium influx is occurring. A

comparison of the translocation of this C2 domain during phagocytosis with another C2 domain with a lower calcium affinity would give a more definitive answer as to where in the plasma membrane calcium influx was occurring first and how long the calcium channels remained open.

3.4.3 Activity of C2 domains in apoptotic cells

The data presented in this chapter show that in apoptotic cells C2 domain translocation is diminished but not completely abolished in apoptotic neutrophils. During the process of apoptosis ~50% of the total phosphatidyl serine is externalised from the inner leaflet to the outer leaflet of the plasma membrane (Martin et al., 1995). Despite this, upon pharmacologically stimulated cytosolic calcium influx, YFP tagged C2 domain is still seen to translocate to the plasma membrane. However, unlike in normal healthy cells where all of the YFP-C2 domain translocates from the cytoplasm to the plasma membrane, only a small percentage of the total C2 domain in the cell translocates in the phosphatidyl serine externalised cells. This is most likely due to there simply being less phosphatidyl serine on the inner leaflet of the plasma membrane for the C2 domain to bind and as such the rate of translocation remains the same.

3.4.4 Location of calpain in apoptotic neutrophils

The data presented here show that despite translocation of classical C2 domains being diminished but not completely abolished in apoptotic cells μ-calpain (which contains a 'C2 like' domain) is completely absent from the membrane in apoptotic neutrophils. This suggests that calpains 'C2 like' domain does not act in the same manner as a typical C2 domain. Phosphatidyl serine binding and calcium ion binding by C2 domain proteins are intrinsically linked. Phosphatidyl serine binding cannot occur without calcium ion binding (Nalefski and Falke, 1996). As the Kd of phospholipid affinity of μ-calpains 'C2

like domain' and its $[Ca^{2+}]_{1/2}$ is unknown this data suggests that the $[Ca^{2+}]_{1/2}$ of μ-calpains 'C2 like domain' and consequently its Kd of phospholipid affinity must be higher than that of classical C2 domains as it does not bind phosphatidyl serine as readily as classical C2 domains when the phosphatidyl serine is decreased, suggesting that its affinity for calcium and therefore phosphatidyl serine must be lower than that of classical C2 domain proteins because, as previously stated, the affinity of a C2 domain protein for calcium dictates its phosphatidyl serine binding affinity. The $[Ca^{2+}]_{1/2}$ of the C2γ-YFP used in these experiments is 0.7μM and the Kd of its phospholipid affinity is 3.1μM (Kohout et al., 2002). Therefore the $[Ca^{2+}]_{1/2}$ of μ-calpains 'C2 like' domain must be higher than 0.7μM. This is an important point as the calcium concentration required for the activation of μ-calpain is also relatively high, around 30μM (Michetti et al., 1997), as discussed in section 1.7.3. If the $[Ca^{2+}]_{1/2}$ of calpains 'C2 like' domain is higher than 0.7μM it would require higher calcium concentrations to bind phosphatidyl serine and translocate from the cytoplasm to the plasma membrane than that required by classical C2 domain proteins such as that from PKCγ. As a result μ-calpain would only be found at the plasma membrane where calcium concentration is at its highest, this is postulated to be within the neutrophil membrane wrinkles (Brasen et al., 2010). If this were the case then μ-calpain would be very specifically located and activated to cleave its substrates, talin and ezrin (Ivetic et al., 2002, Shcherbina et al., 1999), within the membrane wrinkles. As these calpain substrates act to bind the plasma membrane to the actin cytoskeleton to hold the membrane wrinkles in place (Ivetic et al., 2002, Sampath et al., 1998) then the cleavage of these calpain substrates would allow for unwrinkling of the neutrophil membrane and therefore allow the neutrophil to spread.

3.4.4 General discussion

This chapter has established the usefulness of C2 domain translocation as a marker of calcium influx as well as a model of the activity of other putative C2 domain containing proteins such as calpain.

The results presented here have pointed to the possibility that during phagocytosis, calcium influx occurs first via calcium channels around the phagosome followed by influx through calcium channels in the rest of the plasma membrane. In order to further confirm this it would be useful in the future to compare the translocation of different C2 domain proteins with different calcium affinities in order to establish at what part of the plasma membrane calcium concentration was the highest and therefore give a better idea of where in the plasma membrane the calcium influx was occurring.

It has also been illustrated in this chapter that C2 domain proteins with a high enough calcium affinity can still translocate to the plasma membrane upon cytosolic calcium increase in apoptotic cells in which half of the total phosphatidyl serine has been externalised. However calpain is not found to be located at the plasma membrane in apoptotic cells suggesting that the 'C2 like' domain in calpain has a lower calcium affinity than the classical C2 domain used in these experiments. In the future it would be interesting to look at the translocation of other C2 domains with lower calcium affinities in apoptotic cells to see if a lower calcium affinity decreases the translocation when phosphatidyl serine is decreased as has been suggested is the case for calpain.

CHAPTER 4

Translocation of calpain in human neutrophils and RAW-264 cells

4.1 Introduction

In the previous chapter, it was shown that C2 domain proteins move to the plasma membrane during pharmacological and physiological stimulation, including phagocytosis. Since calpain-1 includes a C2 like domain, it is possible that calpain-1 also translocates. In this chapter evidence relating to this will be presented both in primary human neutrophils and in the mouse model cell. Firstly, however, it is necessary to distinguish the roles of elevated Ca^{2+} in calpain translocation and in its activation (as a protease).

4.1.1 Cytosolic calcium as a trigger for calpain translocation

There is some amino acid sequence homology between domain III from calpain 1 and classic C2 domains from protein kinase C gamma and beta, although they are not highly similar, suggesting that calpains 'C2 like' domain is not the same as a classical C2 domain. It has been shown that calpain is found at the plasma membrane after cytosolic calcium has been increased (Gil-Parrado et al., 2003) and that without phosphatidyl serine it is not possible for calpain to bind the plasma membrane. It is therefore likely that an increase in cytosolic calcium concentration is a trigger for calpain to move from the cytoplasm to the plasma membrane.

4.1.2 Cytosolic calcium as a trigger for calpain activation

The exact mechanism of calpain activation is not yet fully understood. Potential mechanisms for calpain activation including the much debated role of autolysis and calpain activator protein are discussed in detail in section 1.7.3. It is known that calpain 1 requires exceptionally high calcium concentrations of around 30μM in order to be activated (Michetti et al., 1997). This is much higher than the physiological calcium concentrations found in the cytosol in a resting cell and even higher than the calcium concentrations found in the bulk cytosol during calcium influx. However calcium concentrations high enough to activate

calpain 1 have been found near the plasma membrane in the neutrophil membrane wrinkles (Brasen et al., 2010, Davies and Hallett, 1998). This would mean that it is possible for calpain 1 to encounter calcium concentrations high enough to be activated in neutrophils.

4.1.3 Potential role of calpain translocation in neutrophil spreading

The calpain substrates ERM proteins talin and ezrin are proteins that are associated with the actin cytoskeleton and are found at the plasma membrane in resting cells (Ivetic et al., 2002, Shcherbina et al., 1999). It has been proposed that these proteins hold the wrinkles on the neutrophil membrane in place and that release of these wrinkles is essential for neutrophil spreading (Hallett and Dewitt, 2007). If calpain were able to translocate from the cytosol where it is found in resting cells to the plasma membrane it would be in the right location to be able to cleave these proteins and trigger neutrophil spreading. Another reason that it is necessary for calpain to translocate to the plasma membrane is that it is here only that it may encounter calcium concentrations high enough to trigger its activation (Brasen et al., 2010).

4.1.4 Aims of the chapter

The work presented in this chapter aims to extend the work presented in the previous chapter to the more difficult question of calpain in human neutrophils. The specific objectives of this work were:

- to establish the location of calpain in neutrophils during spreading and phagocytosis

- to establish whether calpain translocation occurs during cytosolic calcium influx

These aims will be achieved using immunofluorescent staining for calpain in primary human neutrophils and using physiological and pharmacological

techniques to increase cytosolic calcium concentration in RAW-264 cells which have been transfected with YFP tagged calpain 4 in order to visualise its location in the cell.

4.2 Materials and Methods

4.2.2 Immunofluorescent staining for calpain in human neutrophils.

4.2.2a Neutrophils spread on glass

Neutrophils were isolated from human blood as described in section 2.4.1. 100µl of the total cells isolated were placed in a glass bottom dish and incubated at 37°C for 15 minutes to allow the cells to adhere to the glass. Cells were washed 2 times in Krebs-BSA before being fixed in 100ul 3.7% formaldehyde for 10 minutes at room temperature. Cells were washed 3 times in PBS and then permeabilised in 0.1% triton for 4 minutes after which they were washed a further 2 times in PBS. Non specific binding sites were blocked by incubating the cells in 4% horse serum for 1 hour before the addition of the primary antibody. Primary antibody was monoclonal anti-calpain 1 (6C-12) (Santa Cruz – sc32327). Primary antibody was diluted 1 in 100 (in 4% horse serum) before use and cells were incubated in primary antibody overnight at 4°C. The following morning cells were washed thoroughly 3 times in PBS before secondary antibody was added. Secondary antibody was goat anti-mouse IgG FITC (Santa Cruz santa cruz – sc-2010). Cells were incubated in secondary antibody for 1 hour at room temperature in the dark. Secondary antibody was removed by washing thoroughly twice with PBS. Microscopic visualisation of the cells, once stained, was carried out using the CLSM.

4.2.2b Neutrophil phagocytosis

Neutrophils were isolated from human blood as described in section 2.4.1. 100µl of the total cells isolated were incubated for 30 minutes in 0.5mg/ml serum opsonised zymosan particles before being allowed to adhere to glass coverslips to enable fixation and viewing. Cells were then fixed and stained as described in the previous section.

4.2.3 Localised Calpain-YFP Photo bleaching

RAW-264 cells were transiently transfected with calpain4-YFP as described in section 3.2.1b. Cells were analysed using confocal microscopy. Localised photo bleaching was achieved using the restricted area laser scanning mode ("zoom") so that the area to be photo bleached could also be visualised. The laser power was increased during the localised scanning and the effect on local YFP fluorescence monitored as bleaching in that area proceeded. Once significant bleaching had been achieved, the full image acquisition was restored at normal laser power, and extent the calpain YFP bleaching outside the exposed area established. This procedure (FLAP - fluorescence loss after photo bleaching) gives an indication of the mobility of the fluor. A time course of frames was then taken at a rate of 1 frame every 2 seconds to establish the extent of fluorescence recovery (FRAP) as fluorescent molecules diffuse into the exposed zone to replace those which were bleached.

4.2.4 Calcium elevating experiments in RAW-264 cells transiently expressing calpain4-YFP

RAW-264 cells were transiently transfected with calpain4-YFP as described in section 3.2.1b. Pharmacologically stimulated cytosolic calcium increase was achieved by treating the cells with a cocktail containing 10μM ionophore, 5μM thapsigargin 13mM $CaCl_2$ as described in section 2.9. Physiologically stimulated cytosolic calcium influx was achieved by treating the cells with mouse serum. Cytosolic calcium concentration was monitored using fura red calcium indicator as described in section 2.8. All experiments were imaged using a Leica SP2 confocal microscope.

4.3 Results

4.3.1 Calpain-YFP in living cells during phagocytosis and shape change

RAW-264 cells were initially used as a surrogate myeloid cell to investigate the properties of endogenous calpain in a myeloid cell line. The strategy was to express a fluorescent analogue subunit of calpain which does not contain a C2-like subunit (calpain4-YFP). Its translocation would only be by virtue of it partnering endogenous calpain-1. In this way, we can study the movement of endogenous calpian-1 without disturbing its expression levels or increasing the amount of C2 domain protein.

4.3.1.1 Location of calpain-YFP in cells spread on coverslips

Calpain-4 –YFP expressed in RAW 246 cells was located in the cytosol. There was no significant calpain4-YFP at the plasma membrane in resting RAW246 cells. In order to establish whether a small proportion of calpain-4 was attached to the plasma membrane which was not possible to detect because of the larger amount in the cytosol, localised photo bleaching of the cytosol was undertaken. This showed that none of the calpain was permanently bound to the membrane as the total fluorescence decreased and it revealed no fluorescent protein at the cell edge (figure 4.3.1.1.1)

4.3.1.2 Calpain-YFP activity in response to pharmacological cytosolic calcium concentration increase and cell shape change

A pharmacologically stimulated cytosolic calcium increase was stimulated by treating the cells with 10μM ionophore, 5μM thapsigargin 13mM $CaCl_2$. Upon the addition of the stimulus cytosolic calcium concentration increased sharply and remained high (figure 4.3.1.2.1a). However no calpain4-YFP translocation was observed even when cytosolic calcium concentration was at its highest (figure 4.3.1.2.1b)

4.3.1.3 Calpain-YFP activity in response to physiological cytosolic calcium concentration increase and shape change

Physiologically stimulated cytosolic calcium increase was achieved through treating the cells with mouse serum. Upon the addition of mouse serum the cytosolic calcium concentration increased sharply before quickly returning to resting levels (figure 4.3.1.3.1a). This cytosolic calcium concentration increase was not sufficient to trigger calpain4-YFP translocation (figure 4.3.1.3b).

4.3.2 Calpain in human neutrophils

In order to establish the relevance of the finding of C2 domain translocation shown in chapter 3 and the earlier work here, it was decided to use immunohistochemistry to determine the location of calpain-1 in primary human neutrophils.

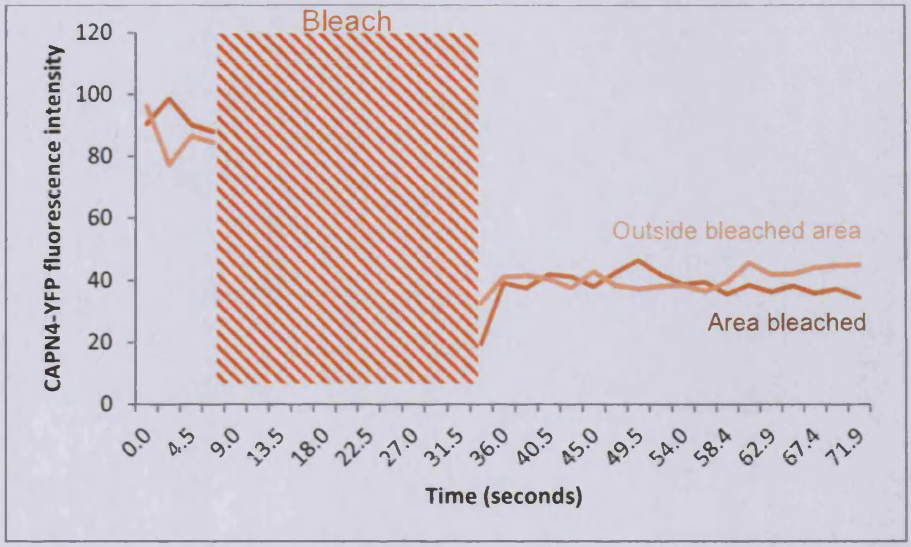
4.3.2.1 Calpain location in spread neutrophils

Calpain in primary human neutrophils is mainly cytosolic. In order to investigate its location during spreading, neutrophils were allowed to spread onto glass coverslips before being fixed, permeablised and probed with anti-calpain. This procedure showed that calpain-1 was concentrated at the cell edge in cells that had spread (figure 4.3.2.1.1). These data are consistent with the translocation of calpain-1 during neutrophil spreading. In spread cells which had polarised and begun the process of chemotaxis, calpain was highest at the leading edge of the cell (figure 4.3.2.2.4), suggesting the possibility that calpain localisation may be important in active cell shape change.

4.3.2.2 Calpain location during and after neutrophil phagocytosis

In order to establish whether translocation of calpain-1 occurred during phagocytosis, primary human neutrophils were allowed to phagocytose human serum opsonised zymosan particles before probing with anti-calpain antibody. Under these conditions, calpain-1 was concentrated around the phagocytic cup when cells were fixed at the start of phagocytosis (figure 4.3.2.2.1) or around newly internalised phagosomes when cells were fixed immediately after the particle had been internalised (figure 4.3.2.2.2). However when cells were fixed after phagocytosis was complete, older phagosomes were devoid of calpain (figure 4.3.2.2.3). This was consistent with translocation of calpain to a localised site of Ca^{2+} influx (i.e. the phagosomal membrane) as detected in the early stages of phagocytosis by RAW246 cells (chapter 3).

a)



b)

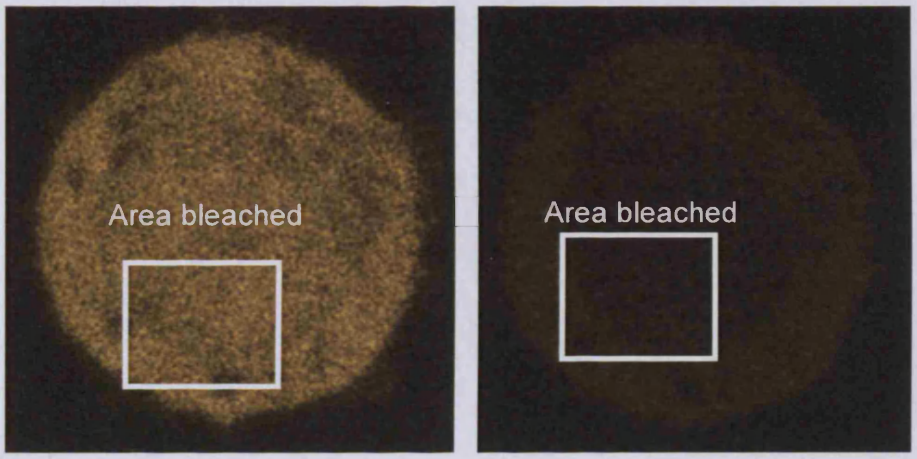
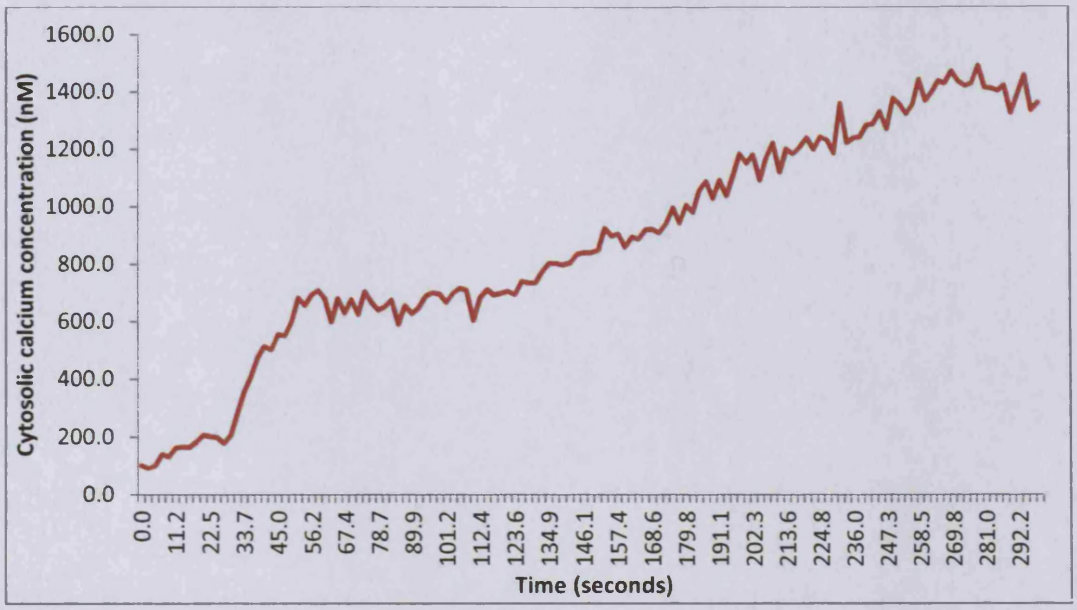


Figure 4.3.1.1.1: Typical result of photo bleaching of calpain 4-YFP in RAW264 cells *(n=3)*. RAW-264 cells expressing YFP tagged calpain 4 were exposed to high intensity laser light in order to bleach the YFP molecules in the cytoplasm of the cell. a) Shows a total loss of YFP fluorescence intensity of the entire cell after photo bleaching, with only a minor recovery. b) shows the cell before (left) and after (right) photo bleaching, showing the area of the cell that was bleached and that the whole of the cell had decreased in fluorescence intensity as molecules from the rest of the cytoplasm moved into the bleached area. Fluorescence at the cell membrane is also decreased indicating that none of the calpain is permanently bound to the plasma membrane in unstimulated cells.

a)



b)

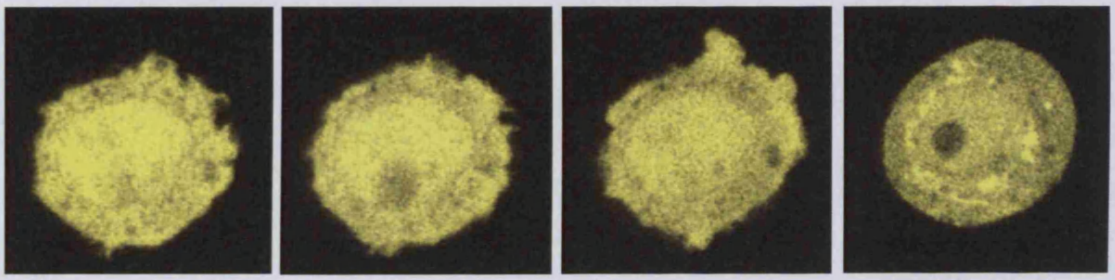
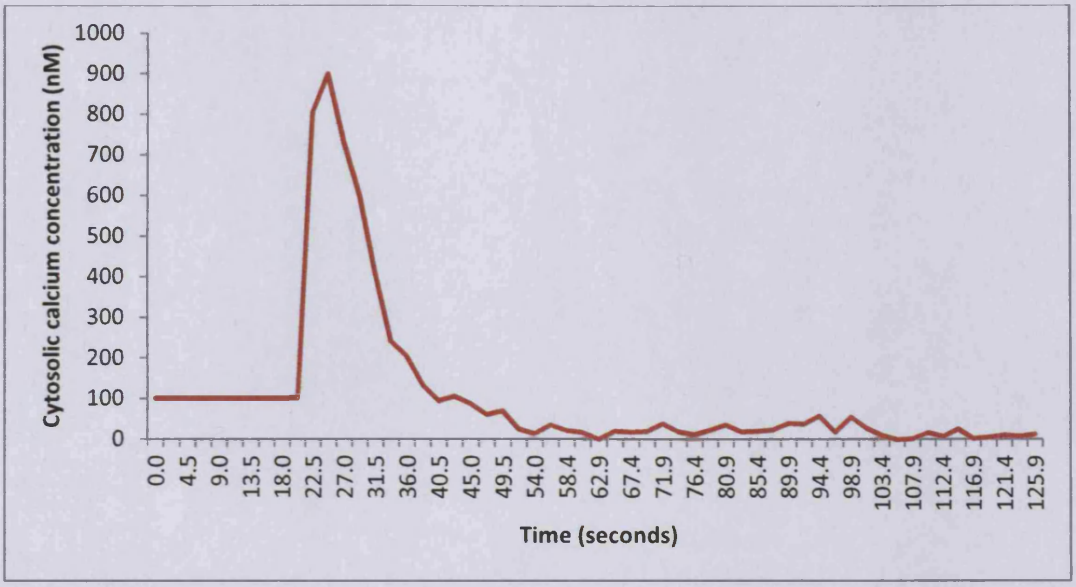


Figure 4.3.1.2.1: Typical response of RAW 264 cells expressing calpain4-YFP to pharmacologically stimulated calcium influx *(n=20)*. RAW cells expressing calpain 4-YFP were treated with 10μM ionophore and 5μM thapsigargin in medium containing 13mM $CaCl_2$ to increase cytosolic calcium concentration in an attempt to stimulated calpain translocation. Cells were also loaded with fura red to measure cytosolic calcium concentration. YFP and fura red fluorescence were monitored simultaneously throughout the experiment. a) Cytosolic calcium concentration was calculated from fura red fluorescence. After the addition of the stimulus cytosolic calcium concentration increased sharply (t~33.7) and remained high. b) Calpain4-YFP did not translocate to the plasma membrane upon pharmacologically induced cytosolic calcium increase.

a)



b)

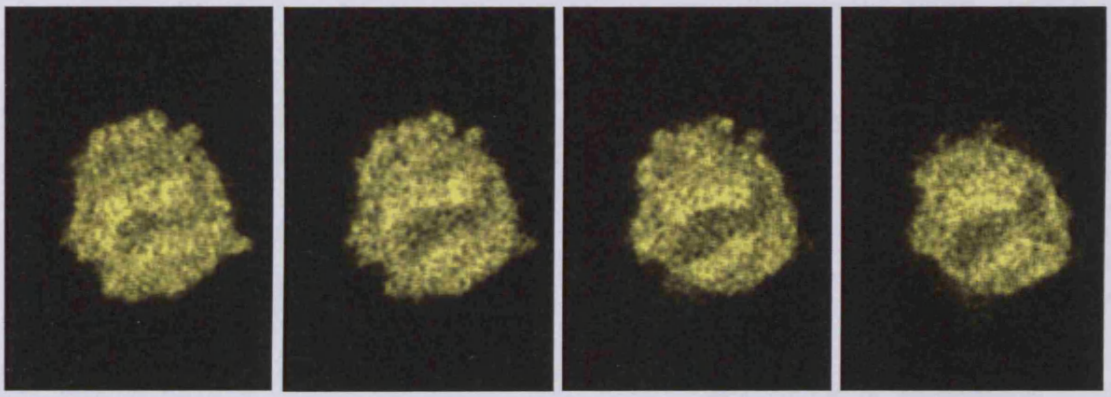


Figure 4.3.1.3.1: Typical response of RAW 264 cells expressing calpain4-YFP to pharmacologically stimulated calcium influx *(n=3)*. RAW cells expressing calpain4-YFP were treated with mouse serum in order to stimulate a physiological calcium influx. Cells were loaded with fura red in order to monitor cytosolic calcium concentration and fura red and YFP fluorescence were monitored simultaneously throughout the experiment. a) Cytosolic calcium concentration was calculated from the fura red intensity. Upon the addition of the mouse serum cytosolic calcium concentration increased sharply (t~18.0s) before returning to normal levels (t~36.0s). b) During this spike in cytosolic calcium concentration no translocation of calpain4-YFP was observed.

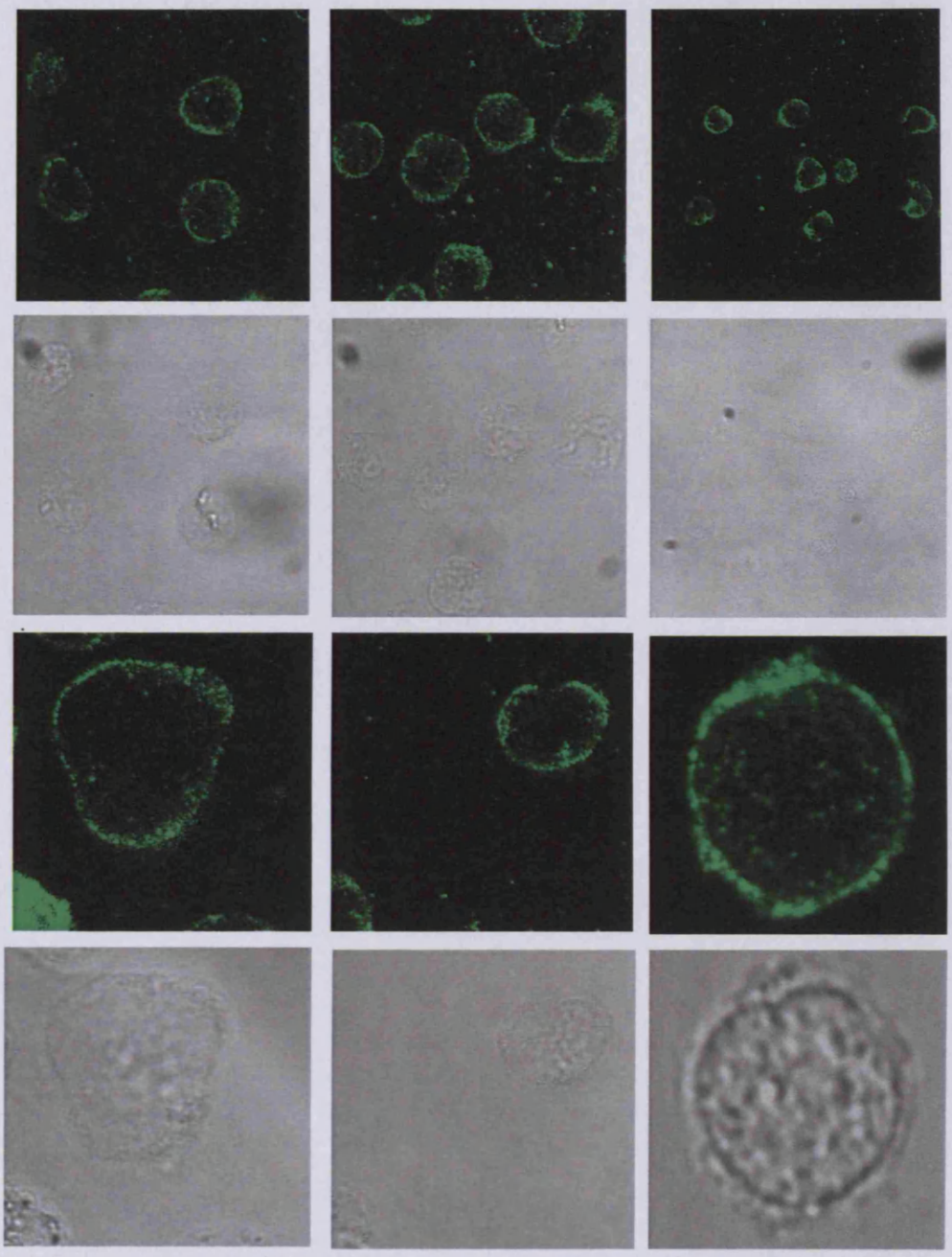


Figure 4.3.2.1.1: Calpain staining in spread neutrophils. Neutrophils were allowed to spread onto glass coverslips before being fixed, permeablised and stained for calpain. Calpain is concentrated at the cell membrane in neutrophils which have spread onto the coverslips (images representative of >200 cells imaged over 4 separate experiments, taken at different magnifications)

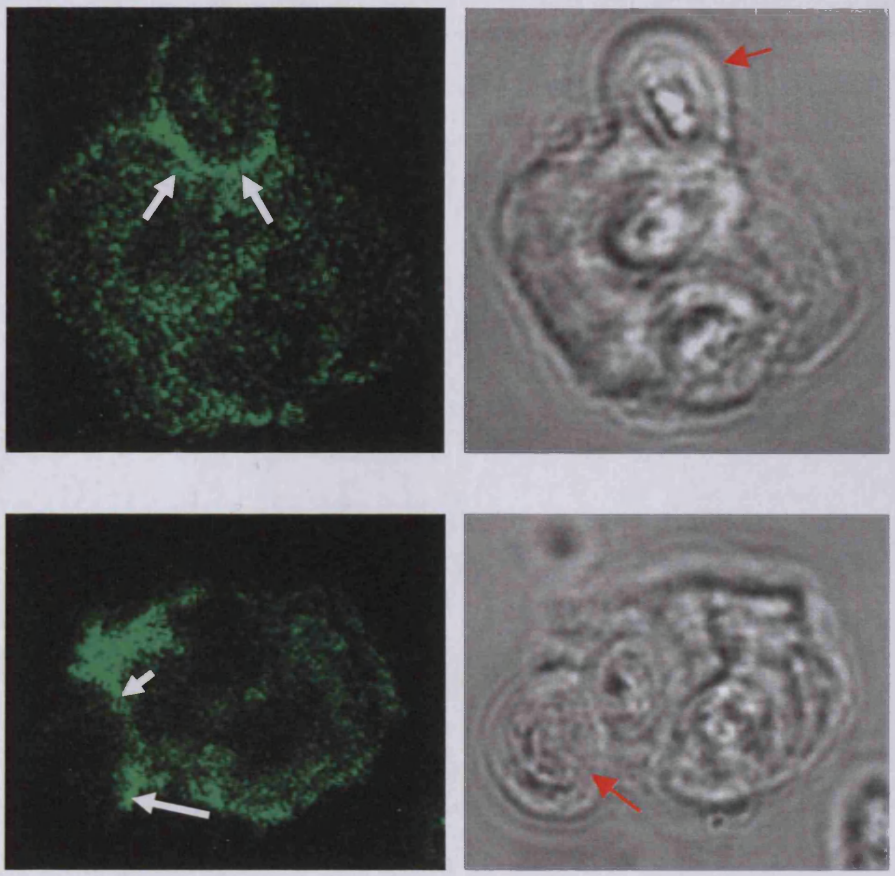


Figure 4.3.2.2.1: Calpain staining in neutrophils during phagocytosis. Neutrophils were allowed to phagocytose human serum opsonised zymosan particles (red arrowhead) before being fixed, permeablised and stained for calpain. The coverslip was then searched for cells which had been fixed at the particle binding stage of phagocytosis. They indicate that calpain is concentrated around the phagocytic cup (white arrows) in neutrophils which are just beginning phagocytosis. (Images are representative of 8 cells caught during the particle binding stage of phagocytosis over 3 separate experiments).

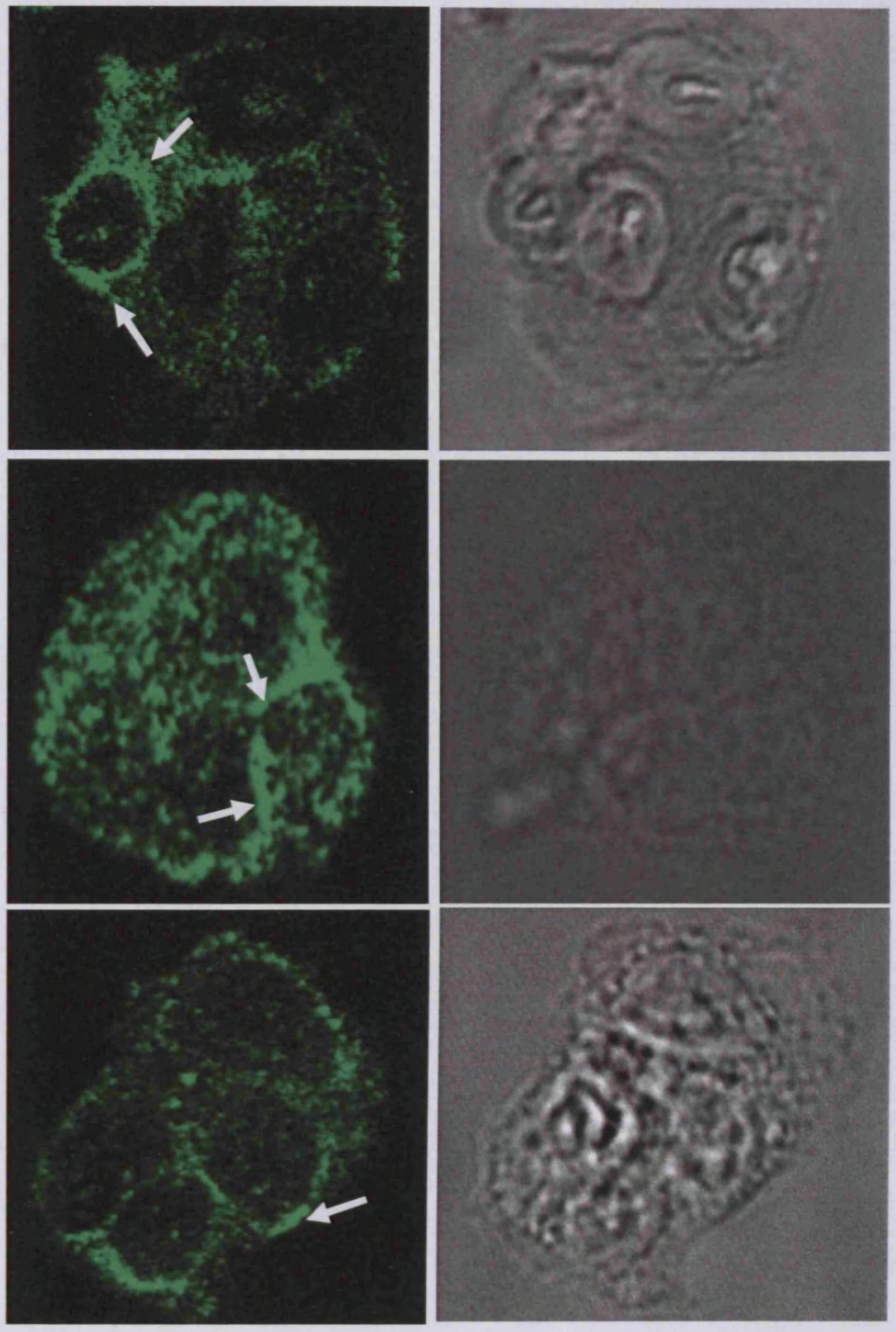


Figure 4.3.2.2.2: Calpain staining in neutrophils immediately after phagosome closure. Neutrophils were allowed to phagocytose human serum opsonised zymosan particles before being fixed, permeablised and stained for calpain. The coverslip was then searched for any cells that still retained calpain staining after phagosome closure. Calpain is concentrated around the internalised particle in neutrophils which have just completed phagocytosis (white arrows). (Images are representative of >11 cells found over 3 separate experiments with calpain staining still around the phagosome after phagosome closure).

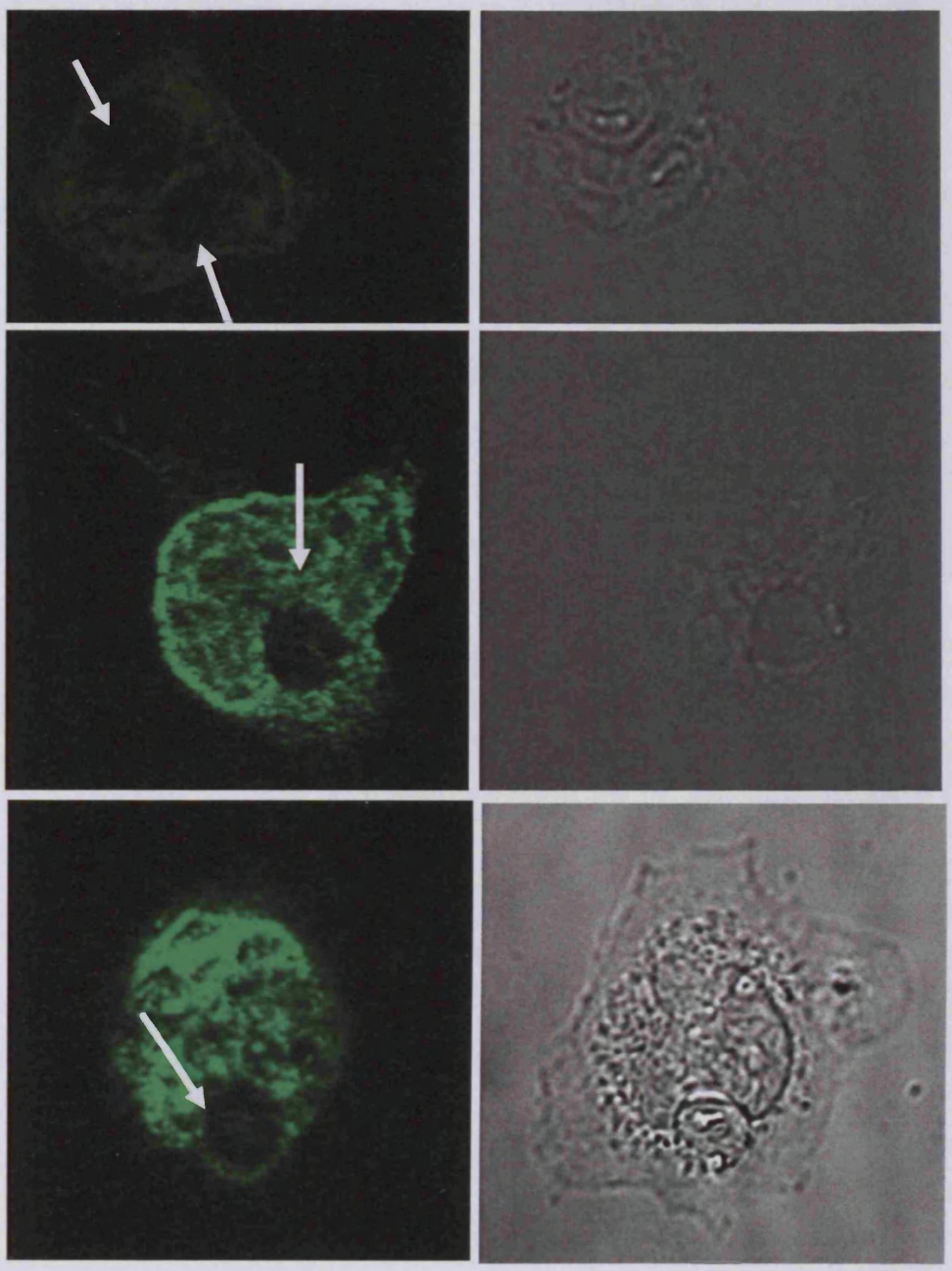


Figure 4.3.2.2.3: Calpain staining in neutrophils several minutes after phagosome closure. Neutrophils were allowed to phagocytose human serum opsonised zymosan particles and then spread onto glass coverslips before being fixed, permeablised and stained for calpain. The coverslip was then searched for any cells which had lost calpain staining around the phagosome once the phagosome had closed. Once phagocytosis is completed calpain staining is no longer found to be concentrated around the internalised particle. (Images are representative of >15 cells over 3 separate experiments found with calpain staining absent from the phagosome after phagosome closure)

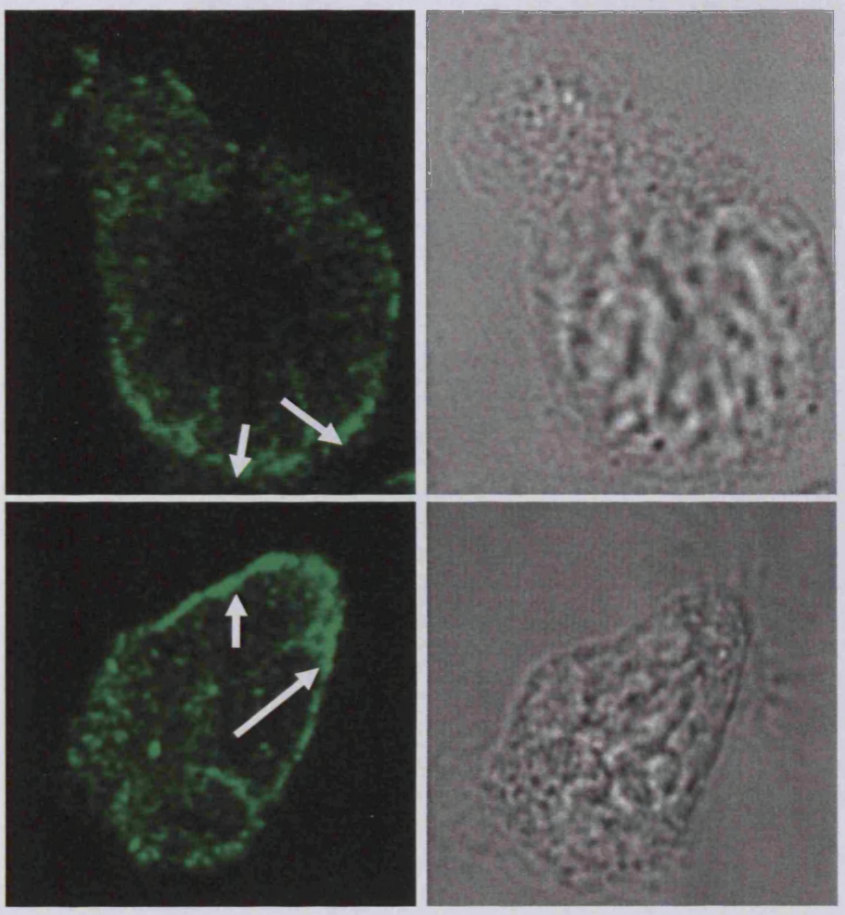


Figure 4.3.2.2.4: Calpain staining in neutrophils during chemotaxis. Neutrophils were allowed to spread onto glass coverslips coated with opsonised zymosan particles before being fixed, permeablised and stained for calpain. Calpain is concentrated at the leading edge in neutrophils which are undergoing chemotaxis (indicated by white arrows). (Images are representative of <20 cells over 3 experiments)

4.4 Discussion

4.4.1 Calpain4-YFP in living cells during phagocytosis and shape change

When calpain 4 was over expressed in RAW264 cells its location was found to be cytosolic, with none found bound to the plasma membrane in resting cells. This was to be expected as calpain is a cytosolic protein (Goll et al., 2003).

Results from chapter 3 indicated that C2 domain proteins of differing calcium affinities are able to translocate readily in response to cytosolic calcium influx, suggesting that by virtue of its 'C2 like' domain, calpain would show translocation to the plasma membrane during cytosolic calcium influx. However, no calpain4-YFP translocation was observed in response to either pharmacologically or physiologically stimulated calcium influx.

Both physiological and pharmacological stimulation of cells resulted in cytosolic calcium concentrations of higher than 500nm which would have been sufficient to stimulate translocation of classical C2 domains to the plasma membrane (section 3.3). It has also been shown in the previous chapter that calpains 'C2 like' domain likely has a much lower calcium affinity than classical C2 domains (section 3.4). It may be the case that the cytosolic calcium concentrations reached in these experiments were not high enough to cause calpain translocation. This is unlikely though, since bulk cytosolic calcium concentrations higher than those shown here would not occur under physiological conditions. It has been shown however that it is possible for micro domains of high calcium concentrations to exist during calcium influx inside the plasma membrane wrinkles on neutrophils (Brasen et al., 2010). It is therefore possible that only the calpain already present within the membrane wrinkles at the time of calcium influx encounters calcium concentrations high enough to bind phosphatidyl serine in the plasma membrane and high enough

to activate the protease. As such any excess calpain in the cytoplasm would remain inactive.

Another possible explanation is that in these experiments only the small subunit of μ-calpain (calpain 4) was over expressed. Calpain 4 does not contain the 'C2 like' domain or the protease domain and is only active when bound to its partner calpain 1 (Goll et al., 2003). This was considered the best approach to these experiments as any response seen would be as a result of endogenous calpain binding to the YFP tagged calpain 4 and could therefore be reliably interpreted as a normal response and not just a consequence of over expression. However it is possible that by only over expressing calpain 4 that any response was masked by an excess of calpain 4 in the cytosol. For example if there was more calpain 4 over expressed than there was endogenous calpain 1 in the cell then not all of the over expressed protein would be bound to calpain 1 and as such there would be an excess of inactive YFP tagged calpain 4 in the cytosol which may have masked any translocation of the active form. It is also possible that the over expressed calpain 4 did not bind with the endogenous calpain 1 and therefore all of the over expressed protein was inactive and thus there was no response.

One final possibility is simply that RAW264 cells don't provide a good model of calpain activity as they are a mouse cell line and they are not neutrophils. However calpain is ubiquitously expressed in all cell types and is highly conserved between mouse and human (figure 4.4.1.1 and 4.4.1.2) and in the experiments conducted here the cytosolic calcium increase was artificial and so any difference in processes between the cells should be irrelevant.

```
CPNS1_HUMAN MFLVNSFLKGGGGGGGGGGGLGGGLGNVLGGLISGAGGGGGGGGGGG--GGGGGGGGTA
CPNS1_MOUSE MFLVNSFLKGGGGGGGGGGG-LGGGLGNVLGGLISGAAGGGGGGGGGGGMGLGGGGGGGGTA

CPNS1_HUMAN MRILGGVISAISEAAAQYNPEPPPPPRTHYSNIEANESEEVRQFRRLFAQLAGDDMEVSAT
CPNS1_MOUSE MRILGGVISAISEAAAQYNPEPPPPPRSHYSNIEANESEEVRQFRKLFVQLAGDDMEVSAT

CPNS1_HUMAN ELMNILNKVVTRHPDLKTDGFGIDTCRSMVAVMDSDTTGKLGFEEFKYLWNNIKRWQAIY
CPNS1_MOUSE ELMNILNKVVTRHPDLKTDGFGIDTCRSMVAVMDSDTTGKLGFEEFKYLWNNIKKWQAIY

CPNS1_HUMAN KQFDTDRSGTICSSELPGAFEAAGFHLNEHLYNMIIRRYSDESGNMDFDNFISCLVRLDA
CPNS1_MOUSE KRFDTDRSGTIGSHELPGAFEAAGFHLNEHLYSMIIRRYADESGNMDFDNFISCLVRLDA

CPNS1_HUMAN MFRAFKSLDKDGTGQIQVNIQEWLQLTMYS  268
CPNS1_MOUSE MFRAFKSLDKNGTGQIQVNIQEWLQLTMYS  269
```

Figure 4.4.1.1: Amino acid sequence comparison of mouse calpain 4 and human calpain 4. Sequence are 95% identical. Differing amino acids are highlighted.

```
CAN1_HUMAN      MSEEIITPVYCTGVSAQVQKQRARELGLGRHENAIKYLGQDYEQLRVRCLQSGTLFRDEA
CAN1_MOUSE      MTEELITPVYCTGVSAQVQKKRDKELGLGRHENAIKYLGQDYETLRARCLQSGVLFQDEA

CAN1_HUMAN      FPPVPQSLGYKDLGPNSSKTYGIKWKRPTELLSNPQFIVDGATRTDICQGALGDCWLLAA
CAN1_MOUSE      FPPVSHSLGFKELGPHSSKTYGIKWKRPTELMSNPQFIVDGATRTDICQGALGDCWLLAA

CAN1_HUMAN      IASLTLNDTLLHRVVPHGQSFQNGYAGIFHFQLWQFGEWVDVVVDDLLLPIKDGKLVFVHS
CAN1_MOUSE      IASLTLNETILHRVVPYGQSFQDGYAGIFHFQLWQFGEWVDVVIDDLLLPTKDGKLVFVHS

CAN1_HUMAN      AEGNEFWSALLEKAYAKVNGSYEALSGGSTSEGFEDFTGGVTEWYELRKAPSDLYQIILK
CAN1_MOUSE      AQGNEFWSALLEKAYAKVNGSYEALSGGCTSEAFEDFTGGVTEWYDLQKAPSDLYQIILK

CAN1_HUMAN      ALERGSLLGCSIDISSVLDMEAITFKKLVKGHAYSVTGAKQVNYRGQVVSLIRMRNPWGE
CAN1_MOUSE      ALERGSLLGCSINISDIRDLEAITFKNLVRGHAYSVTGAKQVTYQGQRVNLIRMRNPWGE

CAN1_HUMAN      VEWTGAWSDSSSEWNNVDPYERDQLRVKMEDGEFWMSFRDFMREFTRLEICNLTPDALKS
CAN1_MOUSE      VEWKGPWSDSSYEWNKVDPYEREQLRVKMEDGEFWMSFRDFIREFTKLEICNLTPDALKS

CAN1_HUMAN      RTIRKWNTTLYEGTWRRGSTAGGCRNYPATFWVNPQFKIRLDETDDPDDYGDRESGCSFV
CAN1_MOUSE      RTLRNWNTTFYEGTWRRGSTAGGCRNYPATFWVNPQFKIRLEEVDDADDYDNRESGCSFL

CAN1_HUMAN      LALMQKHRRRERRFGRDMETIGFAVYEVPPELVGQPAVHLKRDFFLANASRARSEQFINL
CAN1_MOUSE      LALMQKHRRRERRFGRDMETIGFAVYQVPRELAGQP-VHLKRDFFLANASRAQSEHFINL

CAN1_HUMAN      REVSTRFRLPPGEYVVVPSTFEPNKEGDFVLRFFSEKSAGTVELDDQIQANLPDEQVLSE
CAN1_MOUSE      REVSNRIRLPPGEYIVVPSTFEPNKEGDFLLRFFSEKKAGTQELDDQIQANLPDEKVLSE

CAN1_HUMAN      EEIDENFKALFRQLAGEDMEISVKELRTILNRIISKHKDLRTKGFSLESCRSMVNLMDRD
CAN1_MOUSE      EEIDDNFKTLFSKLAGDDMEISVKELQTILNRIISKHKDLRTNGFSLESCRSMVNLMDRD

CAN1_HUMAN      GNGKLGLVEFNILWNRIRNYLSIFRKFDLDKSGSMSAYEMRMAIESAGFKLNKKLYELII
CAN1_MOUSE      GNGKLGLVEFNILWNRIRNYLTIFRKFDLDKSGSMSAYEMRMAIEAAGFKLNKKLHELII

CAN1_HUMAN      TRYSEPDLAVDFDNFVCCLVRLETMFRFFKTLDTDLDGVVTFDLFKWLQLTMFA 714
CAN1_MOUSE      TRYSEPDLAVDFDNFVCCLVRLETMFRFFKLLDTDLDGVVTFDLFKWLQLTMFA 713
```

Figure 4.4.1.2: Amino acid sequence comparison of mouse calpain 1 and human calpain 1. Sequence are 89% identical. Differing amino acids are highlighted.

4.4.2 Calpain in human neutrophils.

Despite no calpain translocation being observed upon cytosolic calcium increase in RAW264 cells expressing calpain4-YFP, when human neutrophils were fixed and stained for calpain expression calpain was found to be at the plasma membrane in spread neutrophils, around the phagosome during but not after phagocytosis and at the leading edge of neutrophils during chemotaxis and shape change. In all of these circumstances calpain is present in the right place and at the right time to play a role in neutrophil shape change. It may be the case that it is easier to see calpain enrichment at the plasma membrane in these cells than in living cells as these cells have been fixed and permeabalised before staining and as such any unbound and excess protein in the cytoplasm is washed away leaving only the material that is bound to the membrane thus making it easier to observe.

4.4.3 General Discussion

Despite the lack of evidence for the ability of calpain to translocate from observing YFP tagged calpain 4 in RAW264 cells, the results of the immunofluorescent staining for the location of calpain in human neutrophils provided compelling evidence that calpain is indeed present in the right place and at the right time to play a role in neutrophil shape change. Calpains substrates talin and ezrin are found in the neutrophil membrane wrinkles (Ivetic et al., 2002, Sampath et al., 1998) and cleavage of these substrates by calpain would allow the membrane to 'unwrinkle'. As calpain is present at the plasma membrane during and immediately after neutrophil shape and calcium micro domains where calcium concentrations are high enough to trigger calpain activation could exist in these membrane wrinkles (Brasen et al., 2010) it is very likely that calpain cleavage of these substrate is causing the unwrinkling of the plasma membrane. However it still remains for this to be observed in real time and in living neutrophil like cells as these cells are notoriously difficult to transfect. These obstacles are addressed in the next chapter.

CHAPTER 5

Expression of fluorescent proteins in neutrophil-like cells

5.1 Introduction

It is clear from the preceding chapters that definitive evidence for an involvement of calpain-1 in neutrophil-like activities will only be achieved by studying neutrophil-like cells. In the earlier chapters, transfection has provided real time data of intracellular events. However, transfecting neutrophil-like cells is a major challenge.

In this chapter, the issues surrounding transfection of neutrophil-like cells are discussed and the attempts to solve the problem are presented.

5.1.1 Established methods of transfection

5.1.1a Calcium phosphate transfection

Calcium phosphate mediated transfection relies on the precipitates which form spontaneously between DNA and calcium phosphate when mixed in a supersaturated solution. The precipitate is then added to the cellular medium where it is taken up by the cells. The stability of the precipitate formed is influenced by a number a number of factors including the concentration of the calcium and phosphate in the solution, the temperature of the reaction, the DNA concentration and the reaction time. Less than one minute reaction time is usually suitable to bind all of the DNA in the solution and extending the reaction time can lead to the formation of aggregates which decreases the transfection efficiency when the precipitate is added to the cells (Jordan et al., 1996). The pH of the medium when the precipitate is added to the cells is also critical as this influences the saturation of the medium and whether or not the precipitate re-dissolves before it is taken up by the cells (Jordan and Wurm, 2004). Osmotic shock of the cells once the precipitate has been added has been

shown to increase transfection efficiency but this is not through increased precipitate uptake into the cells (Jordan and Wurm, 2004).

5.1.1b Electroporation

Batch electroporation involving large external electric fields passed through the cells via two large external electrodes was first described by Neumann et al in 1982 who used a current passed through a solution of mouse myeloma cells and DNA using two electrodes to transfer DNA into the cells (Neumann et al., 1982). This induces a high transmembrane potential which leads to electropermeabalisation of the cell membrane (Weaver and Chizmadzhev, 1996). The exact mechanism of electropermeabalisation is not fully understood but in order for large molecules such as DNA to pass through the membrane the theory exists that electropermeabalisation must create pores in the lipid bilayer. Although there is much theoretical evidence for the existence of these pores in the membrane they have never actually been imaged (Weaver and Chizmadzhev, 1996). The pores form within 1μs but can take anywhere between 1s and 100s to close again (Weaver and Chizmadzhev, 1996). These pores are formed in the cell membrane only at particular voltages (1-20kV cm^{-1}) and lengths of electrical pulses (1-5μs) (Sugar and Neumann, 1984). If either of these criteria are exceeded then complete breakdown of the membrane occurs and the cell ruptures (Weaver and Chizmadzhev, 1996). Higher voltages and longer durations of pulses lead to larger pores being formed in the membrane which are ultimately irreversible (Joshi and Schoenbach, 2000). Once the pores have formed in the membrane, DNA can then be transported into the cell via electrophoresis.

The exact molecular mechanisms which lead to gene expression through normal electroporation are still unknown. It is commonly believed that transient expression of the inserted gene can occur whilst the plasmid is in the cytoplasm of the cell but for the cell to become stably transfected, cell division must occur

in which pores form in the nuclear membrane allowing the plasmid to enter the nucleus and potentially become integrated into the cells genome.

5.1.1c Nucleofection

Normal electroporation acts by forming pores across the plasma membrane when the voltage across the membrane reaches a set strength. This allows the DNA to enter the cell. However DNA cannot enter the nucleus until cell division occurs and the nuclear membrane becomes temporarily more permeable. Nucleofection through a combination of specialised electrical parameters and specific reagents means that DNA can pass through the plasma membrane and then through the nuclear membrane without having to wait for cell division to occur, resulting in a higher transfection rate. Also as the DNA is not held in the cytoplasm for a great length of time it is less susceptible to degradation. The exact molecular mechanism underlying nucleofection remains, however, unknown (Hamm et al., 2002). Despite this nucleofection has proved to be an efficient method of transfection for previously transfection resistant cell types such as stem cells (Aluigi et al., 2006) and immune cells (Maasho et al., 2004)

5.1.1d Lipofection

Lipofection utilises the complexes which are formed between cationic lipids and polyanionic DNA. These interact to form a liposome containing the DNA to be introduced into the cell. The liposome is taken up by the cell through endocytosis and during this process or once inside the cell the liposome becomes destabilised and releases its DNA into the cytoplasm. Destabilisation of the liposome is the critical step in this process as for a successful transfection the DNA must be released from the carrier once inside the cell. It is not known exactly how the liposome becomes destabilised as it is internalised but it is most likely the result of neutralisation by cellular ionic lipids, probably from fusion of the cell membrane with the liposome complexes as they

are internalised (Koynova et al., 2005). This hypothesis is strengthened by the observation that liposomes containing lipid compositions mimicking the cytoplasmic facing monolayer of the plasma membrane release their DNA more quickly into cells (Xu and Szoka, 1996). It is suggested that after the liposome is internalised via endocytosis it destabilises the endosomal membrane which induces flip flop of the lipids in the endosome which form complexes with the liposome and neutralises the lipids in the liposome resulting in the release of the DNA.

5.1.1e Virus mediated gene transfer

The majority of viral mediated DNA transfer systems are based on retroviruses. Retroviruses are enveloped RNA viruses which contain 2 copies of a single stranded RNA genome. The key features of retroviruses which make them good vectors for use in transfection are; their reverse transcriptase enzyme which converts the viral RNA into double stranded DNA and their integrase enzyme which can insert the double stranded DNA that has been created into the host cells genome. Integration of viral DNA into the host cell genome occurs as part of the normal retrovirus life cycle (figure 5.1.1e).

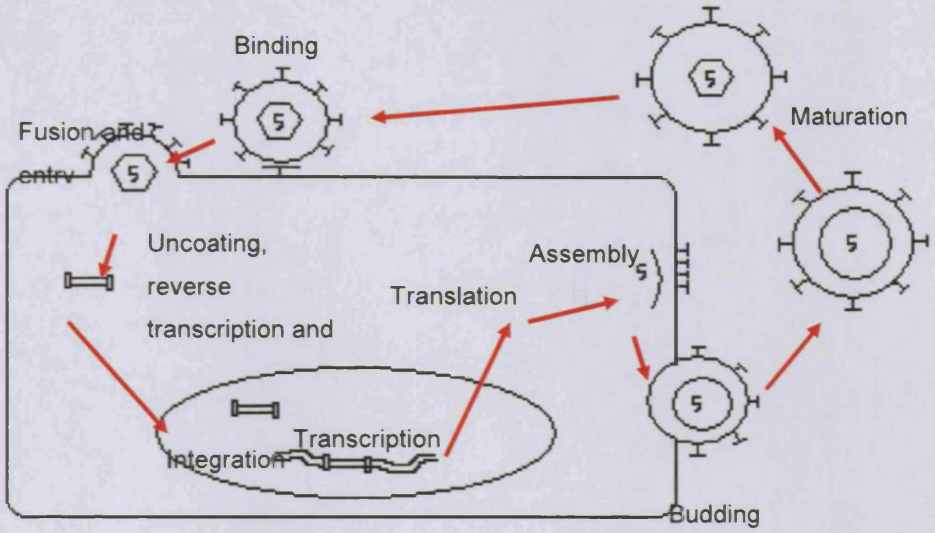


Figure 5.1.1.e: Schematic representation of the retroviral life cycle (adapted from http://www.nimr.mrc.ac.uk/virology/bishop/ (accessed 01/12/09). After initial binding the virus fuses itself to the host cell membrane and enters the cell. The virus then uncoats and reverse transcription and trafficking of DNA into the nucleus occurs. Once the DNA is in the nucleus integration of the viral DNA into the host cells genome occurs to create a pro virus. The viral DNA is then transcribed along with the cells other DNA. The cells own enzymes translate the RNA produced into new viral DNA. New virus is then assembled before the virus buds off from the cell. Further maturation then occurs before the virus can infect a new cell.

Integration of the viral DNA into the host cells genome occurs via removal of dinucleotides from both the 3' ends of the linear double stranded viral DNA, followed by a strand transfer reaction in which the 3' hydroxyl ends of the viral DNA make staggered breaks in the host cells chromosomal DNA, the gaps between the viral DNA and the host cells chromosomal DNA are then repaired by the host cells own DNA ligase enzymes resulting in integration of the viral DNA into the host cells genome. This is the key step in achieving stable transfection through a retroviral vector (Coffin et al., 1997)

Retroviruses have 3 core genes that they require in order to replicate. These are the gag, pol and env genes. The gag gene encodes the core proteins of the virus, the pol gene encodes the viral reverse transcriptase, DNA polymerase and integrase and the env gene encodes the viral envelope proteins. A retroviral vector for use in transfection encodes replication incompetent retrovirus. Most of the coding region of the retroviral genome is replaced by the gene of interest. A packaging cell line that expresses the gag, pol and env genes is used to produce replication incompetent retrovirus that is carrying the gene of interest.

The packaging cell line is transfected with the retroviral vector in order to produce high titres of replication incompetent retrovirus. The virus does not need to be replication competent as there is no need for it to continue to replicate once it has infected the target cells and integrated its DNA into the target cell genome. The packaging cell lines contain the gag, pol and env genes on 3 different plasmids. This provides the necessary genes for the virus to be able to assemble and the bud from the packaging cells but the virus produced would still be replication incompetent. 3 separate recombination events would need to occur between the plasmids which encode the required genes and the viral genome in order to produce replication competent

retrovirus. Once the retrovirus has been produced it can be used to transfect actively dividing target cells.

There are two main subtypes of the retrovirus family which are used for transfection. The most commonly used are the gamma retroviruses. These viruses can only transfect readily dividing cells as breakdown of the nuclear envelope is required for integration of the viral genome to occur. The other commonly used retroviral subtype is the lentivirus. These vectors are usually based on the HIV-1 virus and are able to transfect quiescent cells as they do not require cell division for integration of their genome to occur (Lindemann and Schnittler, 2009, Naldini et al., 1996).

Another type of virus used experimentally for transfection is the adenovirus. These viruses have a double stranded linear DNA genome and are less commonly used than the retroviruses (Lindemann and Schnittler, 2009).

5.1.2 Desirable cellular characteristics for successful transfection

Most transfection methods rely on cells being in an actively growing and dividing state. If a stable transfection is to be achieved then the genetic material introduced into the cell needs to get into the nucleus to stand a chance of being integrated into the genome. This can only be achieved during cell division when the nuclear envelope becomes more porous, with the exception of nucleofection. It is not just a stable transfection that requires active cells. Levels of protein synthesis in the cell have to be high enough that the gene that has been introduced into the cell is transcribed and translated and the desired protein is then expressed by the cell. Most methods of transfection are quite invasive to the cell and result in some level of damage whether it is through creating pores in the membrane through electroporation or introducing a virus into the cell. As such for a cell to be suitable for transfection it must be able to

withstand these insults. This makes more robust and established cell lines more suitable than for example primary cells.

5.1.3 The problem with neutrophils...

Neutrophilic cells, such as primary neutrophils or neutrophillically-differentiated myeloid cell lines, do not synthesise significant amounts of protein and are terminally differentiated. They are also primary cells and are very sensitive to any manipulation. They are not able to survive common transfection techniques which involve forming pores in the cell membrane. They also do not survive for long enough once isolated from fresh blood for retroviral transfection to be successful. Even if it were possible to successfully introduce foreign genetic material into neutrophils, it would still be highly unlikely that they would go on to express the protein that the material encoded as they are terminally differentiated and are no longer synthesising significant amounts of protein, also they do not live long enough once isolated from the blood to synthesise any significant amounts of a new protein.

5.1.5 Aims of the chapter

The aim of this chapter is to establish a method of successfully expressing fluorescently tagged calpain in neutrophil-like cells such as differentiated myeloid cell lines and for the transfection to be stable enough that the gene is still expressed at sufficient levels so that useful fluorescence can be imaged using the confocal laser scanning microscope for experiments once the cells have differentiated.

5.2 Materials and Methods

5.2.1 Transfection of human neutrophils using nucleofection

Neutrophils were isolated from healthy volunteers as previously described (section 2.3). Neutrophils were transfected using the Amaxa nucleofector device as previously described (Johnson et al., 2006). $1.84x10^6$ cells were centrifuged at 1800rpm for 5 minutes and re-suspended in 90µl nucleofector solution (kit T) and transferred to a cuvette. 5µg pmaxGFP plasmid was added to the cells to make the final volume in the cuvette 100µl and electroporated using programme T-27 on the Amaxa nucleofector device. Cells were recovered in 500µl pre-warmed RPMI and transferred to 6 well plates containing coverslips and 1ml pre-warmed RPMI per well (250µl transfected cells per well). Cells were incubated at 37°C in 5% CO_2 for 1 hour or 3 hours before they were observed under the confocal laser scanning microscope (CLSM) to assess the transfection frequency.

5.2.2 Transfection of myeloid cell lines using nucleofection

$2x10^6$ cells were transfected with Amaxa pmaxGFP using the Amaxa nucleofector device according to the manufacturer's protocol. Cells were centrifuged at 2000rpm for 10mins and re-suspended in 100µl nucleofector solution (kit V). 2µg plasmid was added to the cells which were then transferred to a cuvette and electroporated using programme X-001 on the Amaxa nucleofector device. Cells were recovered in 500µl pre-warmed RPMI before being transferred to coverslips in a 6 well plate containing 500µl pre-warmed RPMI per transfection. Cells were incubated at 37°C in 5% CO_2 for 3 hours before they were observed under the CLSM to assess the transfection frequency.

5.2.3 Generation of retrovirus and retroviral transduction

5.2.3.1 Transformation of phoenix ecotropic packaging cell line to generate retrovirus suitable for transducing murine cell types

Phoenix ectropic packaging cells were prepared by seeding into a 6 well plate at a density of $5x10^5$ cells/ml and incubated at 37 °C overnight. The next day the medium on the cells was replaced with fresh medium and cells were transfected with calpain 1-GFP plasmid using Fugene 6 transfection reagent according to manufacturer's instructions. 6µl fugene 6 reagent, 1µg plasmid and 94µl serum free DMEM was used per well. Fugene was pipetted directly into DMEM with care taken not to touch the sides of the tube and incubated at room temperature for 5 minutes. Plasmid was added to the fugene mix and incubated at room temperature for at least 15 minutes. Fugene-DNA mix was added to each well and swirled so that it covers all the cells and cells are incubated at 37°C for 2 days. After 2 days the viral supernatant was harvested and centrifuged to remove any contaminating cells. The supernatant was filtered through a 0.45µm filter and 5µg/ml polybrene was added. Virus was frozen at -80°C for long term storage.

5.2.3.2 Transformation of phoenix amphotropic packaging cell line retrovirus suitable for transducing human cell types.

Phoenix ampotrophic packaging cells were seeded at a density of $1x10^6$ cells/ml in a 10ml flask and incubated at 37°C overnight. Cells were transfected with calpain 1-GFP plasmid using calcium phosphate transformation. Briefly, calcium phosphate precipitate was prepared with 45µg plasmid DNA, 45µl $CaCl_2$, 450µl HeBs and sterile H_20 to a final volume of 900µl. The DNA, $CaCl_2$ and H_20 were prepared in one microcentrifuge tube and added to the HeBs one drop at a time whilst bubbling the HeBs. The mixture was vortexed and then incubated at room temperature for 20 minutes. The medium on the cells was replaced with fresh medium and 25µM chloroquine was added to the culture for 5 minutes followed by the precipitate and the culture was incubated at 37°C

overnight. The chloroquine decreases the pH in the endosomes that take up the DNA-calcium phosphate precipitate so that any enzymes in the endosomes which might degrade the DNA are inhibited. This increases the efficiency of the transfection. The following day the 15ml medium was replaced with 7.5ml fresh medium and the culture was incubated at 33°C overnight. This step is a compromise between getting the virus concentrated and creating optimal conditions for viral stability whilst at the same time maintaining the growth of the cells. On the final day the viral supernatant was harvested and centrifuged at 1200rpm for 5 minutes to remove any contaminating cells. Supernatant was pipetted into 1ml aliquots in cryovials and snap frozen on liquid nitrogen. Virus was stored at -80 for long term storage.

5.2.3.3 Transduction of the MyPH8B6 cell line with μ-calpain-GFP retrovirus

Mouse myeloid progenitor cells were transduced with the calpain retrovirus produced by the packaging cell lines. $1.5x10^6$ cells were centrifuged at 1500 rpm for 5 minutes and re-suspended in 4.5ml calpain 1-GFP viral supernatant (and polybrene) containing SCF (10ng/ml) and oestrogen (1μM). Polybrene is a small positively charged molecule that when it comes into contact with the cell binds to the surface and neutralises the surface charge. This is helpful when infecting the target cells with retrovirus as it allows the viral glycoproteins to bind more efficiently to their receptors because it decreases the repulsion between the sialic acid containing molecules on the cell surface and the virions. Cells were seeded into a 6 well plate at 1.5ml per well and plate was centrifuged at 1500xg for 90 minutes. 2mls fresh medium was added and cells were incubated at 37°C overnight. The next day cells were harvested into a flask and fresh medium was added. GFP expressing cells were sorted using the MoFlo florescence activated cell sorter. Cells were cultured in medium containing oestrogen (1μM) and SCF (20ng/ml) and stocks were frozen in heat inactivated FCS containing 10% DMSO for long term storage at -80°C.

5.2.3.4 Differentiation of the MyPH8B6 cell line into neutrophils

$7x10^5$ cells were centrifuged at 350xg for 5 minutes and washed 3 times in PBS. Cells were seeded at $7x10^4$ cells/ml in 10ml OptiMem medium (Invitrogen) supplemented with 10% heat inactivated FCS, 1% glutamine, 100µg/ml streptomycin, 100µg/ml penicillin, 30µM β-mercaptoethanol, SCF (20ng/ml) and G-CSF (20ng/ml). Cells were maintained in this medium for 4 days until differentiation was complete. Additional medium was added daily to renew the growth factors.

5.2.3 Maturation and transfection of human cord blood derived neutrophils.

5.2.3.1 Isolation of CD34+ cells from neonatal cord blood

CD34+ cells are haematopoietic stems cells which can be isolated from umbilical cord blood. Whilst still in their immature state these cells can be stably transfected using retroviral transduction. CD34+ cells were isolated from frozen mononuclear cord blood cells using a MiniMACS™ cell separator according to the manufacturer's protocol. The MACS cell separation system works by labelling cells with specific monoclonal antibodies conjugated to magnetic microbeads. The MACS column consists of a spherical steel matrix and a high magnetic field is induced in the column when it is placed in the magnetic cell separator. The cells that are labelled with the magnetic antibody remain in the column and any other cells are washed out (figure 5.2.3.1). In the case of CD34+ cell isolation the cells are labelled with mouse anti-human CD34 antibodies conjugated to magnetic microbeads. When the column is placed in the magnetic field the CD34+ cells are enriched in the column and all other cells are washed out. The column is then removed from the magnetic field and the CD34+ cells are eluted from the column.

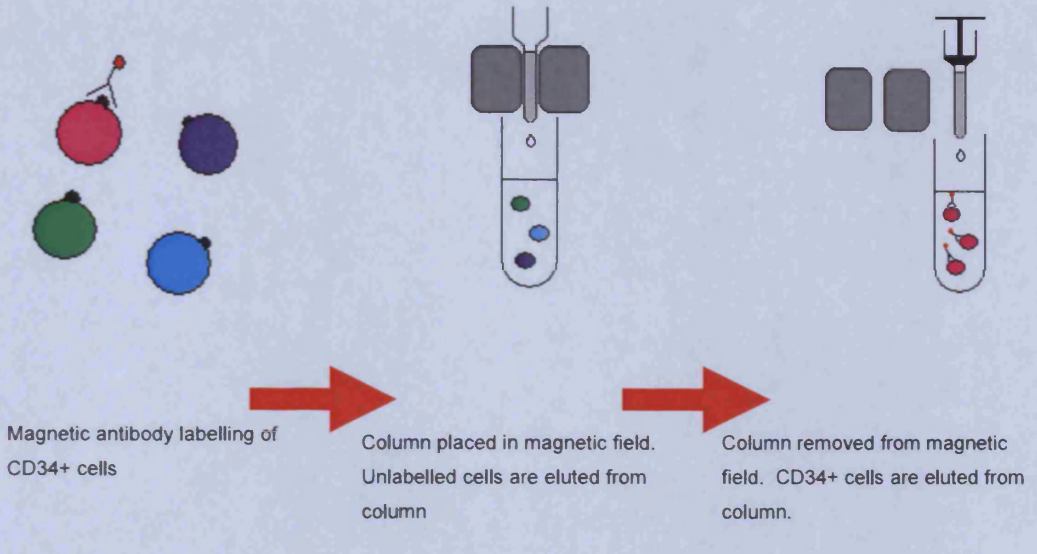


Figure 5.2.3.1: MACS separation of CD34+ cells from mononuclear cord blood cells. CD34+ cells are labelled with magnetic antibodies. A magnetic field is applied to the column so that magnetically labelled CD34+ cells become enriched in the column and all other cells are eluted. The column is then removed from the magnetic field and a plunger is applied to the column in order to elute the magnetically labelled CD34+ cells. (Adapted from MACS website)

$7x10^5$ cells were isolated in 1ml medium supplemented with IL-3, G-CSF, GM-CSF, Flt3 (5µg/ml), IL-6 (10µg/ml) and SCF (20µg/ml). Cells were cultured at a density of $2x10^5$ cells/ml at 37°C/5%CO_2 overnight.

5.2.3.2 Transduction of CD34+ cells with µ-calpain-GFP retrovirus

On the next 2 days of culture cells were infected with calpain 1-GFP retrovirus by transferring them to a RetroNectin® coated 24 well plate which had been presorbed with retrovirus as described previously (Darley et al., 2002). Briefly, an untreated 24 well dish was coated with 150µl RetroNectin® per well and was incubated for 2 hours on a shaker at room temperature. The RetroNectin® was removed and replaced with 1% BSA solution and plates incubated at room temperature for a further 30 minutes. In the meantime 100µl polybrene solution was added to each 1ml vial of virus and the virus was then incubated at 37°C for 20 minutes. The BSA was then removed from the plate and immediately replaced with the retrovirus (1 well at a time so the well doesn't dry out). The retrovirus was presorbed onto the plate by spinning at 4000rpm in a refrigerated centrifuge for 2 hours. After spinning the viral supernatant was removed from the plate and replaced with 1ml serum containing medium and incubated at room temperature for 5 minutes. The medium was then removed and replaced with CD34+ cells and the plate was incubated at 37°C overnight. The infection was repeated the next day with fresh virus.

5.2.3.3 Culture and maturation of transduced CD34+ cells into neutrophils

Cells were re-suspended at a density of $5x10^4$ cells/ml in medium containing IL-3, G-CSF (5ng/ml) and SCF (20ng/ml). SCF promotes the survival of the stem cells in the culture whilst IL-3 stimulates the proliferation of all cells in the myeloid lineage. G-CSF specifically stimulates the survival, proliferation and differentiation of neutrophils and their precursors (Metcalf, 1985). Cells were maintained at a density of approx $4x10^5$ cells/ml for 21-24 days until there were

mature neutrophils present in the culture. Excess cells were frozen in high serum medium containing 10% DMSO to be kept for future use.

5.2.4 Single cell electroporation

As normal electroporation involves electroporating a batch of cells between two large external electrodes the entire cell membrane becomes permeabalised. This can be very damaging to the cell especially considering the length of time it can take for the pores to form. Using single cell electroporation electrical pulses are passed through a DNA filled micropipette to one small area of the cell membrane which the micropipette is in contact with. Using this technique only one small area of the cells membrane becomes permeabalised, as such this technique is much less damaging to cells than normal batch electroporation.

0.25mm silver wire was inserted through a glass capillary in the micropipette holder so that one end of the wire sits in the solution in the micropipette and that the other end of the wire is connected to the negative terminal (anode) of the Grass SD9 stimulator. A second 0.25mm silver wire acts as the ground electrode and was inserted into the cell solution and connected to the positive terminal (cathode) of the stimulator. This creates a circuit in which the negatively charged plasmid DNA will be pushed out of the micropipette and into the cell when the current is switched on (figure 5.2.4.1).

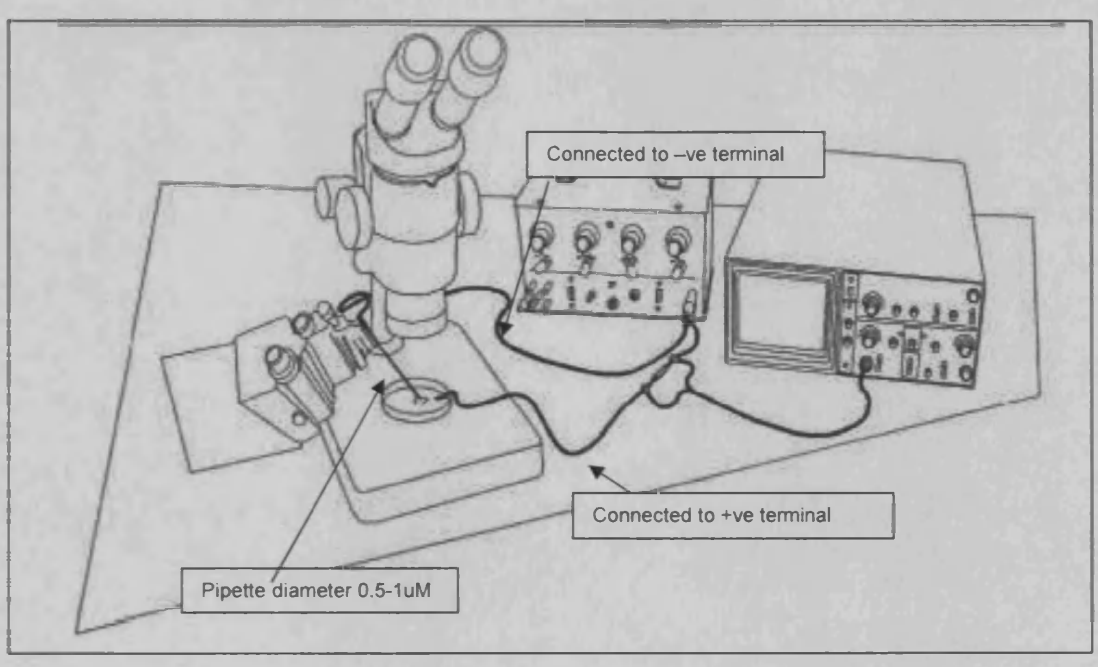


Figure 5.2.4.1: Diagrammatic representation of the setup of the apparatus for single cell electroporation (Haas et al., 2001). A 0.25mm silver wire inserted through the glass micropipette is connected to the negative terminal whilst a second silver wire attached to the positive terminal acts as the ground electrode. As plasmid DNA is negatively charged when the current is switched on it will be forced out of the pipette into the cell through the pores that are created in the membrane by the electric charge.

The micropipette was filled with 2µl of 1µg/µl pmax GFP plasmid or 1µl Lucifer yellow or 1µl fura2 or fura-dextran (figure 5.2.4.2) for visualisation.

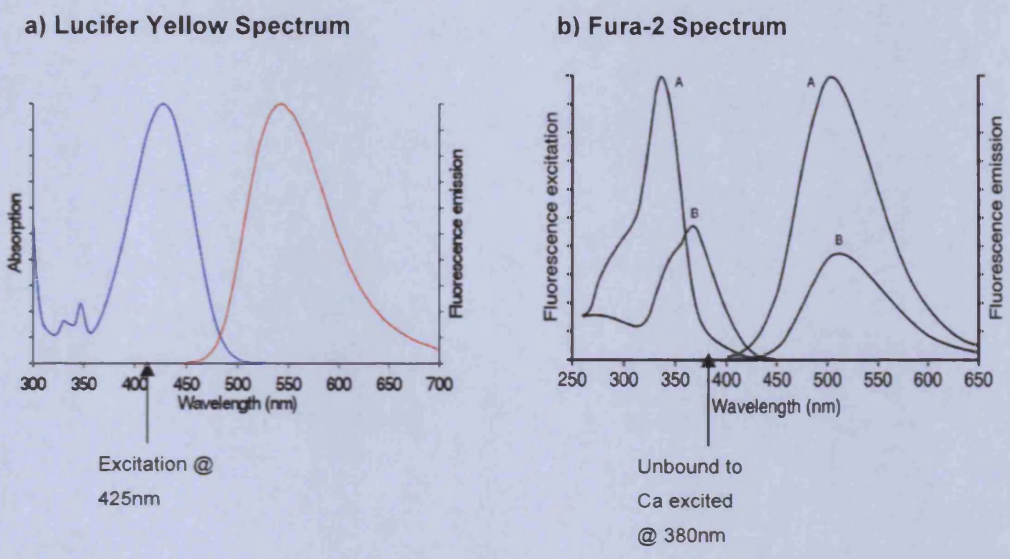


Figure 5.2.4.2: Fluorescent spectrum of Lucifer yellow and cell impermeant fura-2. (a) Lucifer yellow is excited by wavelengths of light at 425nm and emits fluorescence at 550nm. (b) The Ca^{2+} unbound form of fura-2 is excited at 380nm and emits fluorescence at 550nm.

3T3 cells which had been seeded onto a glass bottomed dish the previous night were used to test the system. The pipette was pushed against the cells so that it was in contact with the cell but not piercing the membrane. Cells were shocked with 0.5-1 second trains of 1ms long square pulses at 10V, 8V, 6V or 4V and 200Hz (figure 5.2.4.3).

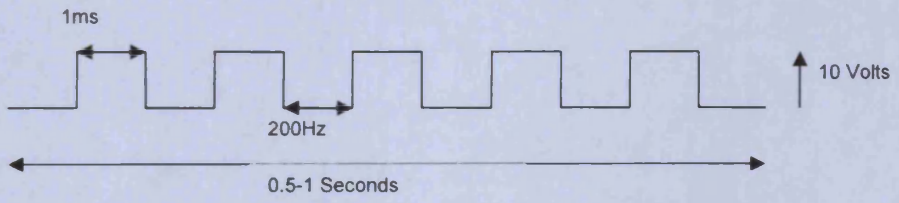


Figure 5.2.4.3: Diagrammatic representation of the electrical parameters used for the electroporation of single cells. The frequency and the length of the pulses remained the same for all experiments (200Hz and 1ms). The voltage was changed to either 10, 8 ,6 or 4 Volts depending on the experiment.

5.2.5 Transduction of human neutrophils and myeloid cell lines with GFP lentivirus.

Neutrophils were isolated from healthy volunteers as previously described (section 2.3). Neutrophils were transduced with a GFP encoding lentivirus as previously described (Dick et al., 2009). Cells were transduced with 50, 100, 200 or 300µl GFP virus at either 4°C or 37°C and incubated for 4 hours or 24 hours. Cells were fixed and analysed for GFP fluorescence using the Acuri flow cytometer. Live cells were imaged using the CLSM.

$2x10^5$ HL60 cells were transduced with 50 or 200µl GFP virus at 37°C for 48 hours. Cells were fixed and analysed for GFP fluorescence using the Acuri flow cytometer. Live cells were imaged using the CLSM.

5.2.5.1 Imaging of transfected cells

Confocal microscopy of live GFP expressing cells was performed using the resonant laser scanning Leica SP2 or SP5 confocal microscope system. Images were acquired with a 63x oil immersion objective and the GFP was excited with a 488nm laser and the emitted light was collected between 510-560nm.

5.3 Results

5.3.1 Nucleofection electroporation of human neutrophils

In order to establish the efficacy of conventional nucleofection for the expression of proteins in primary human neutrophils, a GFP-plasmid was used as an easily detectable fluorescent protein signal. However, no primary human neutrophils expressed GFP either 1 or 3 hour post transfection. This was in contrast to a previous report by (Johnson et al., 2006) despite using similar electroporation conditions and transfection reagents. The most notable effect of electroporation was neutrophil necrosis. It was concluded that primary human neutrophils could not be transfected by this method.

5.3.2 Nucleofection of myeloid cell lines NB4 and HL60

Given the zero-efficacy of transfection in primary neutrophils, two myeloid cell-lines were investigated, NB4 and HL60. In contrast with primary neutrophils, approximately 40% of *NB4 cells* expressed GFP one day after transfection. This fell to around 2% by day 7 (figure 5.3.2.1). Similarly, approximately 5-10% of the HL60 cells expressed GFP one day after transfection but no cells expressed GFP by day 7 (figure 5.3.2.1). Neither of the cell lines became stably transfected, even with antibiotic selection. This meant that the cells could not be transfected before they were differentiated into neutrophil like cells (which itself takes 7-10 days, in which time the protein is lost).

Therefore the key technical challenge involved with this approach was being able to transiently transfect myeloid cells which have already differentiated into neutrophil-like cells. Post-differentiated HL60 cells failed to express GFP. However, it was possible to find a compromise between differentiation and high levels of transfection. It takes approximately 5-7 days for HL60 cells to

differentiate in DMSO. When the cells were transfected at a time that they retained some of their protein synthesis function and were not yet fully differentiated, i.e. on day 2 or 3 of differentiation, some cells on day 5 expressed GFP. However, as only 5% of undifferentiated HL60s cells expressed GFP on day 1 the further lowering of this on differentiation (fig 5.3.2.1) meant that it was difficult to find cells which were both successfully transfected and fully differentiated. This method was thus unreliable at best and most commonly the cells that were successfully transfected in this manner were those which had not fully differentiated.

5.3.3 Retroviral transfection of murine myeloid progenitor cells and human cord blood derived myeloid stem cells

An alternative approach was to transfect myeloid progenitor cells, which retained full protein synthetic capacity and which were available from murine and human sources. The aim was to transform these progenitor cells to mature neutrophils while retaining sufficient GFP for imaging.

5.3.3.1 Expression of calpain1-GFP in mouse myeloid progenitor cells

Flow cytometry analysis revealed that immediately after transduction with the calpain1-GFP retrovirus only 2% of the cells expressed GFP. Cells were therefore sorted on the MoFlo florescence activated cell sorter to produce a cell population with greater than 90% of cells expressing the protein (figure 5.3.3.1.1). As cells underwent differentiation, expression of the calpain1-GFP protein decreased resulting in very low fluorescence in the fully mature neutrophils (figure 5.3.3.1.2)

5.3.3.2 Expression of calpain1-GFP in human cord blood derived myeloid stem cells (CD34+ cells).

Approximately 40% of CD34+ cells expressed GFP after cells had been transduced (figure 5.3.3.2.1a). The GFP expression varied considerably between individual cells in the population (figure 5.3.3.2.1). This may be due to differences in where the retrovirus integrates into the host cell genome. For example, if it integrates near a highly expressed gene with a strong promoter, expression will be high and vice versa. Another factor influencing the levels of GFP expression in the cell is at what stage of maturation the cell is at (see later).

CD34+ cells are immature myeloid stem cells. The cells were re-suspended at a density of $5x10^4$ cells/ml in medium containing IL-3, G-CSF (5ng/ml) and SCF (20ng/ml) in order to differentiate into mature neutrophils. This process took approximately 21-23 days. The CD34+ cells have the potential to mature into any cell in the myeloid lineage. The G-CSF added to the culture medium "encourages" the cells to differentiate down the neutrophil pathway but other myeloid cell types are still present in the culture. Also the cells do no mature simultaneously so at any one time there are several stages of neutrophil development present in the culture. Once the cells have fully matured into neutrophils they only live for 2-4 days before they undergo apoptosis. This makes it difficult to predict the time point at which the highest number of neutrophils will be present in the culture.

Cytospin and wright-giemsa staining were used to observe nuclear morphology in order to find the number of mature neutrophils present in the culture. After 23 days maturation the culture of calpain-GFP transduced cells contained 8.3% polymorphonuclear cells. The control culture contained 13.3% polymorphonuclear cells (figure 5.3.3.2.2). There was no significant difference

in the maturation of calpain-GFP transduced cells and control cells (t-test: p=0.16).

The number of cells expressing GFP decreased as the cells matured towards neutrophils (figure 5.3.3.2.1b). Flow cytometry analysis revealed that after a 21day maturation period, approximately 11% of cells were expressing GFP (figure 5.3.3.2.3a). This combined with the low numbers of neutrophils present in the culture made it difficult to find cells which were expressing GFP and were mature functioning neutrophils. Analysis of the side scatter found that less than 1% of the total population of cells in the culture were both expressing GFP and had the high granularity which is characteristic of mature neutrophils (figure 5.3.3.2.3b).

5.3.4 Single cell electroporation

In order to establish whether or not single cell electroporation was a viable technique for introducing foreign genetic material into hard to transfect cells such as myeloid cell lines and neutrophils, several different parameters were tested in order to optimise the technique. Initial optimisation of the technique was carried out using mouse 3T3 fibroblast cells and the fluorescent dye Lucifer yellow. This is because these cells are more robust and easy to transfect using normal established techniques. Therefore it was sensible to optimise the new technique using a cell type which has been shown to be easy to transfect in the past as it would be logical to assume that any problems with cell viability and transfection frequency would likely be a consequence of the method of transfection rather than the cell line being transfected. Lucifer yellow was used instead of plasmid DNA in the micropipette as it is fluorescent, therefore allowing instant visual confirmation of whether or not the contents of the micropipette had been delivered into the cell.

There were several key features of the technique which needed to be investigated in order to find the optimal conditions for single cell electroporation. Firstly, a compromise had to be found between the applied voltage that the cell could survive and the successful introduction of fluorescent material into the cell. Secondly, the impact that the area of the cell membrane that was permeabilised had on cell survival and transfer of material into the cell was examined using different pipette tip diameters to deliver the electric charge to the cell. Different extracellular environments were also examined to see if an optimum environment could be established. And finally, different size molecules were introduced into the cell to confirm that the pores created in the membrane would be large enough for plasmid DNA to pass through.

5.3.4.1 Optimising single cell electroporation voltage

The advantage of using single cell electroporation over normal batch style electroporation is not only that the smaller area of the cell membrane is permeabalised but also that much lower voltages can be used. Different voltages were examined to see which was most efficient at introducing material into the cell whilst still maintaining cell viability.

Lucifer yellow was successfully introduced into 3T3 cells electroporated using a 0.5-1 second train of 50Volt, 1ms, 200Hz square pulses and this did not cause immediate cell lysis (figure 5.3.4.1.1) However when cells were checked several minutes after the procedure, all cells which had been electroporated had necrosed, suggesting that this voltage was too high to maintain cell viability.

It was still possible to successfully introduce Lucifer yellow into 3T3 cells which were electroporated with 0.5-1 second trains of 10Volt, 1ms, 200Hz square pulses (figure 5.3.4.1.2). In order to establish long term cell viability which

would be necessary should the technique be used for the introduction of foreign DNA into the cell the cells were monitored over a 24 hour period. All of the cells which had been electroporated had necrosed by the end of the 24 hours. The fluorescence levels in the cells was used as an indicator as to how much Lucifer yellow had been introduced into the cell and how much remained in the cytoplasm over the 24 hour period. The fluorescence levels of the cells that had been electroporated decreased exponentially after the procedure. Most cells were no longer fluorescent 1 hour after the procedure and all of the cells which had been electroporated were no longer fluorescent 3 hours after the procedure (figure 5.3.4.1.3). This suggested that the pores formed in the cell membrane by the electroporation did not close immediately after the cell had been electroporated.

When 3T3 cells were electroporated using just a single pulse of 10Volts it was still possible on occasion to successfully introduce Lucifer yellow into the cells. It was necessary to hold the pipette to the cell for several seconds after the pulse had been delivered in order to allow particles to travel from the pipette into the cell through the presumably smaller hole in the membrane that was created with this technique. This technique was not nearly as reliable at introducing material into the cell and a much smaller amount of material was introduced with each attempt (figure 5.3.4.1.4).

5.3.4.2 The effect of the membrane permeabilised area on cell viability after single cell electroporation.

Pipette tip diameters of 1µm and 0.5µm were used to electroporate cells using 0.5-1 second trains of 8V, 6V and 4V, 1ms, 200Hz square pulses to investigate whether the size of the area of membrane permeabilised had an effect on cell viability after electroporation. Lower voltages were used as it had been established in section 5.3.4.1 that the lowest voltage possible should be used to maintain cell viability.

When delivering the electric charge through a pipette tip with a diameter of 1µm there was no change in cell survival and viability between the three different voltages (figure 5.3.4.2.1a). As with previous experiments, there were no cells containing visible amounts of Lucifer yellow after 3 hours (figure 5.3.4.2.1c), suggesting that even with these lower voltages the pores created were not closing immediately after the procedure.

Delivering the electric charge through a pipette tip with a smaller diameter of 0.5 µm led to an increase in cell survival when compared to previous experiments. Some of the cells electroporated using 6V or 8V pulses survived until 24 hours after the procedure (figure 5.3.4.2.2a). Cell survival did not show any relationship to the voltage used for the electroporation. Great care had to be taken with this technique as in order for the electroporation to be successful using lower voltages the contact between the cell and the micropipette had to be as close as possible. Care must be taken to ensure that the contact is as close as possible without puncturing the cell membrane with the micropipette and bursting the cell. As with the previous parameters examined, most cells were no longer fluorescent after 3 hours. In one experimental run, a single cell (8V) was still fluorescent and alive after 24 hours (figure 5.3.4.2.2c). However, this cell had significantly less Lucifer yellow fluorescence than it had when it was initially electroporated. This had decreased exponentially over the initial 3 hours post electroporation period but remained constant for the next 21 hours (figure 5.3.4.2.2c). This indicates that in this particular cell the pores formed in the membrane by the electroporation took 3 hours to close. Once pore closure had occurred, the cell was both viable and retained some electroporated material.

5.3.4.3 The effect of different extracellular environments on cell viability after single cell electroporation.

As the pores formed in the cell membrane by the electroporation took several hours to close, the components of the extracellular medium can pass into the cytoplasm as well as cytoplasmic component 'leaking' out into the extracellular medium. In order to minimise this and hopefully improve cell survival of the procedure the extracellular environment is very important. Ideally the extracellular environment would mimic that of the cytoplasm in order to minimise the exchange of materials between the cytoplasm and the extracellular medium whilst the pores are open. Creating a solution that is exactly identical in composition to the cytoplasm is not practical due to the number of different molecules all present at different concentrations in the cytoplasm, so several different solutions were tried in order to try and find the best compromise. The solution that the plasmid or Lucifer yellow is dissolved in is also important as this solution is transferred directly into the cell during the electroporation.

During nucleofection, a type of batch electroporation, the cells are bathed in a special nucleofector solution to maximise transfection frequency and cell survival. As this solution was limited in supply the cells were not bathed in the solution during the procedure but the Lucifer yellow in the pipette was diluted in nucleofector solution V before being introduced into the 3T3 cells by electroporation with 0.5-1 second trains of 10Volt, 1ms, 200Hz square pulses. Cells were monitored over 24hrs to see how long the cells survived for after the procedure and how long it took for the pores formed in the membrane to close. All of the cells electroporated died after 24hours. In most cases the fluorescence in the cells decreased exponentially indicating that the pores in the membrane were still open (figure 5.3.4.3.1). Only one cell still contained any visible Lucifer yellow after 1 hour and there was no visible Lucifer yellow in this cell after 3 hours (figure 5.3.4.3.1). This suggests that that the inclusion of nucleofector solution in the pipette did not increase the speed of pore closure.

Based on a previous report stating that electroporation was successful when cells were bathed in mammalian saline during electroporation and tissue culture medium was added after the cells had been electroporated (Rae and Levis, 2002), it was decided to attempt the single cell electroporation using these conditions. The Lucifer yellow in the micropipette was diluted in mammalian saline and the cells were bathed in the mammalian saline solution during the procedure. Cells were then electroporated with 0.5-1 second trains of 6Volt, 1ms, 200Hz square pulses. The mammalian saline was then replaced with normal tissue culture medium after the cells had been electroporated. No cells survived more than 5 hours after the procedure and as with previous experiments, fluorescence levels of the cells decreased exponentially suggesting it took several hours for the pores in the cell membrane to close (figure 5.3.4.3.2).

As an alternative to minimal saline lucifer yellow was diluted in Krebs medium and the cells bathed in Krebs during the procedure. The cells were electroporated with 0.5-1 second trains of 6Volt, 1ms, 200Hz square pulses. This produced similar results to those shown when cells were bathed in mammalian saline. No cells survived more than 5 hours after electroporation, and fluorescence decreased exponentially indicating the pores in the cell membrane remained open for several hours after the procedure (figure 5.3.4.3.3). Neither of these experiments suggest that saline or Krebs solution is a suitable extracellular environment to maximise cell survival during single cell electroporation.

5.3.4.4 Introducing different molecules into cells using single cell electroporation.

As electroporation did not result in transfer of material from the pipette into the cell 100% of the time it would be necessary to include a fluorescent marker as well as the plasmid DNA in the micropipette when attempting to introduce DNA

into the cell to confirm that the electroporation had been successful. As Lucifer yellow emits fluorescence at the same wavelength as GFP (which is the fluorescent tag on the plasmids intended for use with this technique), it was not a suitable marker for use when the plasmid was being introduced into the cells. It was decided instead to use cell impermeant fura2 as this emits fluorescence at a UV wavelength which is sufficiently different to be distinguished from the fluorescence emitted by the GFP. This would allow cells which had been electroporated but were not expressing the protein of interest to be differentiated from those that were. Fura2 was successfully introduced into 3T3 cells using a 0.5-1 second train of 10Volts, 1ms, 200Hz square pulses (figure 5.3.4.4.1). As had been previously observed when introducing material into cells using single 10V pulses, the transfer of material from the pipette into the cell was improved when the pipette was held to the cell for several seconds after the electroporation.

Using the same parameters optimised in 3T3 cells fura2 was introduced into differentiated HL60 cells using a 0.5-1 second train of 10Volts, 1ms, 200Hz square pulses. However after the initial peak in fluorescence upon electroporation the fluorescence levels proceeded to decline in an exponential manner after which the cell died (figure 5.3.4.4.2). This again suggests the pore formed in the cell membrane during the electroporation procedure is not closing immediately afterwards therefore allowing the cytosolic contents of the cell to leak out and the surrounding medium to leak in resulting in cell death. This was observed using 3T3 cells but the cells survived much longer after the procedure than the HL60 cells.

Earlier experiments indicated that using a single pulse as opposed to a train of pulses to electroporate the cell resulted in a smaller hole forming in the membrane (section 5.3.4.1). It was hypothesised that a smaller hole in the membrane would be less damaging to the more sensitive HL60 cell. Fura2 was

successfully introduced into differentiated HL60 cells using a single pulse of 10Volts. However, this was again followed by an exponential decrease in fluorescence of the cell once the pipette was removed and the cells died within 20 minutes of being electroporated (figure 5.3.4.4.3).

When attempting to introduce plasmids into 3T3 cells using single cell electroporation Fura2 or fura dextran was loaded into the pipette as well as the plasmid to act as a marker to make sure that the cells had been successfully electroporated. None of the cells that were electroporated were alive 24hours later and as a consequence no GFP expression was seen. Fura dextran was used as it is a larger molecule and would be a good indicator as to whether the pores created in the cell membrane by the electroporation were large enough to allow something as large as a plasmid to pass through. It was harder to introduce fura dextran into cells as it is a large molecule and tended to aggregate when an electric charge was applied and get blocked in the pipette. It was possible on some occasions to introduce fura dextran into cells so it was decided that the pores created by the electroporation were big enough for a large molecule such as a plasmid to enter the cell. It was therefore concluded that the plasmid was entering the cells but the cells were not surviving the procedure.

Separate batches of cells were electroporated with the GFP plasmid and fura2 and were monitored over 3 hours to see if the expression of the GFP was quick and transient before the cells died. Most cells died by 2hrs and no GFP expression was observed.

It was concluded that single cell electroporation would not be suitable for transfection of myeloid cells as long term survival of cells was poor.

5.3.5 Lentiviral transfection of human neutrophils

A previous report has stated that it is possible to transiently transfect primary human neutrophils using lentiviral mediated transduction (Dick et al., 2009). Lentiviral transduction seemed plausible as lentiviruses to not require actively dividing cells in order to integrate their DNA into the cells genome and are readily able to infect quiescent cells. Dick et al used flow cytometry to analyse the transfection frequency after the neutrophils had been transduced with the lentivirus. As such a similar protocol was followed in these experiments where neutrophils were transduced with a GFP lentivirus for 4 hours before analysis. Flow cytometry analysis revealed a higher number of neutrophils to be apparently GFP positive as the volume of lentivirus used for transduction was increased. (figure 5.3.5.1). However no GFP positive cells could be found when the transduced neutrophils were analysed with confocal microscopy.

It has also previously been noted that lentiviral transduction efficiency is higher when cells are transduced at lower temperatures (Pearce and Brennan 2010 unpublished personal communication). Subsequently cells were transduced at both 4°C and 37°C both with the 4 hour incubation times as used by Dick et al and 24 hour incubation times. Flow cytometry analysis revealed that a higher number of neutrophils were apparently GFP positive when cells were transduced at 37°C and that the number of cells that were apparently GFP positive decreased when the incubation time was increased to 24 hours (figure 5.3.5.2). Again there were no GFP positive cells identified when cells were observed using confocal microscopy for any of these conditions.

These results lead to the possibility that the 'GFP positive' cells identified by the flow cytometry analysis are necrosed cells exhibiting auto fluorescence. This would follow as a higher volume of lentivirus used for transduction is likely to lead to more cell necrosis. Also neutrophils gradually start to undergo apoptosis immediately after they are released into the blood stream. As such

neutrophils rarely survive for more than 24 hours after they have been isolated from fresh blood. This process of apoptosis occurs more quickly at 37°C than it does at 4°C. This would explain the results seen when the cells were incubated at different temperatures.

Dick et al reported donor variation in the transduction efficiency of neutrophils. This was also observed with neutrophils from three different healthy volunteers which were transduced with GFP lentivirus for 4 hours at 4°C (figure 5.3.5.3). This however probably resulted from neutrophils from different donors undergoing apoptosis at different rates after they are isolated from the blood.

5.3.6 Lentiviral transfection of myeloid PLB-985 cells

As the lentiviral transduction method was unsuccessful in primary human neutrophils, it was tried in myeloid PLB-985 cells instead. Incubation of PLB-985 cells with GFP lentivirus for 48 hours produced a cell population where 70-90% of the cells were producing GFP (figure 5.3.6.1). The percentage of cells producing GFP increased as the volume of lentivirus that they were incubated with increased.

These cells became stably transfected with the GFP gene carried by the lentivirus. When the cells were differentiated into neutrophil like cells they still expressed the GFP protein (figure 5.3.6.2). So loss of the construct during differentiation was not a problem with this method of transfection as it had been with others (see section 5.3.2). The cells which had been differentiated and still expressed GFP were still able to phagocytose zymosan particles (figure 5.3.6.3) showing that the transfection had not impacted upon the neutrophil like characteristics of these cells. Phagocytosis was complete within around 1 minute, a similar time to that taken by primary human neutrophils.

Differentiated PLB-GFP cells were also able to phagocytose multiple zymosan particles further confirming their neutrophil like characteristics (figure 5.3.6.4).

It was concluded that this approach could generate functionally competent human phagocytes with sufficient GFP signal to permit physiological experiments. This is the method of transfection that will be used for the remainder of the project.

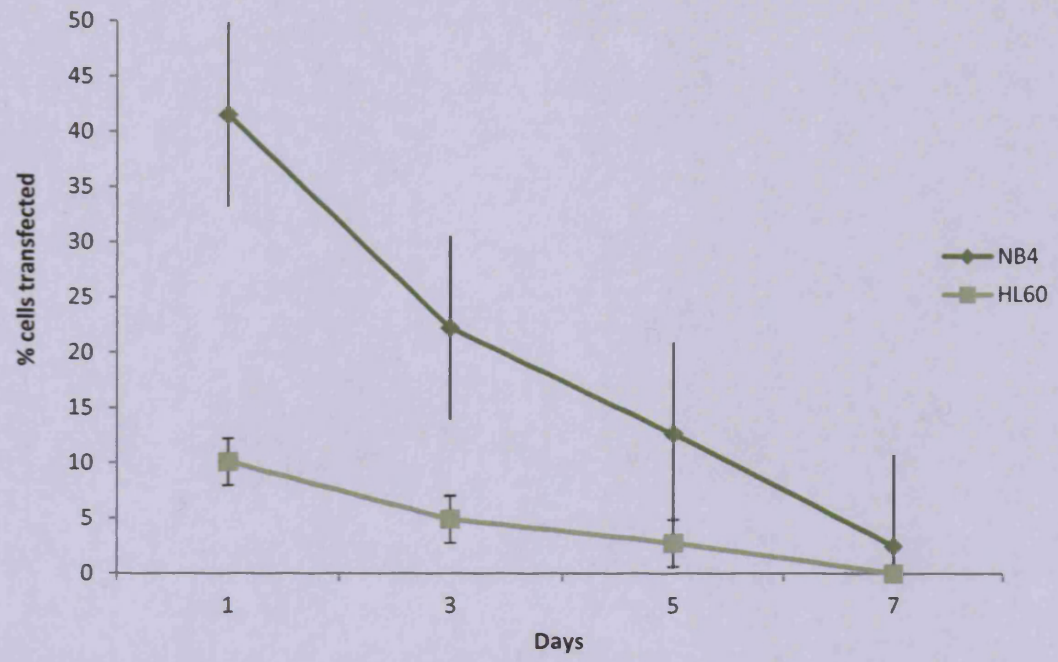


Figure 5.3.2.1: The average transfection frequencies of HL60 cells and NB4 cells when transfected with the pmaxGFP plasmid using the Amaxa nucleofector device. Neither cell line becomes stably transfected, even with antibiotic selection. NB4 cells express GFP at higher levels initially and as a result express the protein for slightly longer than the HL60 cells. (data representative of <300 cells over 3 separate experiments).

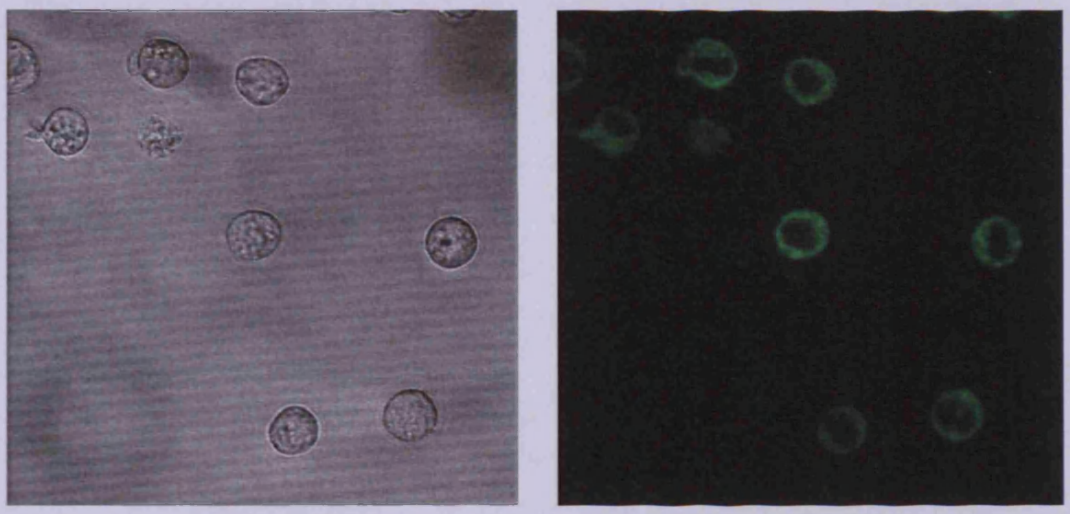


Figure 5.3.3.1.1: MyPH8B6 cells expressing calpain1-GFP. MoFlo cell sorting isolated a population of cells of which approximately 90% of the population were expressing GFP.

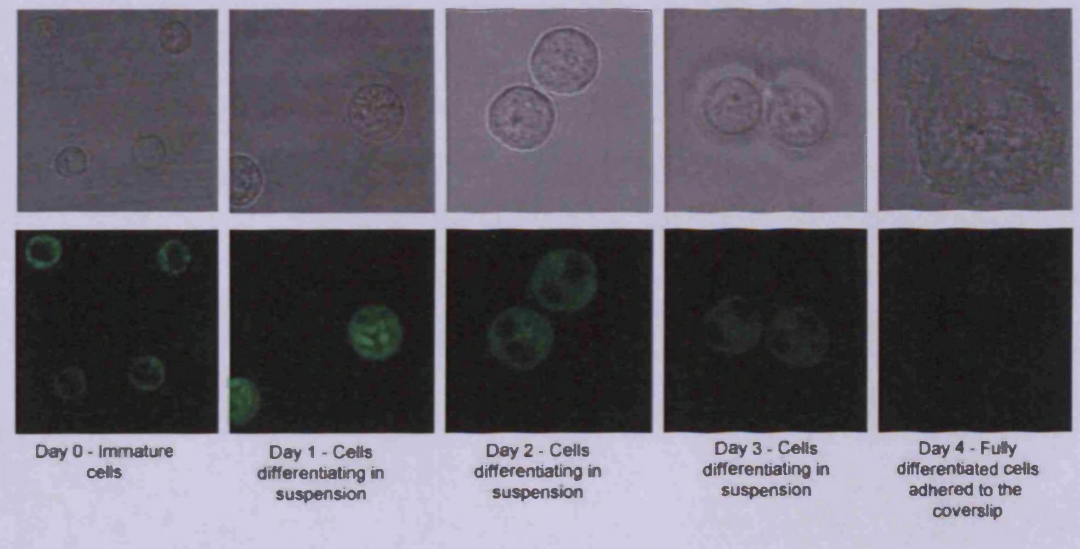


Figure 5.3.3.1.2: MyPH8B6 cells imaged as they undergo differentiation into mature neutrophils. Cells lose GFP expression once they become fully differentiated.

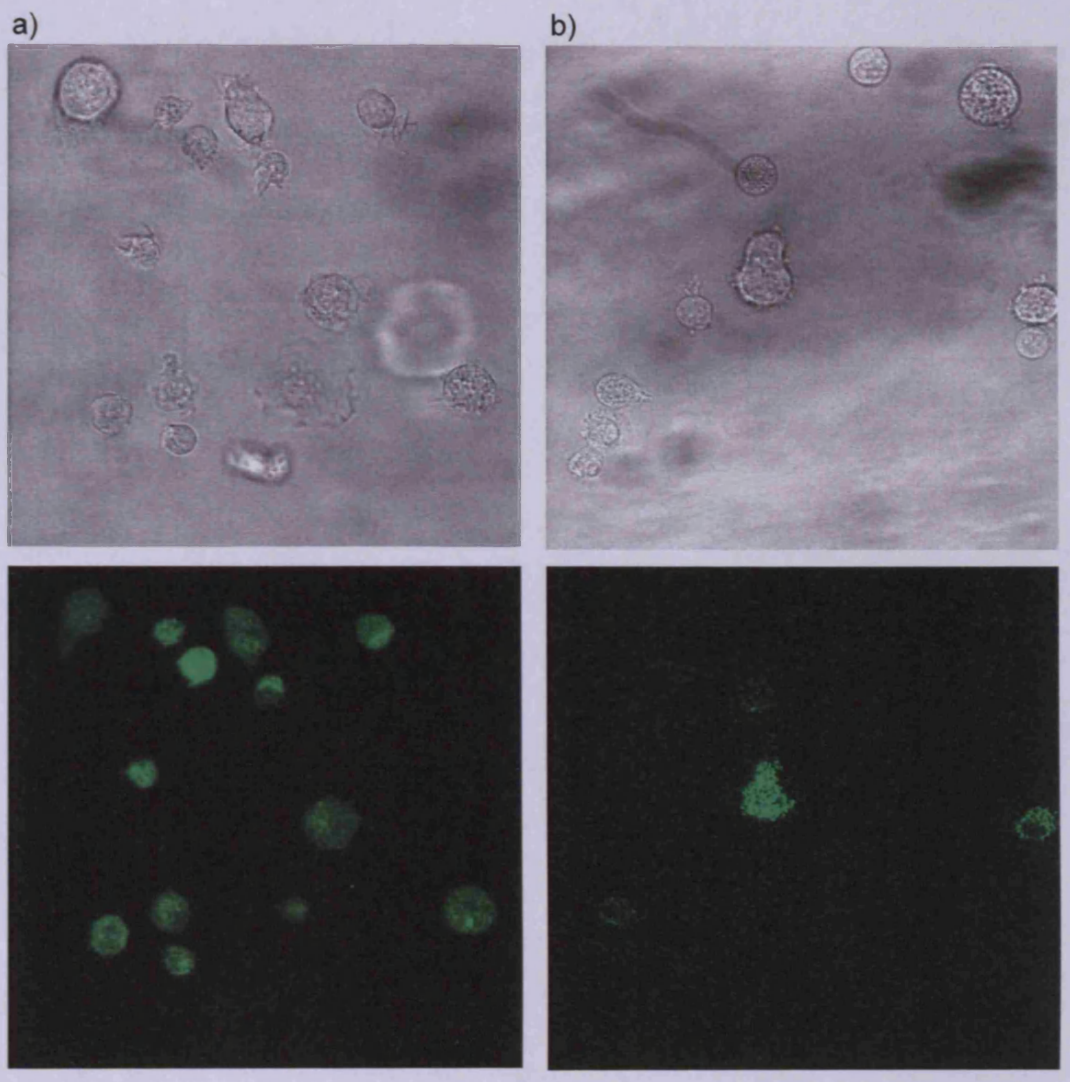
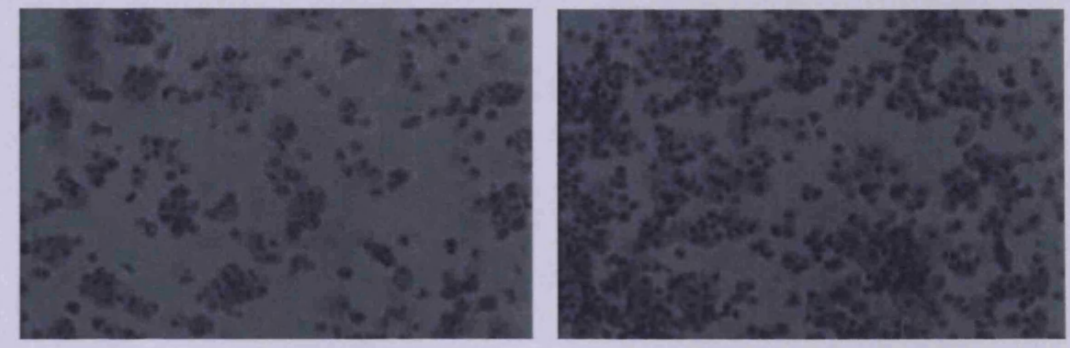


Figure 5.3.3.2.1: Expression of calpain1-GFP in human umbilical cord blood derived CD34+ myeloid stem cells at different time points after transfection. a) GFP expression in CD34+ cells on day 1 after transduction with the calpain1-GFP retrovirus. b) GFP expression in CD34+ cells after 23 days differentiation.



CD34+ control cells CD34+Calpain1-GFP cells

Figure 5.3.3.2.2: CD34+ control cells and CD34+ cells transduced with calpain1-GFP retrovirus after 23 days differentiation. Cells were cytospun and stained with wright-giemsa stain. There is no difference in nuclear morphology between the control cells and the calpain1-GFP transduced cells.

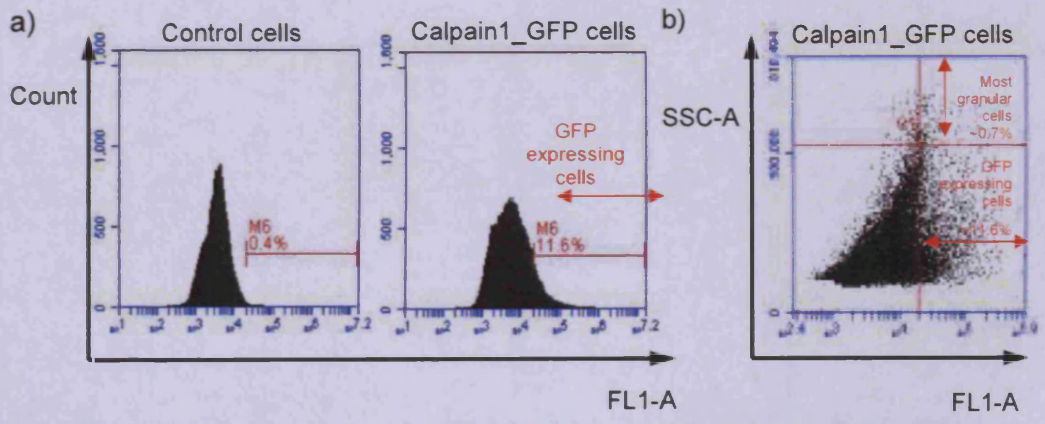


Figure 5.3.3.2.3: Flow cytometry analysis of CD34+ control cells and CD34+ cells transduced with calpain-GFP retrovirus after 21 days of differentiation. a) Approximately 11% of calpain1-GFP transduced cells were expressing GFP after 21 days differentiation. b) Analysis if side scatter showed that less than 1% of the population were granular enough that they could potentially be neutrophils and were also in the group that were still expressing GFP.

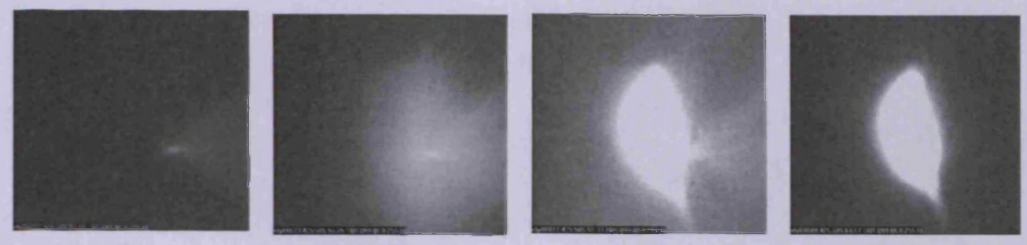


Figure 5.3.4.1.1: An example of lucifer yellow being introduced into 3T3 cells by single cell electroporation using a 0.5-1 second train of 50V, 1ms 200Hz square pulses *(n=5).* Within 0.77 seconds the Lucifer yellow had been introduced into the cell. The cell was still glowing 13 seconds later.

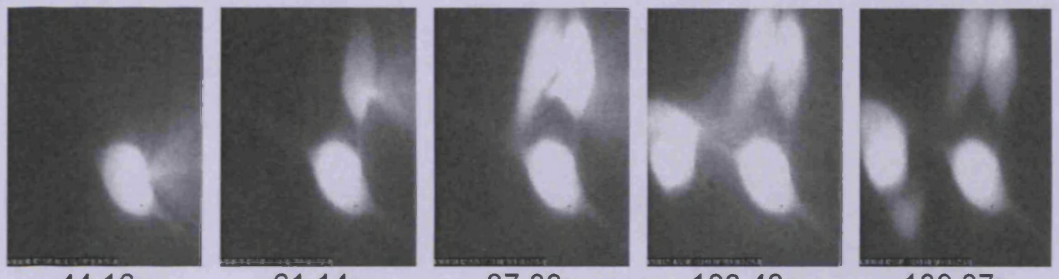
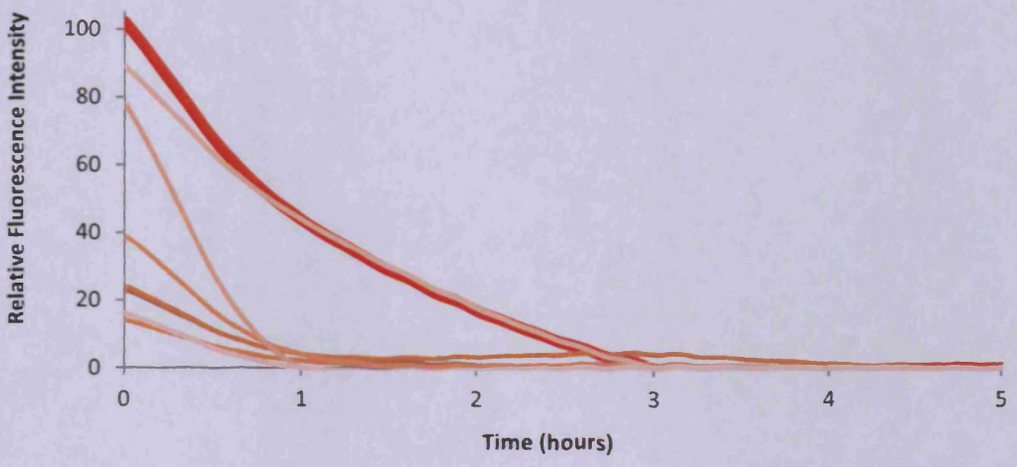


Figure 5.3.4.1.2: An example of 3T3 cells electroporated with 0.5-1 second trains of 1ms long square pulses at 10V and 200Hz *(n=8).* Multiple cells were easily electroporated in a short space of time.

a)



b)

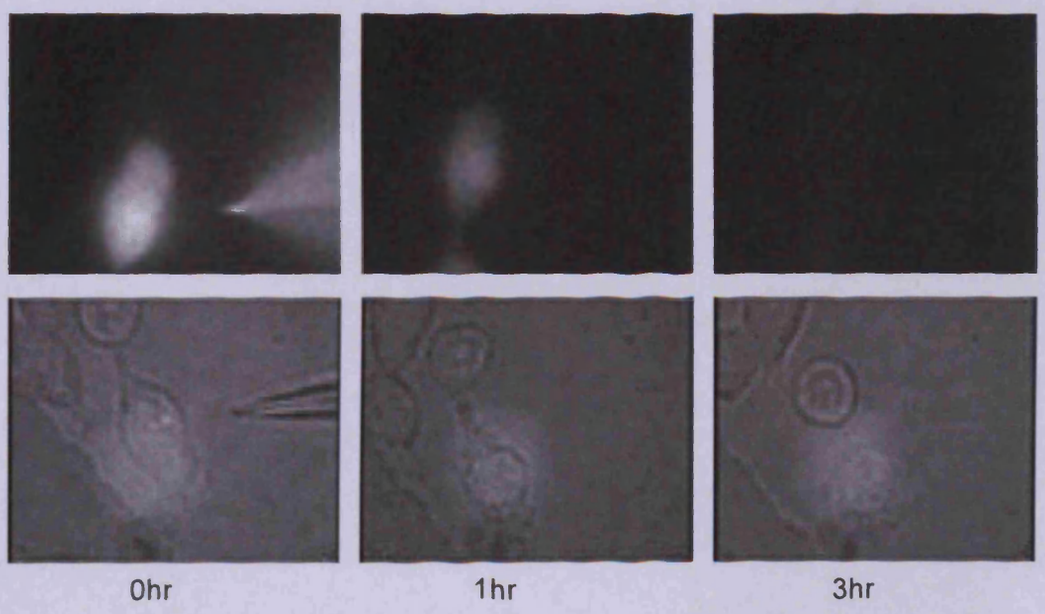
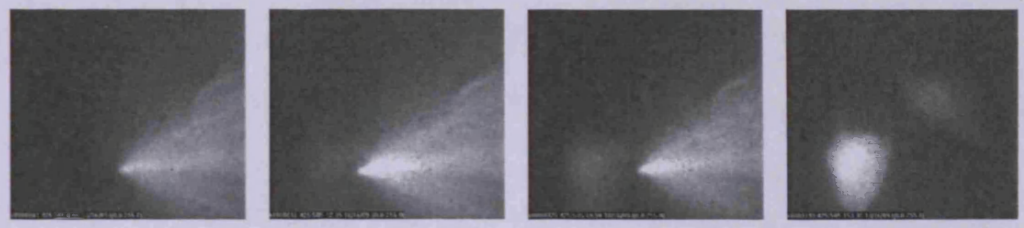


Figure 5.3.4.1.3: Lucifer yellow introduced into 3T3 cells using electroporation by 0.5-1 second trains of 10V, 1ms, 200Hz pulses *(n=8)*. a) Fluorescence of the cells decreased exponentially after electroporation. Most cells were dead within 1 hour, no cells survived longer than 3 hours. Each line represents a single cell electroporated. b) An example of one of the cells shown in the graph (indicated by the bold red line in (a)) showing fluorescence at 0, 1 and 3 hours post electroporation.



0.69s 12.35s 19.21s 153.35s

Figure 5.3.4.1.4: 3T3 cells electroporated using single 10V pulses. In the few cases *(n=3)* where it was possible to introduce Lucifer yellow into the cells using this method much less material was introduced than when using trains of pulses and it was necessary to hold the pipette to the cell for some time after electroporation to allow particles to travel from the pipette into the cell.

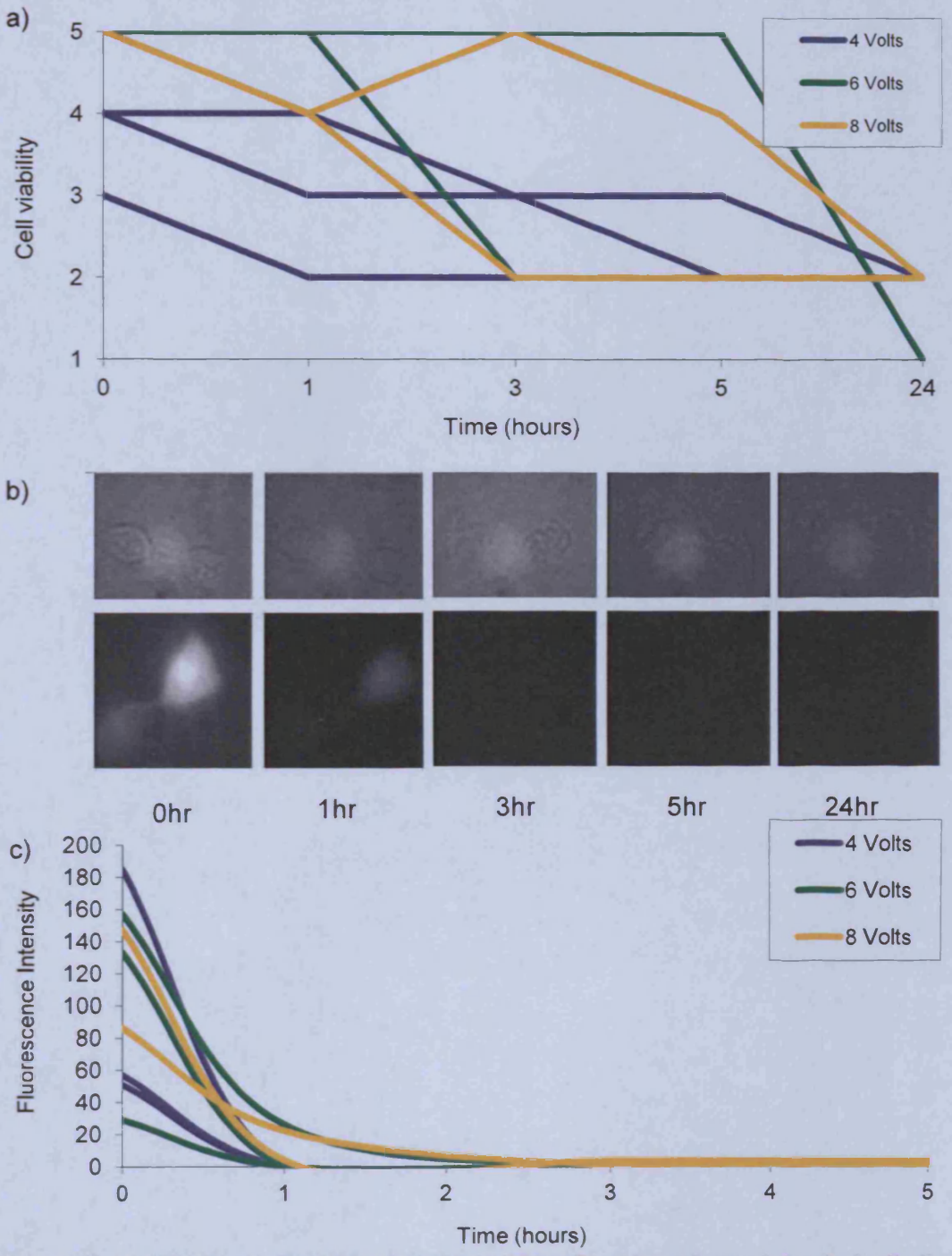


Figure 5.3.4.2.1: Lucifer yellow introduced into 3T3 cells using a larger (~1µm) pipette tip diameter and 0.5-1 second trains of 8V, 6V or 4V, 1ms, 200Hz pulses *(n=7)*. a) Cell viability scored from 1-5 with 1 being 'cell has died and detached from coverslip' through to 5 being a 'healthy cell'. No overall difference in cell viability was observed between the voltages tested. b) Shows an example of a cell electroporated with 6V trains of pulses, illustrating how the fluorescence of the cell decreased and the cell necrosed over time after the procedure. c) Fluorescence intensity of cells electroporated. Lucifer yellow was successfully introduced into the cells but the fluorescent intensity decreased exponentially after electroporation.

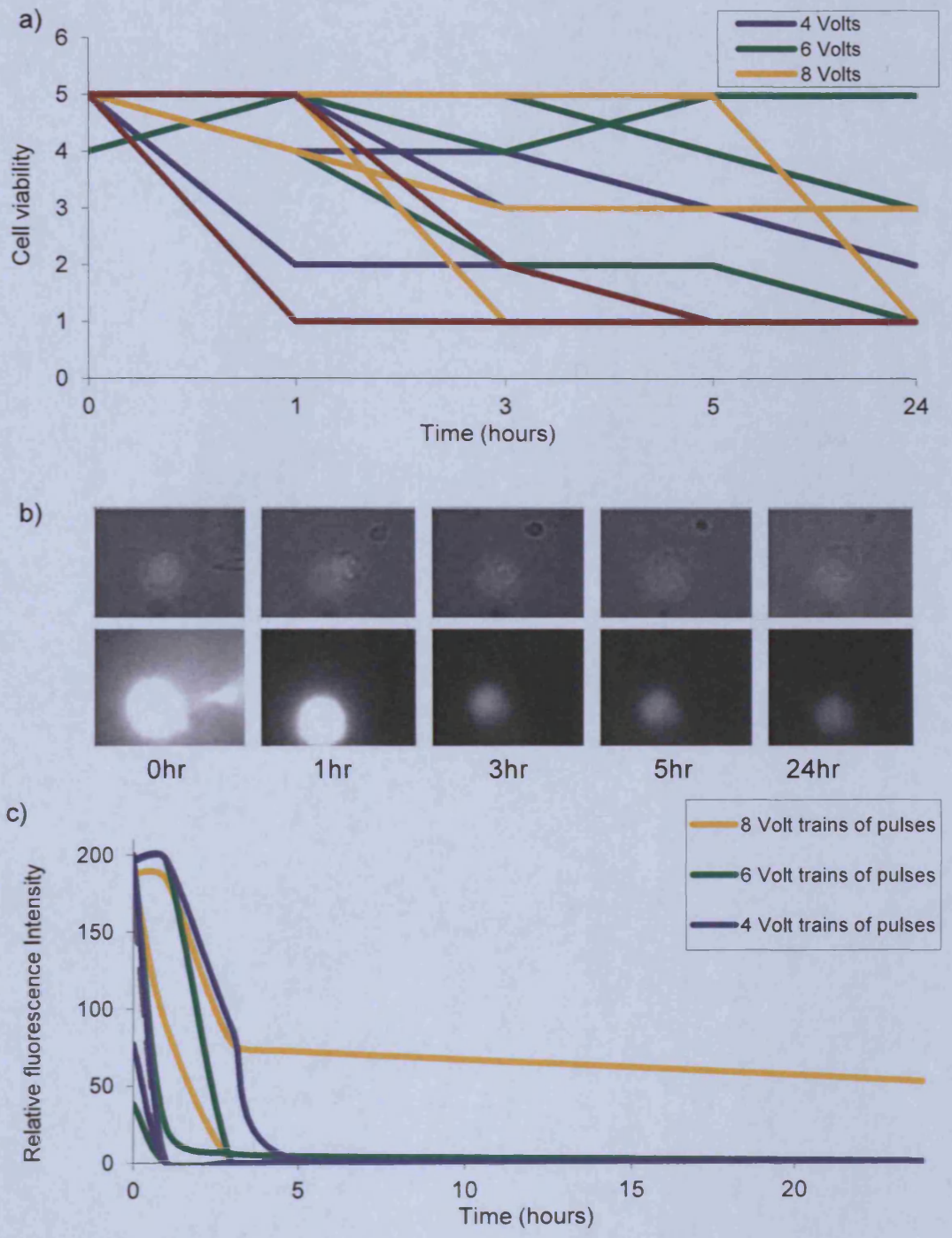


Figure 5.3.4.2.2: Lucifer yellow introduced into 3T3 cells using a smaller (~0.5µm) pipette tip diameter and 0.5-1 second trains of 8V, 6V or 4V, 1ms, 200Hz pulses *(n=8)*. a) Cell viability scored from 1-5 with 1 being 'cell has died and detached from coverslip' through to 5 being a 'healthy cell'. Two of the cells electroporated survived the procedure for 24 hours. This however showed no relationship to the voltage used to electroporate them. b) Shows an example of a cell electroporated with 8V trains of pulses, illustrating how the fluorescence of the cell decreased for the first 3 hours after the procedure before remaining constant. c) Fluorescence intensity of cells electroporated. Lucifer yellow was successfully introduced into the cells. The fluorescent intensity decreased exponentially after electroporation with all cells apart from one showing no fluorescence after 5 hours.

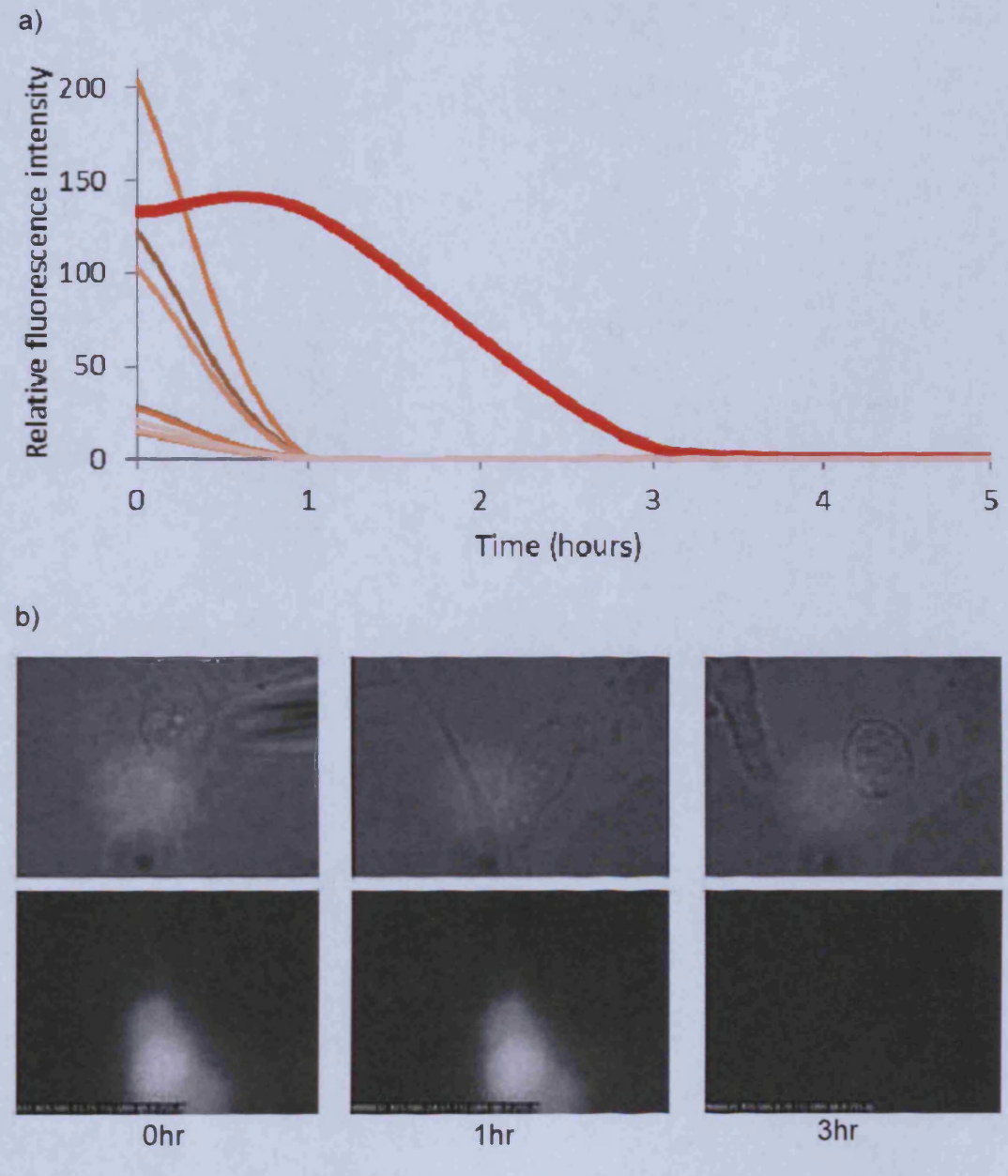
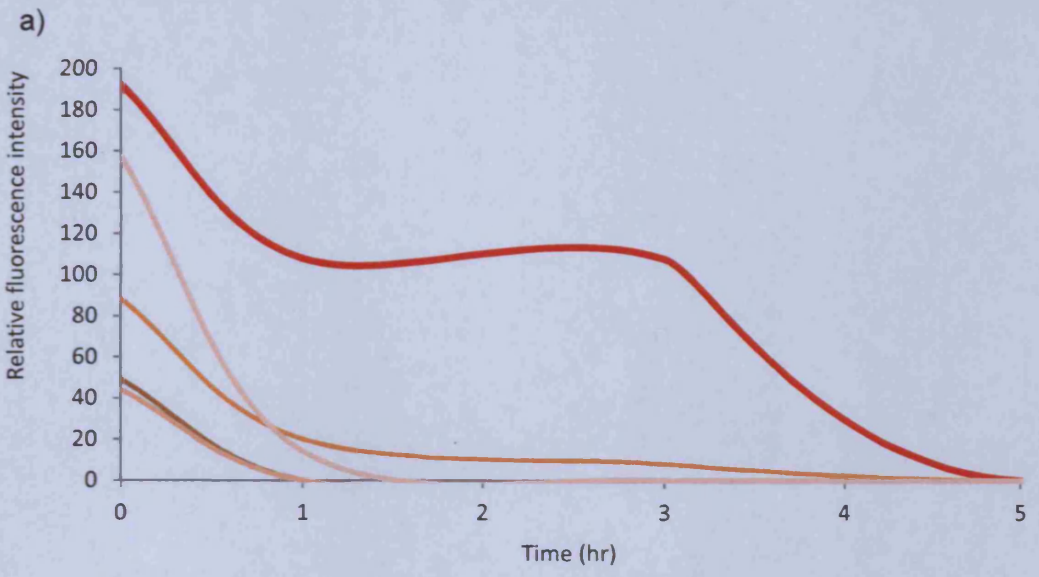


Figure 5.3.4.3.1: Lucifer yellow introduced into 3T3 cells along with nucleofector solution V using electroporation by 0.5-1 second trains of 10V, 1ms, 200Hz pulses *(n=8).* a) Fluorescence of the cells decreased exponentially after electroporation. Most cells were dead within 1 hour, no cells survived longer than 3 hours. b) An example of one of the cells shown in the graph (indicated by the bold red line in (a)) showing fluorescence at 0, 1 and 3 hours post electroporation.



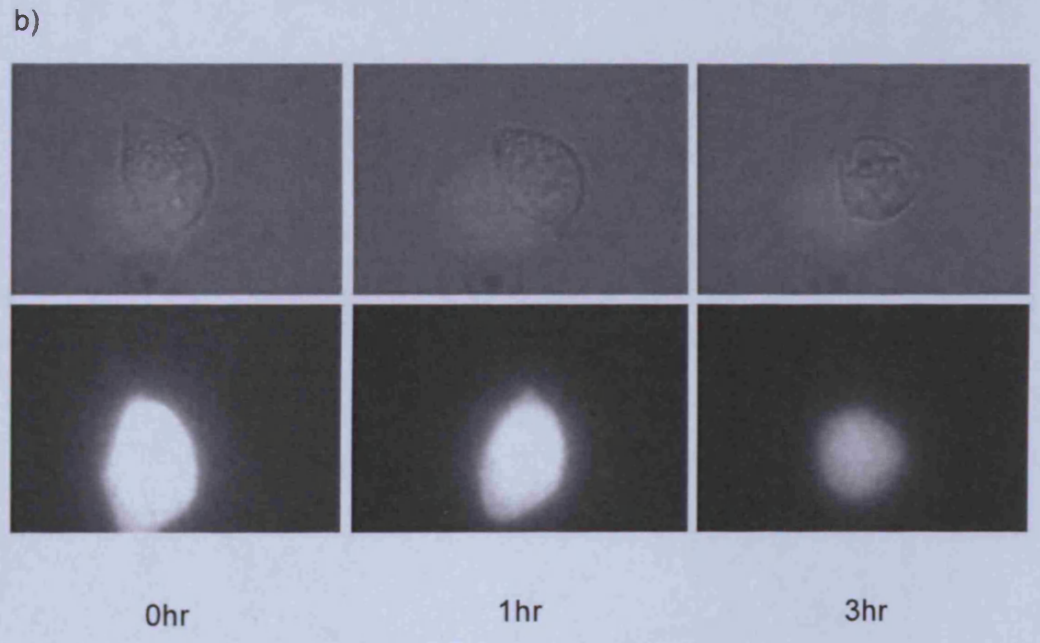


Figure 5.3.4.3.2: Lucifer yellow (diluted in mammalian saline) introduced into 3T3 cells (bathed in mammalian saline) using 0.5-1 second trains of 6V, 1ms, 200Hz pulses *(n=5)*. a) In most cases fluorescence decreased exponentially after the procedure. The one cell that was the exception to this was no longer fluorescent after 5 hours. b) An example of one of the cells shown in the graph (indicated by the bold red line in (a)) showing fluorescence at 0, 1 and 3 hours post electroporation.

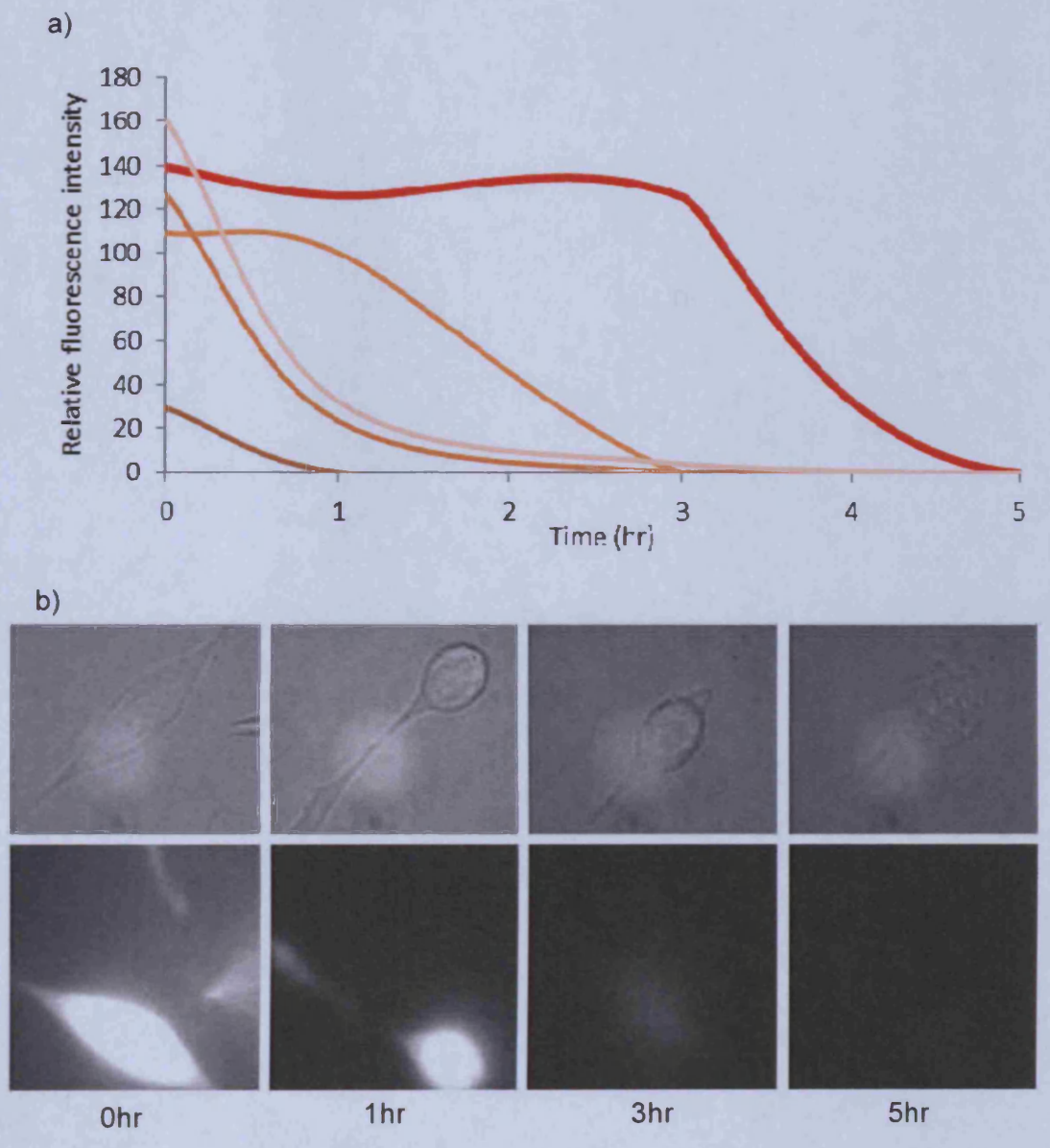


Figure 5.3.4.3.3: Lucifer yellow (diluted in Krebs buffer) introduced into 3T3 cells (bathed in Krebs buffer) using 0.5-1 second trains of 6V, 1ms, 200Hz pulses *(n=5)*. a) In most cases fluorescence decreased exponentially after the procedure. The one cell that was the exception to this was no longer fluorescent after 5 hours. b) An example of one of the cells shown in the graph (indicated by the bold red line in (a)) showing fluorescence at 0, 1 and 3 hours post electroporation.

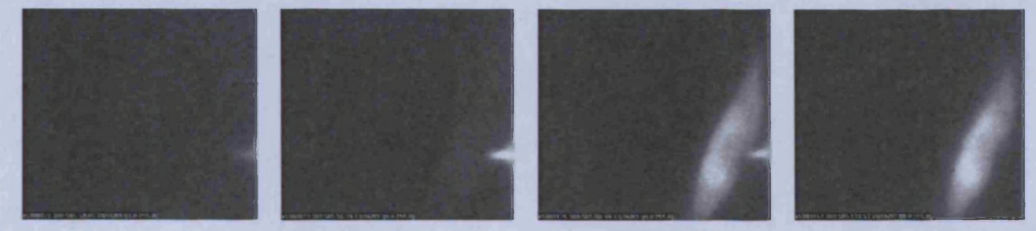


Figure 5.3.4.4.1: An example of cell impermeant fura2 introduced in a 3T3 cell using single cell electroporation using 0.5-1 second train of 10V, 1ms, 200Hz pulses *(n=6).* Fura2 was successfully introduced into the cell. Transfer of material from the pipette into the cell was improved if the pipette was held to the cell for several seconds after electroporation.

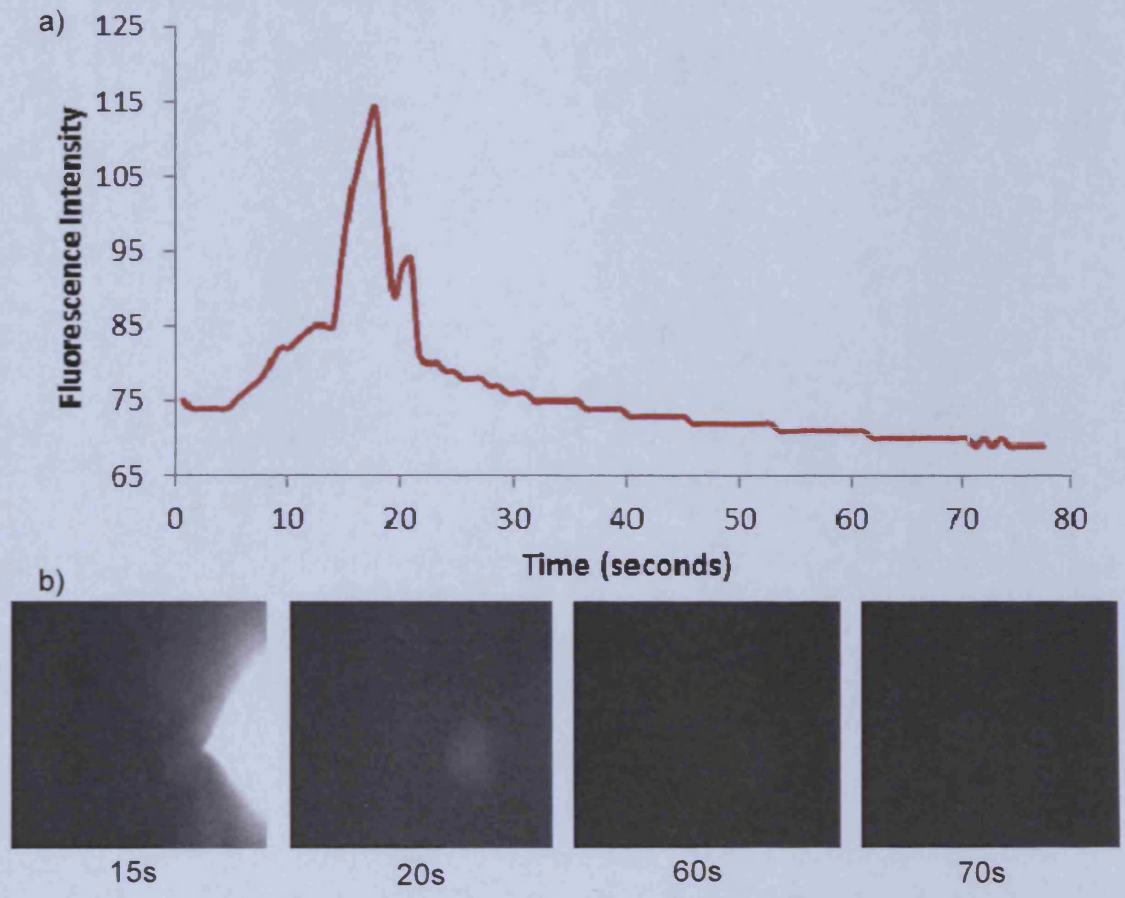


Figure 5.3.4.4.2: Cell impermeant fura 2 introduced into differentiated PLB-985 cells using single cell electroporation with a 0.5-1 second train of 20V, 1ms, 200Hz pulses. The pulse was applied at t=15 seconds. a) Fluorescence decreased in an exponential manner immediately after the electroporation followed by cell death.

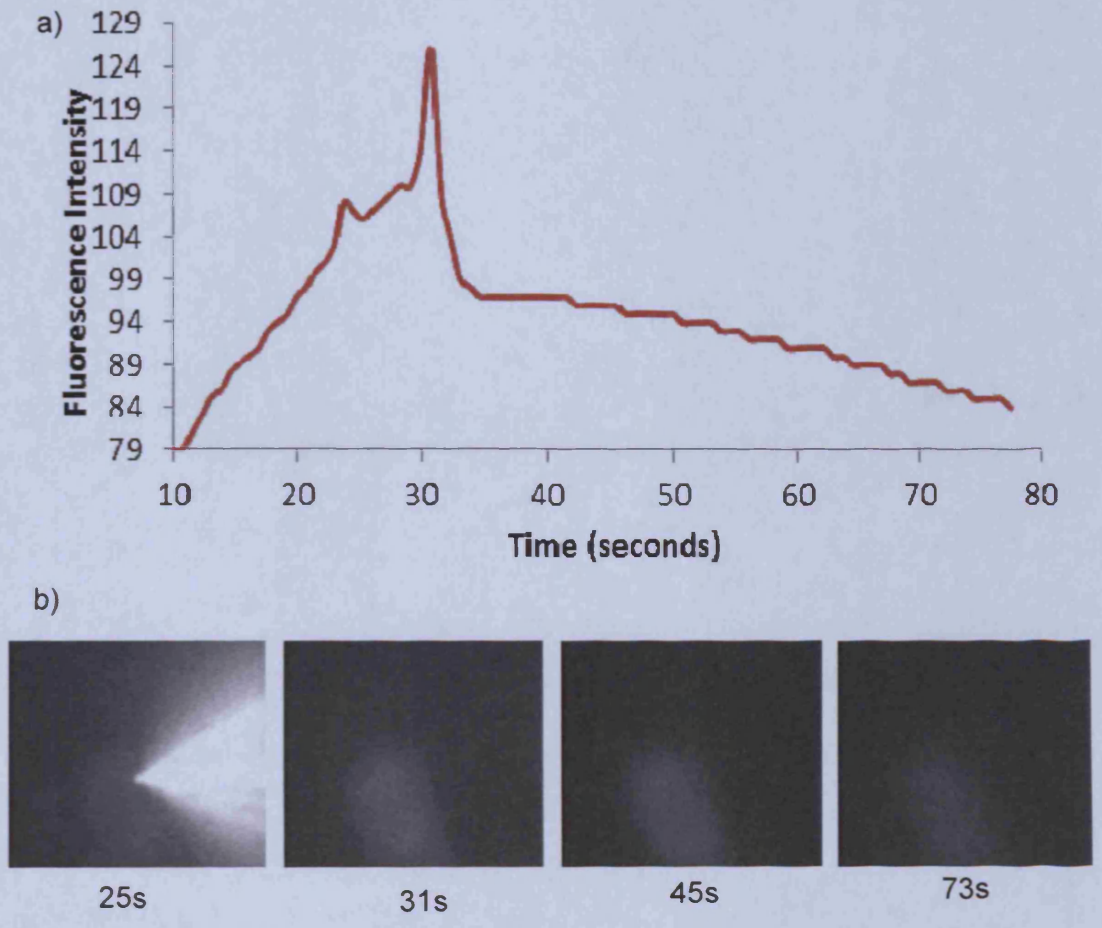


Figure 5.3.4.4.6: Cell impermeant fura2 introduced into differentiated PLB-985 cells using a single pulse of 10V. The pulse was applied at t=28 seconds. Fluorescence decreased exponentially after the pipette was removed.

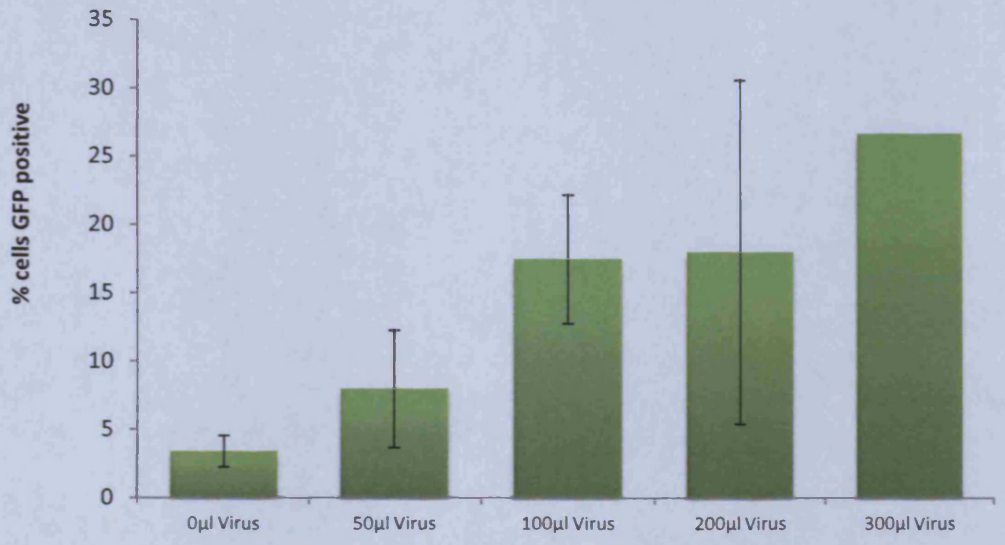


Figure 5.3.5.1: % of neutrophils apparently GFP positive after lentiviral transduction when analysed with flow cytometry. % neutrophils which are apparently GFP positive increases when cells are treated with increasing volumes of GFP virus.

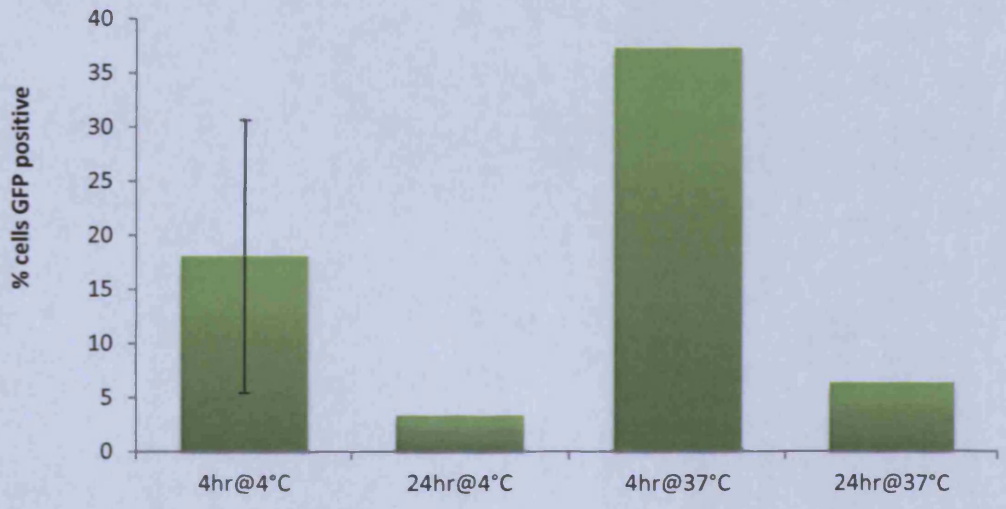


Figure 5.3.5.2: % of neutrophils apparently GFP positive after lentiviral transduction at different temperatures when analysed with flow cytometry. Cells were incubated with 200µl virus for 4 or 24hr at 4°C or 37°C. The % of neutrophils which are apparently GFP positive increases when the cells are incubated with virus at 37°C rather than 4°C. Incubation with the virus for 24hrs leads to a decrease in cells which are GFP positive.

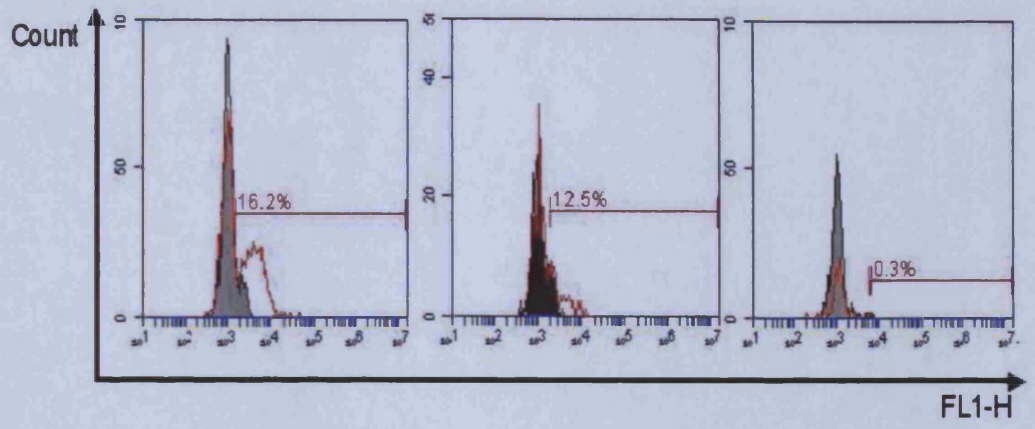


Figure 5.3.5.3: Donor variation in apparent levels of GFP expression in neutrophils as reported by Dick et al. Neutrophils were isolated from three healthy volunteers and transduced with GFP lentivirus for 4 hours at 4°C. Apparent levels of GFP expression when analysed by flow cytometry after 4 hours varied by individual.

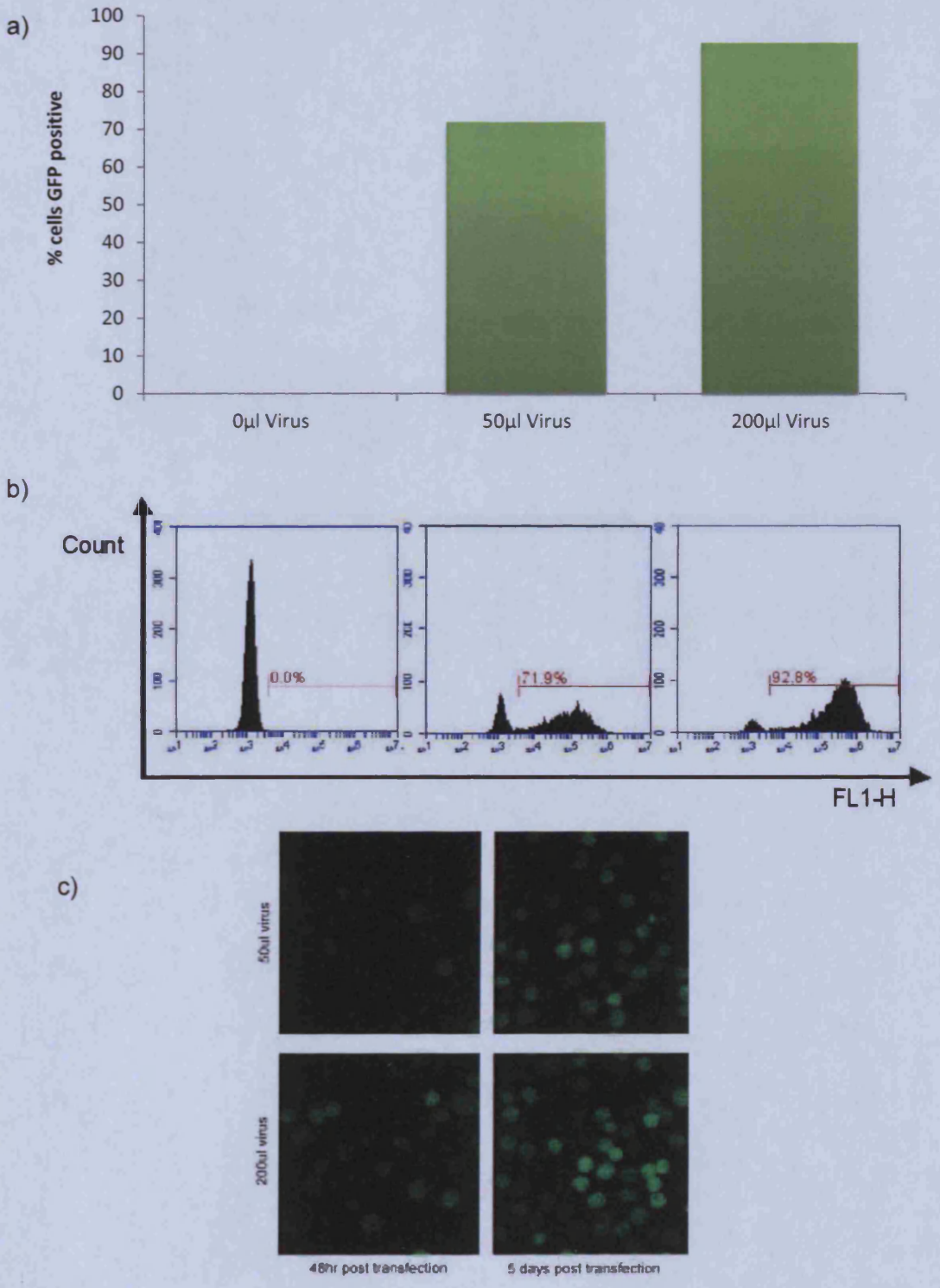
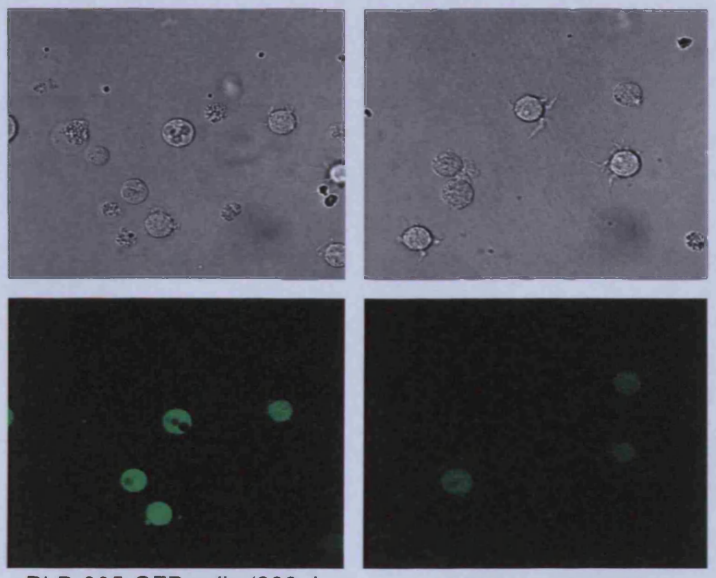


Figure 5.3.6.1: GFP expression in PLB-985 cells transduced with GFP lentivirus. PLB-985 cells were transduced with GFP virus for 48hrs at 37°C. Cells were transduced with either 50µl or 200µl GFP virus. (a&b) Over 90% of the cells transduced with 200µl virus were expressing GFP after 48hrs. (c) Cells were still expressing GFP 5 days post transfection. It was concluded that the cells may be stably transfected.



PLB-985-GFP cells (200µl virus) differentiated in 1.3% DMSO for 9 days

PLB-985-GFP cells (50µl virus) differentiated in 1.3% DMSO for 9 days

Figure 5.3.6.2: GFP expression in PLB-985 cells 9 days after transfection. PLB-985-GFP cells differentiated in 1.3% DMSO for 9 days. Cells are still expressing GFP after differentiation and display characteristics of successful differentiation (i.e. adhesion to glass, irregular morphology and extended membrane processes, multilobed nuclei, phagocytosis)

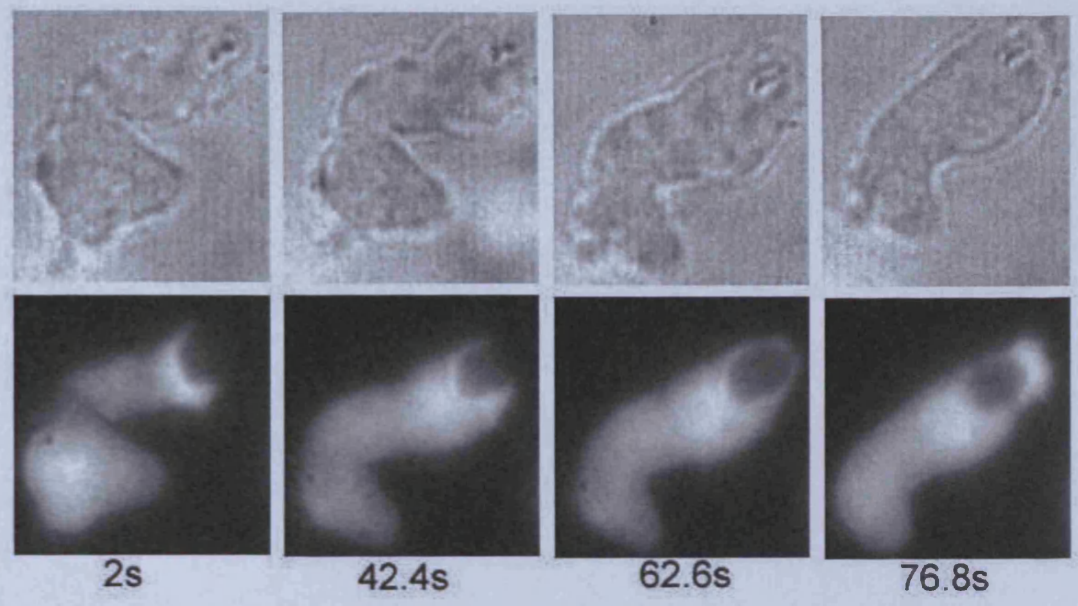


Figure 5.3.6.3: An example of a GFP transduced PLB-985 cell phagocytosing an opsonised zymosan particle after 5 days differentiation in 1.3% DMSO *(n<3)*. HL60 cells which had been transduced with GFP were grown in culture for 2 weeks prior to differentiation for 5 days in 1.3% DMSO. After 5 days differentiation cells were able to phagocytose serum opsonised zymosan particles. This particular example took 1 minute 4 seconds to completely engulf the particle.

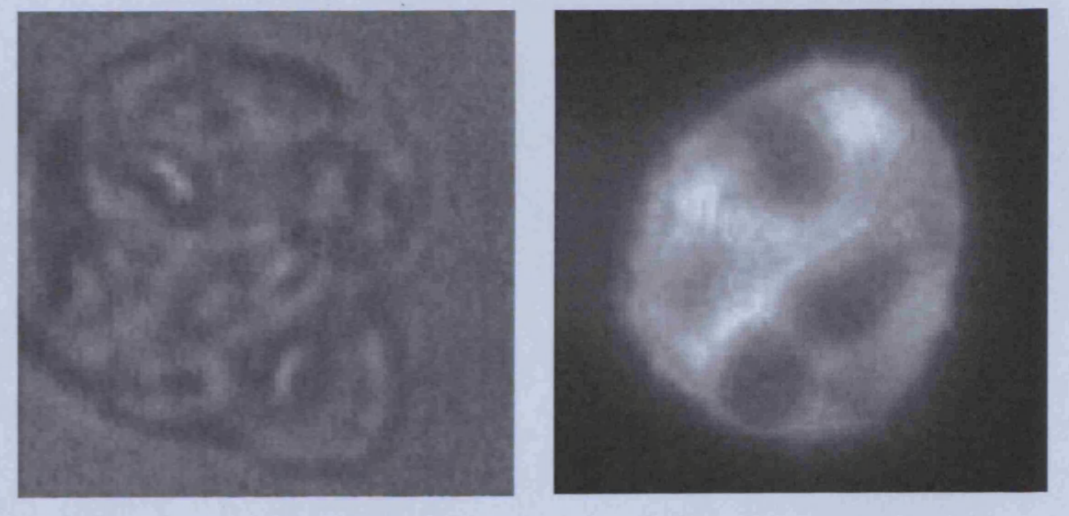


Figure 5.3.6.4: GFP transduced HL60 cell which had phagocytosed at least 4 opsonised zymosan particles after 5 days differentiation in 1.3% DMSO. HL60 cells which had been transduced with GFP virus were grown in culture for 2 weeks before being differentiated for 5 days in 1.3% DMSO. After 5 days differentiation cells were incubated with opsonised zymosan particles for 30 minutes. After 30 minutes most cells had taken up one or more zymosan particles. This cell is an example of a cell which had taken up 4 zymosan particles.

5.4 Discussion

The work in this chapter has established the unsuitability of a number of widely-used methods for transfecting neutrophils and myeloid cells. This is because (i) terminally differentiated cells have few recovery mechanisms and the "insult" of transfection results in their death by necrosis or apoptosis and (ii) both mature human neutrophils and functionally competent phagocytes terminally differentiated from cell lines synthesis very little protein. It was necessary to develop a methodology which gave a highly efficient transfection of the growing precursor cells (cell line) such that sufficient expressed protein remained when protein synthesis was shut-down during terminal differentiation.

5.4.1 Transfection of myeloid cell lines using nucleofection

The work presented in this chapter showed that although undifferentiated HL60 and NB4 cells were more easily transfected than differentiated or partially differentiated cells, the expression of the protein was very transient. It was not possible to achieve stable transfection of these cells, but it was possible to transfect NB4 cells that had been partially differentiated. Although these cells retained the protein when fully mature, they did not display any neutrophil-like characteristics such as phagocytosis or a cytosolic calcium signal in response to fMLP. They therefore, were not suitable to use in this project.

Another myeloid cell line, HL60s expressed transfected protein after differentiation but this was too transient to be able to reliably produce cells that were both differentiated and had the protein. When differentiated though these cells display many neutrophil-like characteristics (Mollinedo et al., 2008). It is possible that HL60 cells were more difficult to transfect than the NB4 cell line as they were slightly further along the maturation pathway towards neutrophils when they were immortalised and as such their potential for producing new proteins is decreased when compared to that of NB4 cells. This may also be

why when differentiated, the HL60 cell line displays many more neutrophil like characteristics than the NB4 cell line.

It has not been possible to successfully transfect mature neutrophils by nucleofection despite previous reports claiming that the technique works (Johnson et al., 2006). This is probably because neutrophils are terminally differentiated cells which are no longer undergoing cell division or producing significant amounts of new protein and these are essential features needed if a cell is to be successfully transfected. These same problems apply when trying to transfect mature (differentiated) HL60 cells.

5.4.2 Retroviral transduction of mouse myeloid progenitor cells and human cord blood derived stem cells

Retroviral transfection was investigated as a more promising method of achieving a more stable level of transfection in neutrophil like cells.

The MyPH8B6 cell line is a mouse myeloid progenitor cell line isolated and immortalised from mouse bone marrow (McDonald et al.). These cells can be cultured in an immortalised state in the presence of oestrogen or can be differentiated into neutrophils when cultured in the presence of G-CSF. CD34+ myeloid progenitors are isolated from human cord blood samples. These are human myeloid progenitor cells which can be differentiated in the presence of IL-3, G-CSF and SCF over 21-24 days. These cells differentiate into all types of myeloid cells including neutrophils, although some reports state that neutrophils produced in this manner are not fully functional (Dick et al., 2008). It was possible to stably transfect both of the above mentioned cell types with the pMX-Mu-calpain-EGFP retroviral vector before the cells were differentiated. However the expression of the protein decreased as the cells differentiated.

This decrease in expression was also observed when the MyPH8B6 cells were transfected with another construct, dectin 1(McDonald et al.).

The decrease in the expression of the gene as the cells differentiate is likely to be dependent upon where in the genome the gene is integrated when the cell is infected with the retrovirus. Many genes are 'switched off' during the differentiation process and if the calpain-GFP construct was integrated into one of these genes then expression of calpain-GFP would also be decreased as the cells differentiate. The calpain-GFP protein which has already been produced by the cell before it started to differentiate would still be free in the cytoplasm, The amount of active protein remaining would depend on the life-time of the protein in the cytosol as it is degraded after differentiation. Although stability of calpain-GFP in the cytosol is not known, this effect would account for the decrease in fluorescence seen during the differentiation process.

5.4.3 Single cell electroporation

Single cell electroporation has been reported to be successful in a number of different cell types (Haas et al., 2001, Rae and Levis, 2002). However, the work here shows that it is only successful under very specific parameters. Cells were sensitive to the voltage applied, the diameter of the pipette tip and the extracellular environment. Although the transfer of material into the cells was very successful, the long term viability of the cells after the procedure was always a problem. Instead of closing the electroporation pores immediately after the electric current had stopped and the pipette was the removed, the pores remained open for a number of hours. This inevitably led to cell death.

5.4.4 Lentiviral transduction of human neutrophils

It has been previously reported that it was possible to transfect neutrophils using the lentiviral method of transfection (Dick et al., 2009). This study used flow cytometry to determine the levels of transfection. When samples of neutrophils which had been transduced with GFP lentivirus were analysed using flow cytometry it did appear that they had increased levels of GFP expression. However, when these same samples were imaged using the CLSM no GFP expressing cells were found. Some of the cells which were dead or necrosed exhibited some auto fluorescence though. The increase in GFP positive cells found by the flow cytometer when cells were incubated with increased amounts of virus could simply be due to increasing numbers of dead cells which are auto-fluorescent. The same explanation can be applied to explain the increase in GFP positive cells found when cells were incubated at 37°C as opposed to 4°C as it is known that once out of the body, neutrophils undergo spontaneous apoptosis when incubated without the G-CSF or GM-CSF (Simon, 2003). As this is a biological process it naturally occurs faster at 37°C than it does at 4°C. It is unlikely that this is a method that can be applied to mature neutrophils as they lack the characteristics that are necessary for a successful transfection as discussed in sections 5.1.3 and 5.4.1.

5.4.5 Lentiviral transduction of myeloid PLB-985 cells

Although lentivirus as a method of transfection was unsuccessful for mature neutrophils, the technique was more successful when used to transduce undifferentiated PLB-985 cells. A PLB-985 cell line stably expressing GFP was generated using this technique and sufficient GFP was retained by the cells after they were full differentiated. There was no decrease in GFP levels during differentiation as had been observed when CD34+ cells or MyPH8B6 cells were transfected with the retrovirus. This may be due to the integration site of the gene in the host cells genome as discussed in section 5.4.4 in relation to retroviruses; or it could be that GFP is more stable in the cytoplasm as the cells

differentiate than is the calpain-GFP expressed in CD34+ cells and MyPH8B6 cells. The ability of lentiviruses to transfect cells which are not dividing may have meant that the efficiency of the transduction before the cells were differentiated was higher than that achieved with the retrovirus and as such the effect of the decrease in expression of the gene as the cells differentiate was minimised. The decrease in expression during differentiation may become more evident when a larger gene is transduced into the cells. However this technique was found to be the most efficient and reliable of all of those that were investigated and is the main technique which will be used to express fluorescently tagged proteins in PLB-985 cells for the future of this project.

5.4.6 Overall discussion

Achieving a level of transfection in myeloid cell lines which is stable enough that the protein of interest is still expressed when the cells are fully matured into neutrophil like cells is a hard task, and this has only rarely been reported. There are just two stably transfected HL60 cell lines generated, one expressing Akt-GFP (Servant et al., 2000) and the other p67-phox-GFP (van Bruggen et al., 2004). In addition to this, some success has been reported in transiently expressing proteins in HL60s. HL60s which have been transiently transfected have been reported by Lacelle et al (Lacalle et al., 2007) and Huttenlocher (Nuzzi et al., 2007). Despite these few successes, these cells are notoriously difficult to transfect and are rarely used in such studies. However, this technique is essential for the success of this project, as it is not possible to transfect mature neutrophils. It is essential to find an amenable and most appropriate model to investigate the importance of calpain in neutrophil spreading. After investigating several techniques, it was decided that the best way to achieve this was through lentiviral transduction of undifferentiated PLB-985 cells. This was the only method which achieved levels of transfection stable enough that the protein was still being expressed by the cells after they were fully differentiated. The reasons for this have been discussed in the previous sections. Nucleoporation, a modified form of traditional

electroporation, did not result in stable or high levels of transfection. Retroviral transfection of human cord blood stem cells and an immortalised mouse neutrophil cell line resulted in a higher level of transfection. However as this was not stable, by the time the cells were fully differentiated and functionally mature, the levels of protein retained in the cytoplasm were too low to be useful. Single cell electroporation proved to be a reliable way of introducing foreign material into cells but long term survival of the cells using this technique was never achieved. Although this technique may not be appropriate for the introduction of genetic material into cells it may still serve a function for more short term studies. Despite a report that lentiviral mediated transduction of neutrophils is possible (Dick et al., 2009), it was not possible to repeat this success. As such lentiviral mediated transduction of undifferentiated PLB-985 cells will be the technique used to express fluorescently tagged proteins in neutrophil like cells for the remainder of this project

CHAPTER 6

Expression of fluorescently tagged calpain in PLB-985 cells

6.1 Introduction

The lentiviral method established in the last chapter was shown to be a successful technique for expressing protein in PLB-985 cells. A stably expressing PLB-985-GFP cell line was generated using this technique and sufficient GFP was retained by the cells after they were full differentiated.

In this chapter, the outcome of adopting this approach will be presented.

6.1.1 PLB-985 cells

PLB-985 cells are a human myeloid leukaemia cell line, grown in suspension which can be differentiated into neutrophil like cells. PLB-985 cells are from the same source and are genetically identical to the more commonly termed HL60 cell line (Drexler et al., 2003). They are thought to have arisen through cross contamination during the establishment of the HL60 cell line. They can be differentiated into neutrophil like cells by incubation in 1.3% DMSO as has been described for the HL60 cell line (Collins et al., 1978, Jacob et al., 2002, Mollinedo et al., 2008). When in their undifferentiated state it is possible to transiently transfect PLB-985 cells. This cannot be achieved in neutrophils. These two features are the two most important features of this cell line for this study as if a stable transfection can be achieved and then the cells differentiated then they provide an ideal model for observing that potential effects of genetic manipulation on neutrophils.

6.1.2 Lentiviral transfection

As established in the previous chapter transfection of the PLB-985 cell line using lentiviral transduction is the only reliable technique to achieve stable levels of transfection in this cell line. This is achieved by exploiting the lentiviruses natural ability to integrate its own DNA into the host cells genome in

quiescent, non rapidly dividing cells. The full molecular basis of retroviral and lentiviral transduction have been discussed in detail in the previous chapter.

6.1.3 Aims of the chapter

The aim of this chapter is to use the lentiviral approach to express calpain constructs in PLB-985 cells.

The objectives are:

- To construct GFP tagged calpain 1, calpain 4 and calpain 1(domain III)
- To clone GFP tagged calpain 1, calpain 4 and calpain 1(domain III) into separate lentiviral expression vectors
- To produce 3 different lentiviruses expressing GFP tagged calpain 1, calpain 4 and calpain 1 (domain III) respectively.
- To produce 3 PLB-985 cell lines stably expressing GFP tagged calpain 1, calpain 4 and calpain 1 (domain III)

These aims will be achieved using a range of standard molecular biological and transfection techniques to produce the GFP tagged calpain, lentiviral expression vectors and the final lentivirus. The 3 lentiviruses produced will then be used to transduce PLB-985 cells with the aim of achieving a stable transfection.

6.2 Materials and Methods

6.2.1 Generation of calpain plasmids

6.2.1.1 Cell line screen for calpain 1 and calpain 4 expression

40 different cell lines and tissues were screened for calpain 1 and calpain 4 expression using CAPN1/C2F1, CAPN1/C2R1, Calp1R1 and Calp4R1 primers. β Actin was used as a control. The general PCR program was followed (figure 6.2.1.1.1), altering the annealing temperature depending on the primers used.

6.2.1.2 RNA isolation from white blood cells

White blood cells were isolated from the blood of healthy volunteers as explained in section 2.4.1. The cells were lysed by repeated pipetting in 1ml TRI reagent, followed by 5 minute incubation at room temperature. 200µl chloroform was added and the cells were vortexed for 15 seconds before being incubated for a further 5 minutes at room temperature. The sample was centrifuged at 12000g for 15 minutes at 4°C. After centrifugation the top phase of the liquid contains the RNA. This top phase was transferred to a fresh eppendorf tube. 500µl isopropanol was added to precipitate the RNA, this was incubated for 10 minutes at room temperature, before being centrifuged at 12000g for 10 minutes at 4°C. The RNA was then found in the pellet at the bottom of the tube. The supernatant was removed and the pellet was washed in 1ml 75% ethanol and vortexed. The pellet was centrifuged for a further 15 minutes at 7500g at 4°C. The pellet was air dried at 55°C before being re-suspended in 50µl pre-warmed DEPC water. RNA concentration was then quantified using a spectrophotometer.

6.2.1.3 Generation of white blood cell cDNA

White blood cell cDNA was generated by reverse transcription PCR from the white blood cell RNA using the Precision qScript™ reverse transcription kit according to the manufacturer's protocol. cDNA was diluted 1:3 in H2O. Quality checking of cDNA was performed using PCR for β actin and calpain expression was checked using CAPN1/C2F1, CAPN1/C2R1, Calp4F1 and Calp4R1 primers

6.2.1.4 TOPO cloning (for generation of GFP tagged calpain)

Calpain 1 and calpain 1 domain III were amplified from white blood cell cDNA using Invitrogen™ Platinum® Taq DNA polymerase high fidelity according to the manufacturer's instructions. Calpain 4 was amplified from HECV cDNA using the same technique. PCR products were then cloned into Invitrogen™ Vivid Colors™ pcDNA™ 6.2 EmGFP/TOPO mammalian expression vectors according to the manufacturers protocol. TOPO cloning takes advantage of the terminal transferase activity if Taq polymerase which adds a single deoxyadenosine to the 3' end of PCR products. The TOPO vector is supplied in a linearised format with a single overhanging 3' deoxythymidine and topoisomerase covalently bound to the vector. The topoisomerase cleaves the phosphodiester backbone of the overhanging 3' deoxythymidine of the vector which covalently links the deoxyadenosine from the PCR product to the deoxythymadine from the vector. This allows for efficient cloning without the need for creating restriction sites on PCR products.

Plasmids were transformed into Invitrogen oneshot® top10 *E.coli* cells according to the manufacturers protocol. Briefly, plasmid is transformed into chemically competent *E.coli* (OneShot® TOP10 cells) by adding the plasmid the cells and then incubating the cells on ice and then heat shocking to permeabalise the cell membrane to allow the plasmid to enter. Nutrient rich

SOC medium is added to increase the efficiency of the transformation. Bacteria were then spread onto Ampicillin selective LB agar plates at different concentrations in order to grow one plate containing evenly spaced colonies. Plates were incubated overnight at 37°C.

The following day 10 colonies were chosen at random and were checked for the presence and orientation of the insert by PCR with T7 forward primer and an insert specific reverse primer. A ~90bp product was observed when the insert was present in the wrong orientation and a larger product was observed when the insert was present in the correct orientation. No product at all was observed when there was no insert present. Colonies which contained the insert in the correct orientation were inoculated into LB medium containing 100ug/ml Ampicillin and were grown overnight at 37°C whilst shaken at 200rpm. Plasmids were then transformed into TOP10 *E.coli* and amplified as described in section 2.4.

6.2.1.5 In-Fusion Advantage PCR cloning (for generation of lentiviral plasmids)

Calpain 1-GFP, calpain 4-GFP and calpain 1 domain III-GFP were amplified from their TOPO vectors using Phusion™ Hot Start High Fidelity DNA Polymerase (New England Biolabs) according to the manufacturer's protocol using primers with 15bp extensions which are homologous to the ends of the linearised vector. The amplified PCR products are then cloned into the linearised Lenti-SxW vector by homologous recombination with the linearised vector using the In-Fusion Advantage PCR cloning kit (Clontech) according to the manufacturer's instructions. Plasmids were then transformed into TOP10 *E.coli* and amplified as described in section 2.4.

6.2.1.6 Plasmid Sequencing

All plasmids for which there were known primer sites before, after or within the insert were sequenced. The resulting sequences were then entered into a BLAST search to confirm that the plasmids contained the correct insert.

6.2.2 Generation of lentivirus

293T packaging cells were prepared by seeding into a T175 tissue culture flasks and incubating at 37°C overnight so that the cells are sub confluent by the morning of transformation. Cells were transformed using Effectene transfection reagent (Qiagen). Briefly, 2µg of plasmid of interest (CAPN1, CAPND3 or CAPNS1-GFP Lenti plasmids), 1.5µg P8.91 plasmid and 1µg pMD2G plasmid were mixed with buffer EC to a final volume of 600µl. Plasmids P8.91 and PMD2G encode the viral DNA and the DNA for the viral envelope. These are transduced on separate plasmids to minimise the chance of recombination occurring and wild type virus being produced. 32 µl of enhancer was added to the plasmid-buffer mixture and the solution was vortexed then incubated at room temperature for 5 minutes. 120 µl effectene reagent was then added to this and the solution was vortexed again and then incubated at room temperature until a precipitate formed. During this time the medium on the cells is replaced with fresh complete medium. 5ml complete medium is then added to the transfection complexes and mixed before adding the transfection complexes to the cells. The cells were then incubated at 37°C for 48hours.

After 48 hours the medium from the cells was harvested and centrifuged at 1500rpm for 10 minutes to remove any debris. The harvested medium was then ultracentrifuged at 26000rpm for 4 hours. The supernatant was removed and the pellet of lentivirus was re-suspended in 300 µl medium. Virus was frozen at -80°C for long term storage.

6.2.3 Transduction of PLB-985 cells

PLB-985 cells were transduced with GFP tagged calpain construct by incubating $2x10^6$ cells with 200µl purified viral supernatant for 48 hours. After 48 hours cells were spun out of the viral supernatant and the culture medium was replaced with fresh medium and cells were allowed to recover.

6.2.4 Protein expression analysis

Protein expression in transduced cells was analysed by confocal microscopy and western blotting

6.2.4.1 Western blotting

Approximately $5x10^6$ cells per sample were spun down and re-suspended in 2ml neutrophil lysis buffer with an additional 2µl protease inhibitor. Samples were incubated on ice for 1hr, vortexing every 15 minutes. Samples were boiled for 10 minutes before being centrifuged at 14000rpm for 5 minutes. The pellet was discarded and the supernatant was retained. Sample buffer and reducing agent were added to the sample before the sample was run for 1hr at 150V, 55mA on a TRIS acetate 3-11% gel.

Following electrophoresis the protein in the gel was transferred to a membrane by western blotting for 1hr at 30V, 200mA in a buffer-tank blotting apparatus.

Non specific binding was blocked by washing the membrane in milk solution. The membrane was washed in 10% milk with tween for 1 hour before being incubated overnight at 4°C. The following day the membrane was washed for a further 1 hour in 10% milk with tween before 3 x 10 minute washes in 3% milk with tween. A 1 in 5000 dilution of anti-GFP-HRP antibody in 3% milk with

tween was incubated with the membrane for 1 hour at room temperature. Unbound antibody was removed by 3 x 10 minute washes in TBS with tween followed by 2 x 10 minute washes in TBS only. For exposure 2ml exposure reagent A and 2ml exposure reagent B was added to the membrane for 2minutes before the membrane was exposed for 1, 5, 7 and 12 minutes respectively.

6.2.5 Cytosolic calcium elevation experiments

Pharmacologically stimulated cytosolic calcium increase was achieved by treating the cells with a cocktail containing 10μM ionophore, 5μM thapsigargin 13mM $CaCl_2$ as described in section 2.9. Physiologically stimulated cytosolic calcium influx was achieved by treating the cells with formylated peptide (fMLP). Cytosolic calcium concentration was monitored using fura red calcium indicator as described in section 2.8. All experiments were imaged using a Leica SP2 confocal microscope.

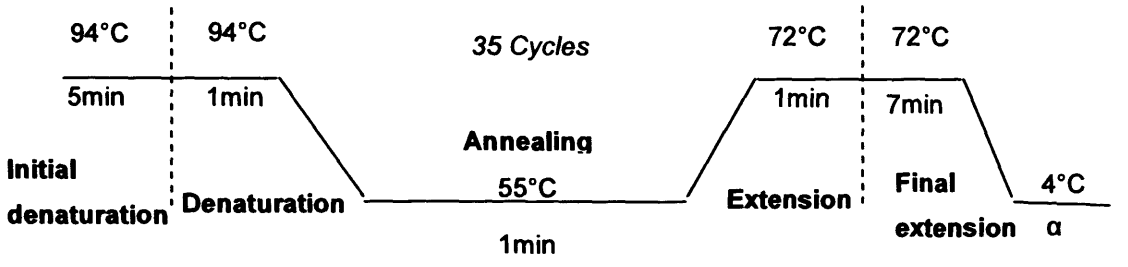


Figure 6.2.1.1.1: Generic PCR program

6.3 Results

6.3.1 Generation of white blood cell cDNA

In order to establish a source of calpain 1 and calpain 4 (calpain small subunit CAPNS1) several different cell lines and tissues were screened for Calpain 1 and Calpain 4 expression. Calpain 1 and Calpain 4 were expressed in a number of cell lines at different levels (figure 6.3.1.1). Interestingly, HL60 cells had detectable mRNA for calpain 1 but no detectable small sub unit and leukocytes (WBC) had very little detectable small sub-unit compared to calpain-1. White blood cell cDNA was successfully generated as described in section 6.2.1.3 (figure 6.3.1.1)

6.3.2 Generation of plasmids containing GFP tagged calpain

Calpain 1, calpain 4 (small subunit) and domain III from calpain 1 were successfully amplified from white blood cell cDNA (figure 6.3.2.1). Different PCR conditions and primer combinations yielded different results so the conditions which produced the highest yield and most specific generation of product were used for the generation of GFP tagged calpain. In order to generate GFP tagged calpain 1, calpain 4 and calpain 1 domain III the PCR fragments generated from the WBC cDNA were cloned into Invitrogen™ Vivid Colors™ pcDNA™ 6.2 EmGFP/TOPO mammalian expression vectors as described in section 6.2.1.4. As these vectors already contain GFP, this generated GFP tagged calpain. 3 plasmids were successfully generated using this strategy:

- CAPN1-GFP TOPO (GFP tagged calpain 1)
- CAPND3-GFP TOPO (GFP tagged domain III (C2 like domain) from calpain 1)
- CAPNS1-GFP TOPO (GFP tagged calpain 4)

Sequencing with T7 forward primers (described in Appendix IV) which bind the TOPO plasmid just before the insert site so that that plasmid is sequenced

forward from there confirmed the presence of the correct insert in the plasmids (See appendix VI)

6.3.3 Generation of lentivirus plasmids containing GFP tagged calpain

CAPN1-GFP, CAPND3-GFP and CAPNS1-GFP were successfully amplified from their respective plasmids as described in section 6.2.1.5 (figure 6.3.3.1). The amplified calpain-GFP fragments were successfully cloned into LentiSxW vectors as described in section 6.2.1.5. This was confirmed by plasmid sequencing (See appendix 9-17). Plasmids were sequenced with LentiSxW Forward and LentiSxW Reverse primers (described in section 2.1.4). These primers bound upstream and downstream of the CAPN-GFP insert and allowed for sequencing from both ends of the insert to confirm the presence of the CAPN and the GFP tag. The sequencing reaction can only reliably sequence ~500bp, therefore in the case of the CAPN1-GFP insert which was greater than 1000bp in length the plasmid was also sequenced with CAPN1/C2 forward and CAPN1/C2 reverse primers (described in section 2.1.4) which bind in the middle of the CAPN1 sequence. Overlapping of these sequences with the sequences from reactions with the LentiSxW primers allowed for sequencing of the entire insert.

6.3.4 Transduction of PLB-985 cells

PLB-985 cells were successfully transduced with CAPN1-GFP and CAPNS1-GFP with nearly all cells expressing some level of GFP 48 hours post transfection, detected by CLSM (figure 6.3.4.1). This level of GFP expression was maintained over several weeks and cells were considered to be stably transfected (figure 6.3.4.2). The distribution of GFP signal within PLB-985 was global for CAPNS1-GFP but CAPN1-GFP was excluded from the nucleus (figures 6.3.4.1 and 6.3.4.2).

In the case of the CAPN1D3-GFP lentivirus, 48hrs post transfected, cells were expressing low levels of GFP, visualised by CLSM (figure 6.3.4.3). However over expression of CAPN1D3 caused cell death and 1 week post transfection all cells were dead. The transduction was repeated on 3 separate occasions with 2 separate batches of lentivirus. In all transfections, the cells died within 1 week.

6.3.5 Analysis of protein expression of PLB-985 cells after transduction

In order to establish whether the stably expressed protein was the desired construct, protein was extracted and analysed by Western blotting using an anti-GFP antibody. Disappointingly, PLB-CAPN1GFP cells were only expressing GFP alone and although there was calpain4-GFP present in the PLB-CAPNS1GFP cells there was also free GFP expressed (figure 6.3.5.1).

6.3.6 Calpain 4 – GFP activity in response to elevated cytosolic calcium

As it had been established that some GFP tagged calpain 4 was present in the PLB-CAPNS1GFP cell line its ability to translocate in response to elevated calcium was analysed. GFP tagged calpain 4 did not translocate in response to elevated cytosolic calcium whether stimulated with fMLP or pharmacologically (figure 6.3.6.1)

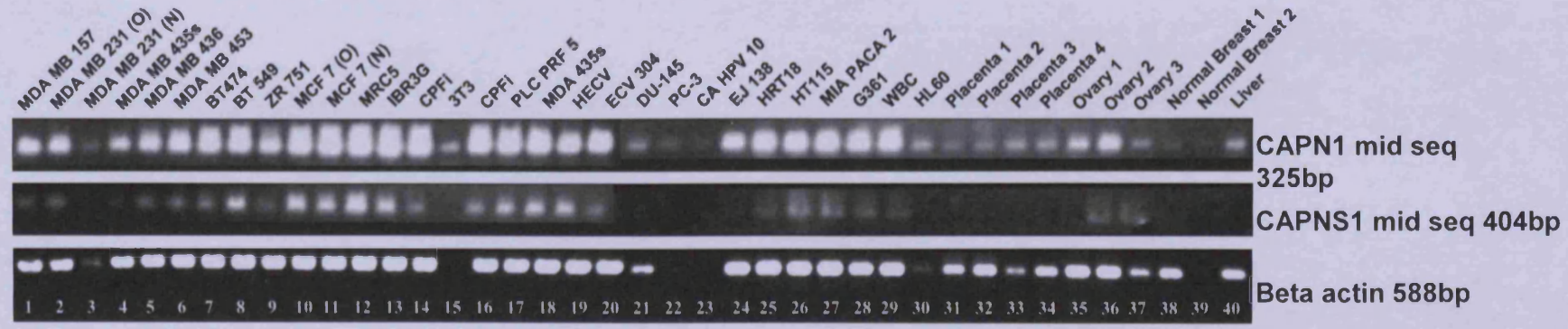


Figure 6.3.1.1: Cell line screen for calpain 1 and calpain 4 expression. A variety of cells lines were screened for calpain 1 and calpain 4 expression. HL60 cells had detectable levels of calpain 1 expression (top panel) but no detectable calpain 4 expression (middle panel) and white blood cells (WBC) had high levels of calpain 1 expression but lower levels of calpain 4 expression

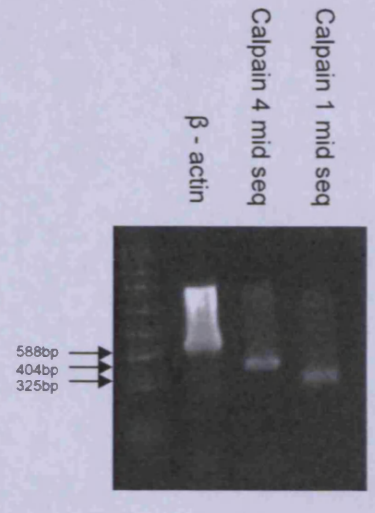


Figure 6.3.2.1: white blood cell cDNA quality check and calpain expression check. The ubiquitously expressed β actin gene was amplified by PCR to check the quality of the white blood cell cDNA (β actin band size ~588bp). A small portion of calpain 1 and calpain 4 were amplified by PCR to confirm expression of the genes of interest in the white blood cell cDNA (calpain 4 band size ~404bp, calpain 1 band size ~325bp). Primers used are described in appendix IV.

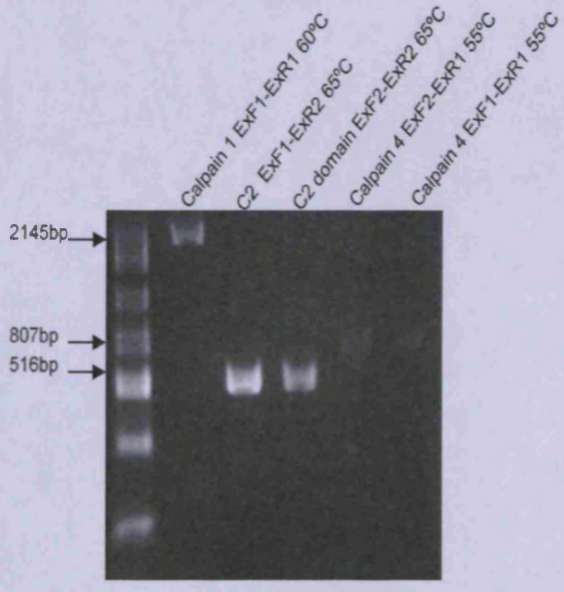


Figure 6.3.3.1: Calpain 1, calpain 4 and calpain 1 domain III (C2) amplified from WBC cDNA. The genes of interest, CAPN1 ((calpain 1) ~2145bp), CAPN1D3 (('C2 like' domain) ~516bp) and CAPNS1 ((calpain 4) ~807bp) were amplified from white blood cell cDNA ready to be cloned into the TOPO GFP plasmid. The primers that were used to amplify the plasmids are indicated in the figure labels and can be found in appendix IV.

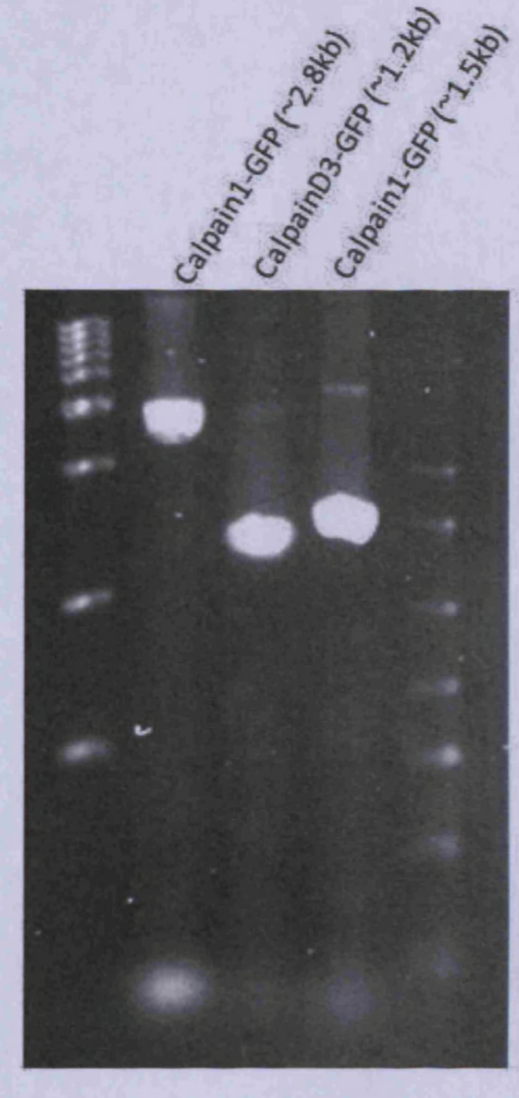


Figure 6.3.4.1: Calpain1-GFP, calpainD3-GFP and calpain4-GFP amplified from TOPO plasmids. GFP tagged calpain 1, calpainD3 and calpain 4 were amplified from the TOPO plasmids so that the gene of interest, already tagged with GFP could be cloned into the Lenti SxW plasmid. Fragments were amplified with primers described in appendix IV with extensions homologous to the plasmid they were to be inserted into.

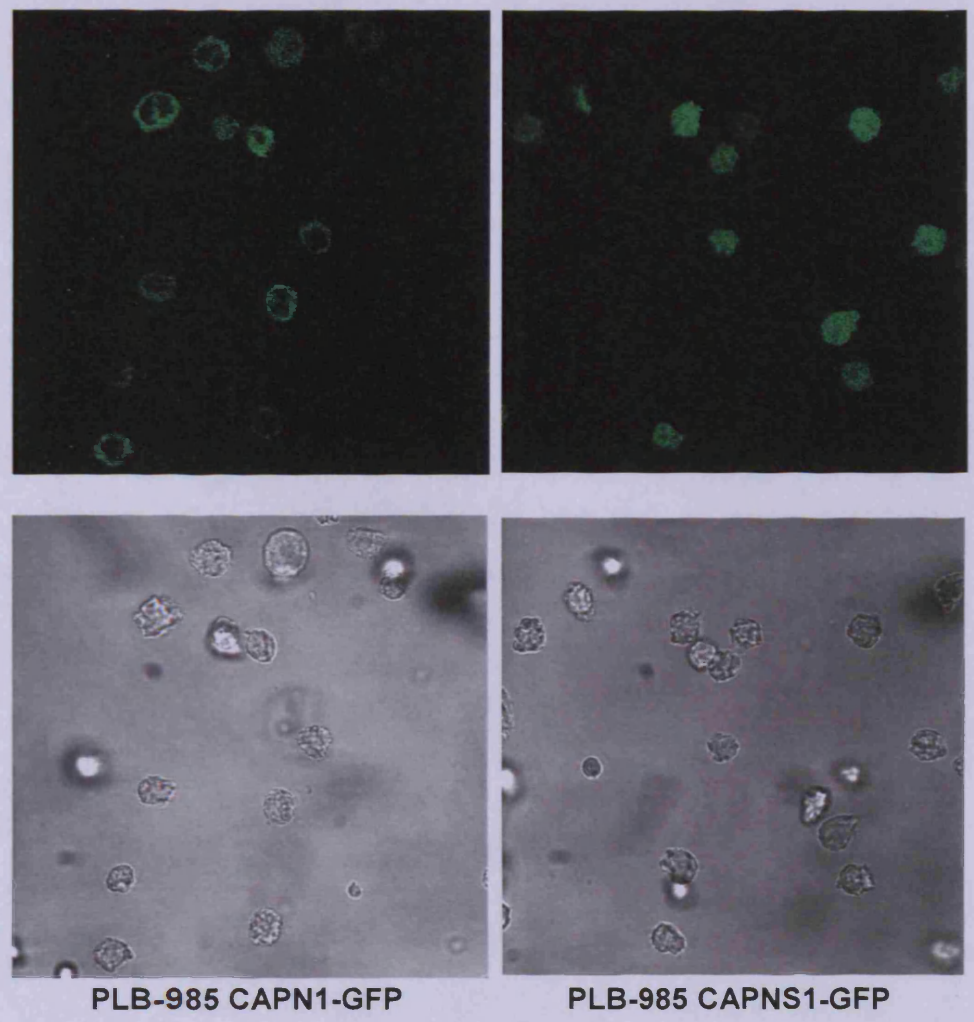


Figure 6.3.4.1: PLB-985 cells 48 hours after transduction with CAPN1-GFP or CAPNS1-GFP lentivirus. All cells were expressing GFP. GFP was excluded from the nucleus in the PLB-985-CAPN1-GFP cells but not in the PLB-985-CAPNS1-GFP cells.

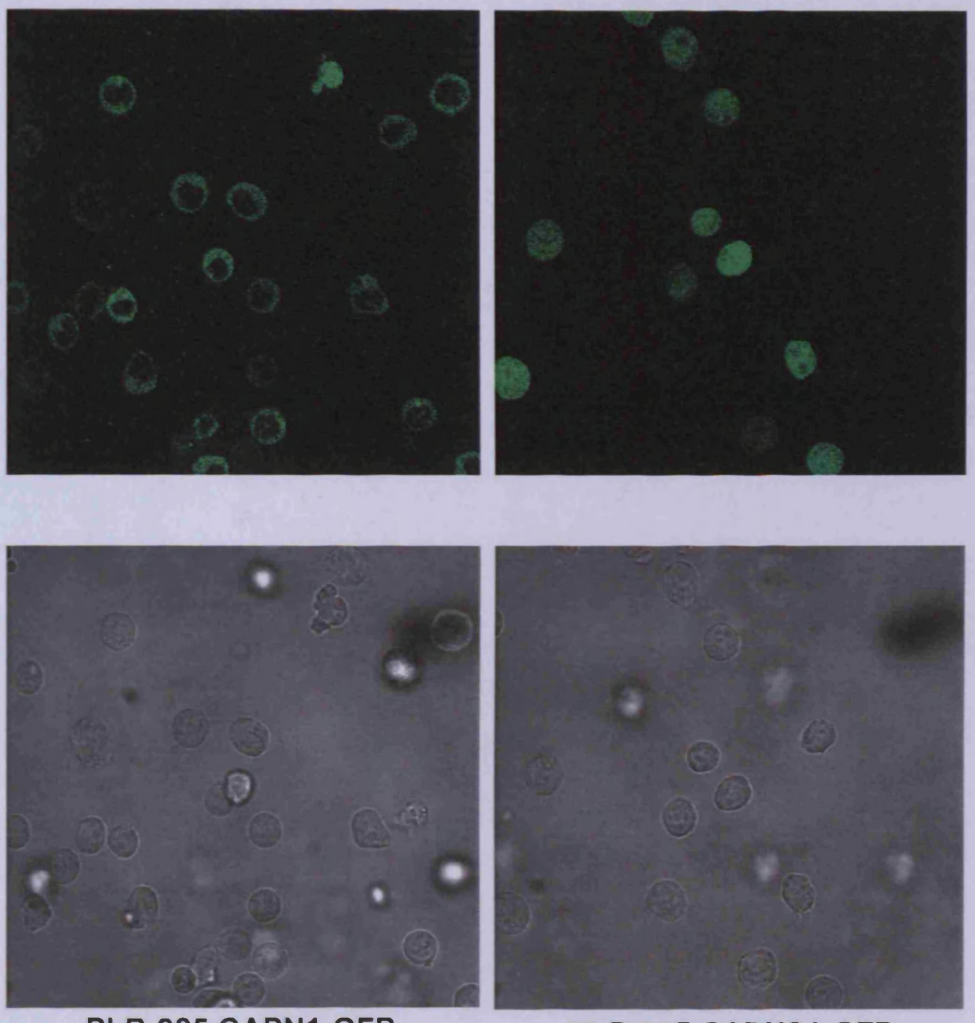


Figure 6.3.4.2: PLB-985 cells 4 weeks after transduction with CAPN1-GFP or CAPNS1-GFP lentivirus. Cells were still expressing GFP 4 weeks post transfection indicating that that cells had become stably transfected.

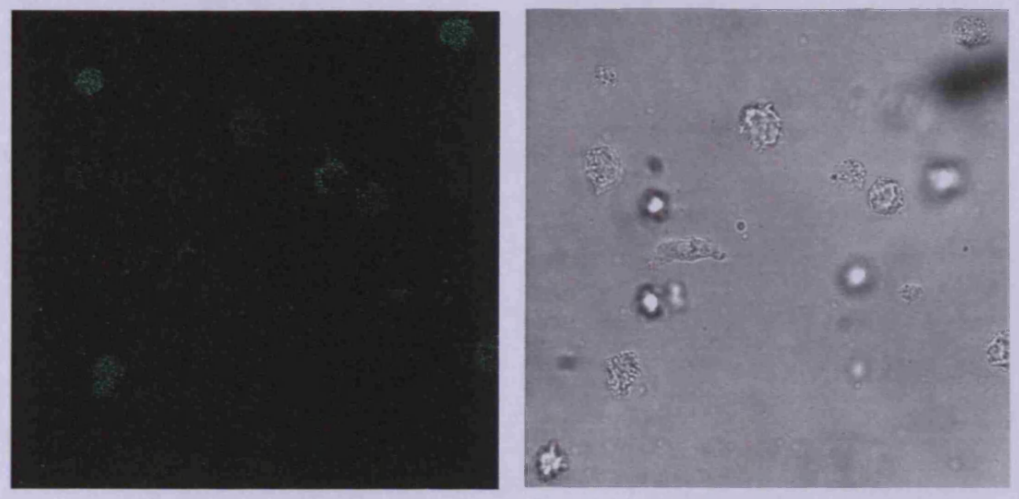
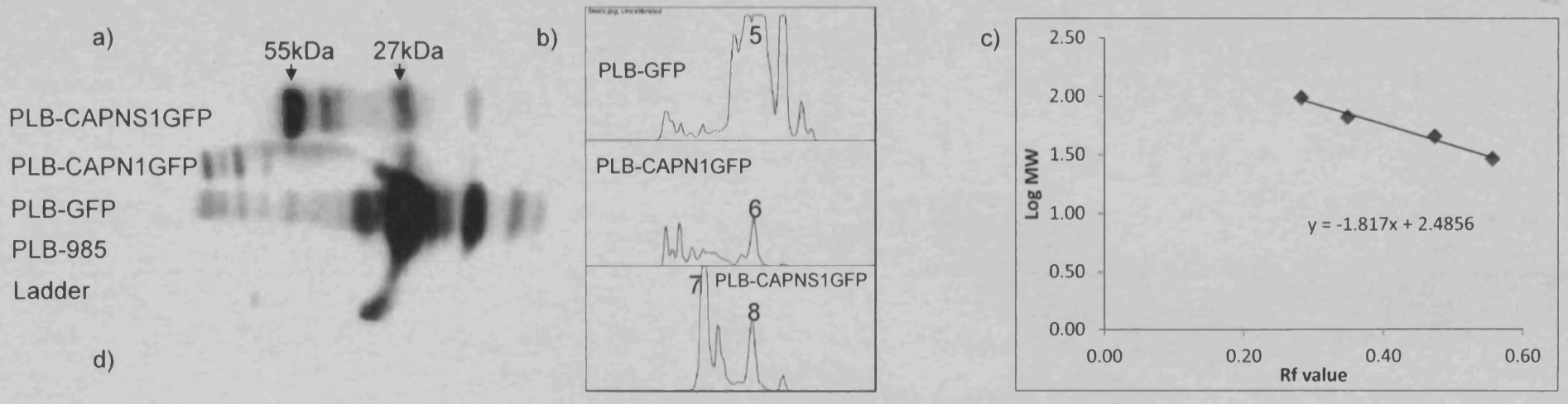


Figure 6.3.4.3: PLB-985 cells 48 hours after transduction with CAPN1D3-GFP lentivirus. Cells were expressing GFP after initial transduction with the CAPN1D3-GFP lentivirus although at quite a low level.



Band	Label	X	Standard MW(kDa)	Rf	Log MW (kDa)	MW of unknowns (kDa)	Expected MW of unknowns
1	MW ladder	140	97.4	0.28	1.99		
2	MW ladder	173	66.0	0.35	1.82		
3	MW ladder	235	45.0	0.47	1.65		
4	MW ladder	276	29.0	0.56	1.46		
5	Lane 3 band 6 (GFP)	297		0.60	1.40	24.98	27kDa
6	Lane 4 band 3 (GFP)	290		0.58	1.42	26.50	27kDa
7	Lane 5 band 1 (CAPN4-GFP)	204		0.41	1.74	54.74	55kDa
8	Lane 5 band 3 (GFP)	287		0.58	1.43	27.18	27kDa
9	Dye front	496		1			

Figure 6.3.5.1: Western blot analysis of protein expression in PLB-GFP, PLB-CAPN1GFP and PLB-CAPNS1GFP cells. a) PLB-GFP, PLB-CAPN1GFP and PLBCAPNS1-GFP had free GFP protein in them. There is evidence of a small amount of CAPN1-GFP present but not enough to be certain. CAPNS1-GFP cells had both GFP bound to calpain4 and free GFP. b,c,d) Further analysis comparing the Rf values of the bands in the gel to the Rf values of the standards in the molecular weight ladder confirmed the correct molecular weights for the free GFP and the GFP bound to calpain4.

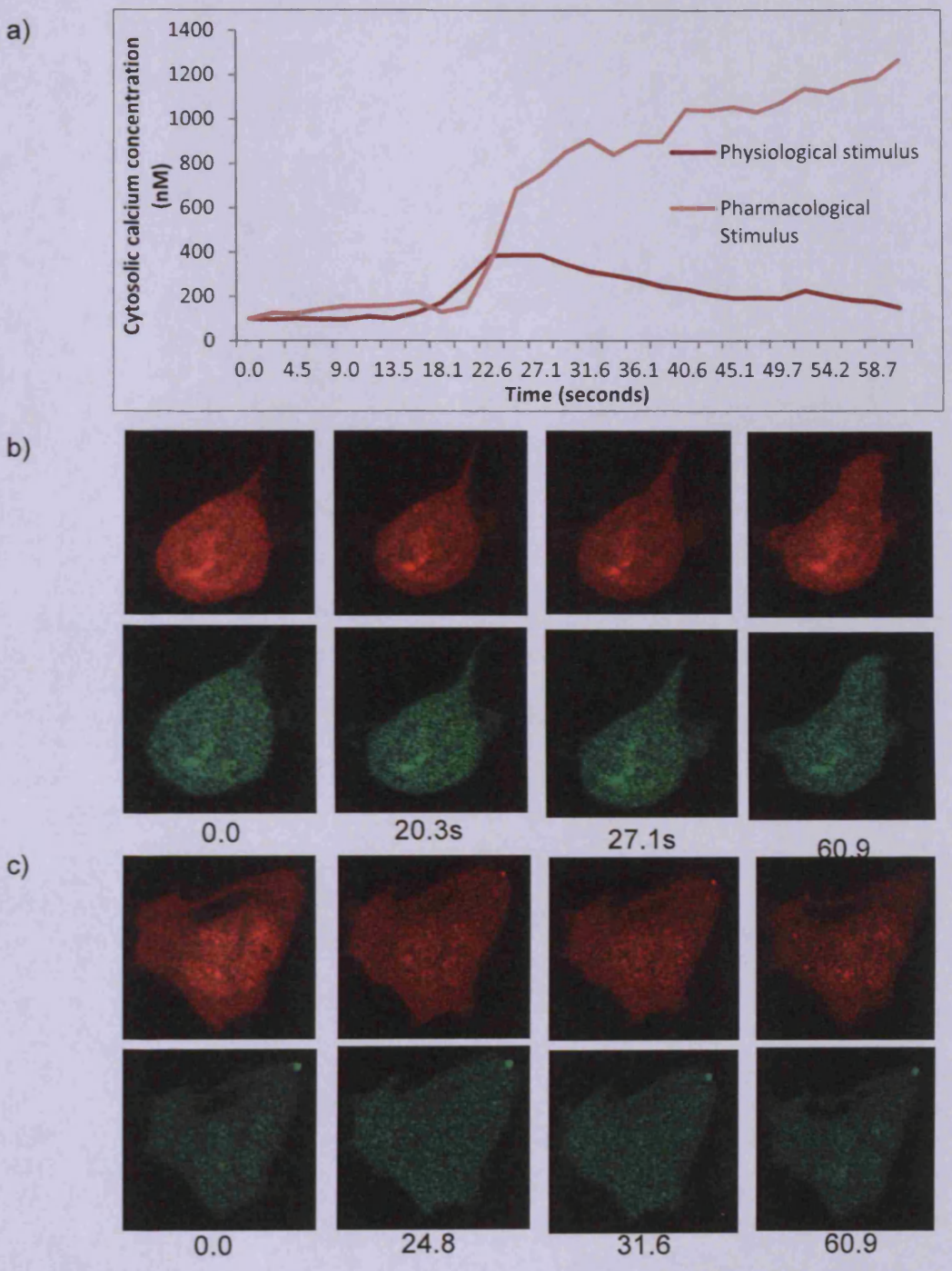


Figure 6.3.6.1: Typical cctivity of calpain4-GFP in response to elevated cytosolic calcium in PLB-985 cells expressing CAPN4-GFP *(n>5)*. PLB-985 cells expressing CAPN4-GFP were treated with either fMLP to stimulate a pharmacological calcium influx (b) or ionomycin and thapsigargin to stimulate a pharmacological calcium influx (c). Cells were also loaded with fura red to monitor cytosolic calcium concentration. GFP fluorescence and fura red fluorescence were monitored simultaneously throughout the experiment. a) Cytosolic calcium concentration was calculated from the fura red fluorescence and was raised through both pharmacological (10μM ionomycin and 5μM thapsigargin in medium containing 13mM calcium) and physiological (fMLP) stimuli. Calpain 4 GFP did not translocate in response to either stimulus. b) fMLP. c) 10μM ionomycin and 5μM thapsigargin in medium containing 13mM calcium.

6.4 Discussion

6.4.1 Effect of over expression of calpains C2 like domain on cell survival

Attempts to produce a cell line that was stably over expressing domain 3 (C2 like domain) from μ calpain failed as over expression of this domain alone was lethal to the cell line used in this study. However over expression of whole μ calpain or whole calpain 4 had no effect on cell viability. Knock out studies in mice have shown that knocking out calpain 4 in mice is lethal as the mice die during embryonic development (Arthur et al., 2000) but knocking out calpain 1 has no effect on mice survival or general phenotype (As am et al., 2001). As such there has been no previous suggestion that this particular domain of calpain plays a crucial role in cell survival. Calpain undergoes a conformational change to become activated (Hosfield et al., 1999). It may be the case that in normal physiological circumstances the 'C2 like' domain of calpain is only exposed when calpain is in its active state and thus not exposed the majority of the time. Expressing the 'C2 like' domain alone would result in the 'C2 like' domain being active in the cytoplasm all the time. It may be the case that having calpains C2 like domain constitutively active is blocking other C2 domain proteins from binding the plasma membrane and thus blocking them from functioning, this would have detrimental effect on the activity of the cell. However this phenomenon was not seen in the short term when classical C2 domains are over expressed in a variety of cell lines so it is therefore unlikely that this is the case here. The exact role of domain 3 ('C2 like' domain) in calpain 1 activation as well as membrane targeting is still unknown. Until more is understood it would be difficult to speculate on why it's over expression is lethal to this particular cell line.

6.4.2 Transduction of PLB-985 cells with CAPN1-GFP and CAPNS1-GFP

Lentiviral transduction of the PLB-985 cell line produced stably transfected cell lines. However the GFP which was expressed by these cell lines was no longer bound to the calpain as intended. DNA sequence analysis showed that the

calpain, the GFP tag and the 'linker molecule' linking the two were all present in the lentiviral vector used to generate the lentivirus. It can therefore be assumed that the linker molecule may be being cleaved by a non specific process when the protein is expressed in the cells. This would be unusual but no other obvious explanation immediately presents itself. The nature of the time constraints imposed on this project meant that this problem could not be fully addressed. If it were the linker molecule between the calpain and the GFP tag being cleaved that is the cause of this problem then one possible solution would be to create the GFP tagged calpain in a different way. i.e. as a direct fusion without the need for a linker molecule. This could be achieved using the In-Fusion Advantage PCR cloning technique used to insert the calpain-GFP products into the lentiviral expression vector and this is a possible direction to move toward in the future. Some GFP tagged calpain 4 was found to be present intact in the PLB-CAPNS1GFP cell line. In this cell line the calpain4-GFP did not translocate in response to elevated cytosolic calcium concentrations. The potential reasons for over expressed calpain 4 not translocating in response to high calcium concentrations were discussed in section 4.4.1. Another possible explanation for the translocation not being visible in this case is that in this cell line there was also a substantial amount of free GFP which had been cleaved from the calpain 4. This GFP free in the cytoplasm would not translocate and would act to further mask any possible translocation of the GFP tagged calpain 4, perhaps to the extent that it is undetectable.

6.4.3 Overall discussion

Despite the setbacks suffered in this chapter lentiviral transfection still remains the most successful and efficient method of stably transfecting myeloid cell lines which as discussed in the previous chapter is still difficult to achieve and has only been successful in a two cases (Servant et al., 2000, van Bruggen et al., 2004). If time allowed for some minor changes to the constructs

themselves then it remains likely that it can be successful for this project as well and it remains a possible technique for the future.

CHAPTER 7

Membrane dynamics during phagocytosis in human neutrophils

7.1 Introduction

Phagocytosis involves an apparent expansion of the surface area of the neutrophil which is sufficient to enclose the particle to be internalised. In addition, the fusion of granules specifically to the phagosome would be expected to increase its surface area. Whatever the mechanism for the psuedopodial membrane expansion, there may be an accompanying process to retrieve membrane and so regulate the amount of surface area during and after the process. Although this has been explored previously, the work presented in this chapter presents data which sheds light on the process.

7.1.1 Phagocytosis

Phagocytosis is the internalisation of foreign pathogens in response to the activation of opsonin receptors on the neutrophil surface. These are Fc receptors or β2 integrins. Activation of these receptors triggers a signalling cascade which ultimately results in actin polymerisation and extensive membrane and cytoskeletal reorganisation leading to the extension of membrane pseudopodia around the pathogen and internalisation of the pathogen (Jutras and Desjardins, 2005, Lee et al., 2003). The pseudopodia extend around the pathogen following the path of receptor activation in what is known as the 'zipper mechanism' (figure 7.1.1.1) (Jutras and Desjardins, 2005). Once the pathogen has been fully engulfed by the phagosome and internalised a process called phagosome maturation occurs. This is the results of 'kiss and run' fusion of granules with the phagosome which add and remove contents from the phagosome (Lee et al., 2003). This process is triggered by a cytosolic calcium increase (Jaconi et al., 1990) and the rank order of granule fusion (i.e. secretory vesicles, gelatinase granules, specific granules and azurophil granules) with the phagosome at a particular time is also calcium dependent (Sengelov et al., 1993). This process leads to the phagosome gaining various degradative enzymes (myeloperoxidases, hydrolases and NAPDH oxidase) all necessary for the final step in phagocytosis, pathogen destruction.

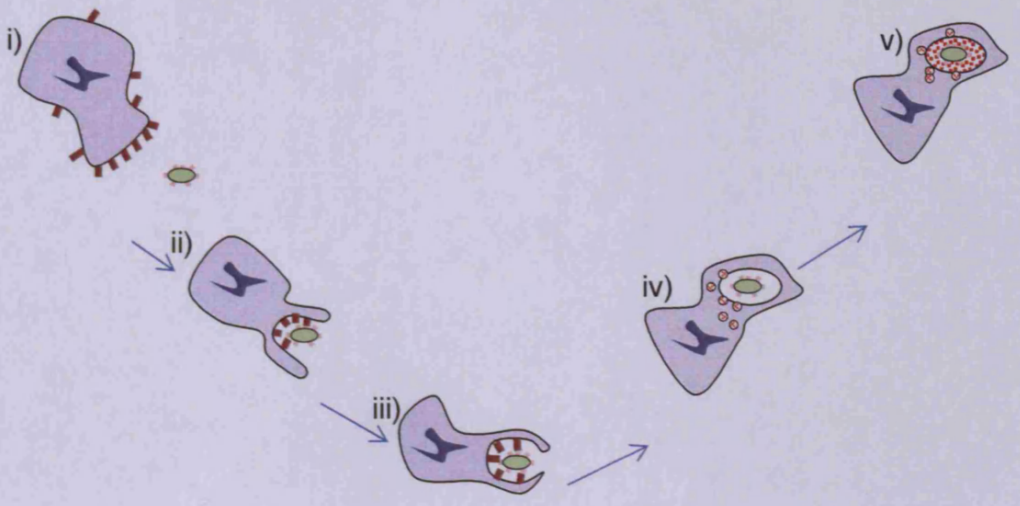


Figure 7.1.1.1: The process of C3bi mediated phagocytosis from pathogen recognition to pathogen destruction. i) Pathogen detection and redistribution of CD11b/CD18 to site of pathogen. ii) Interaction of CD11b/CD18 on neutrophil with C3bi on bacteria induces pseudopodia formation. iii) Pseudopodia extend around the pathogen following the path of receptor activation – 'zipper mechanism'. iv) Phagosome closes and granules begin to release their contents into the phagosome. v) Bacteria is destroyed inside the phagosome.

7.1.2 Membrane expansion and retrieval

The plasma membrane of the neutrophil is covered in surface wrinkles of approximately 300nm in length (Bruehl et al., 1996, Shao et al., 1998), covering 30-40% of the cell surface (Bruehl et al., 1996). During phagocytosis large pseudopodia are able to extend around particles with a surface area of up to ¼ of that of the cell, and when a neutrophil spreads its apparent surface area increases two fold (Hallett and Dewitt, 2007). It has therefore been suggested that these wrinkles provide a source of extra membrane for neutrophil shape change such as spreading and phagocytosis. The focus of this thesis so far has been on how the membrane is unwrinkled to provide this extra plasma membrane but it is also worth noting that a mechanism for membrane retrieval and how the wrinkles are reintroduced into the plasma membrane is yet to be fully addressed. It has been reported that phagocytosis of gram positive non opsonised bacteria by neutrophils is accompanied by the formation of "pinosomal structures" around the phagosome (Bauer and Tapper, 2004).

7.1.3 Aims of the chapter

The aim of this chapter is to investigate this hypothesis the "pinosomal structures" form the basis of excess membrane retrieval after phagocytosis. To do this, it was necessary to observe neutrophil phagocytosis of opsonised zymosan particles in the presence of fluorescent extra cellular fluid phase markers in real time.

7.2 Materials and Methods

7.2.1 Neutrophil phagocytosis in the presence of Lucifer yellow

Neutrophils were isolated from healthy volunteers as previously described (section 2.3). Neutrophils were incubated with 50µg/ml lucifer yellow (50µg/ml) and 100µg/ml opsonised zymosan particles for 30 minutes at 37°C. Cells were then visualised and checked for the presence of pinosomes using the CLSM. Z-stacks were taken to visualise the location of the pinosomes in 3 dimensions. 3D reconstruction of images was performed using IMARIS software.

7.2.2 Neutrophil phagocytosis in real time in the presence of Lucifer yellow

Neutrophils were isolated from healthy volunteers as previously described (section 2.3). 50µg/ml lucifer yellow and 10µg/ml opsonised zymosan particles were added to the cells immediately prior to the experiment. Z-stacks and time series were taken as the cell phagocytosed in order to visualise the appearance of the pinosomes in time and space. 3D reconstruction and analysis of images was performed using IMARIS software.

7.2.3 Neutrophil phagocytosis in real time in the presence of rhodamine dextran.

Neutrophils were isolated from healthy volunteers as previously described (section 2.3). 250µg/ml rhodamine dextran and 10µg/ml opsonised zymosan particles were added to the cells immediately prior to the experiment. Dextran was used to determine if the pinosomes which were being formed were caused by transient binding of granules to the pinosome which only bind long enough to allow small molecules to diffuse from the phagosome into the granule. Rhodamine dextran is 10,000 MWt and is excited at 540nm and emits fluorescence at the red end of the spectrum. Z-stacks and time series were

taken as the cell phagocytosed in order to visualise the appearance of the pinosomes in time and space. 3D reconstruction and analysis of images was performed using IMARIS software.

7.2.4 Neutrophil phagocytosis in real time in presence of fluorescein conjugated BSA

Neutrophils were isolated from healthy volunteers as previously described (section 2.3). Cell membranes were stained with PKH26 red fluorescent cell membrane stain (Sigma). 1% fluorescein conjugated BSA and 10μg/ml opsonised zymosan particles were added to the cells immediately prior to the experiment. BSA is a protein of 68kDa which when tagged with the fluorescein molecule is excited at 488nm and emits light in the green region of the spectrum. Cell membranes were stained with PkH26 red membrane stain (Invitrogen) in order to try and visualise whether the pinosomes were formed from the cells plasma membrane or were derived from existing granules within the cell. Z-stacks and time series were taken as the cell phagocytosed in order to visualise the appearance of the pinosomes in time and space. 3D reconstruction and analysis of images was performed using IMARIS software.

7.2.5 Neutrophil phagocytosis in real time with fluoroscein conjugated BSA added after the phagosome had closed.

Neutrophils were isolated from healthy volunteers as previously described (section 2.3). 10μg/ml opsonised zymosan particles were added to the cells immediately prior to the experiment. Cells were allowed to phagocytose and as soon as the phagosome had closed 1% fluoroscein conjugated BSA was added to the cells to visualise whether the pinosomes were formed from the plasma membrane of the membrane that was surrounding the phagosome. Z-stacks and time series were taken as the cell phagocytosed in order to visualise the

appearance of the pinosomes in time and space. 3D reconstruction and analysis of images was performed using IMARIS software.

7.3 Results

7.3.1 The formation of pinosomes/fluid filled inclusions during phagocytosis

In order to investigate the relationship between phagocytosis and the formation of pinosomes/fluid filled inclusions, Lucifer yellow was used as a fluorescent marker of the extracellular fluid phase. In neutrophils which were incubated for 30 minutes with zymosan particles and Lucifer yellow, Lucifer yellow was found in the phagosome itself as well as in other inclusions throughout the cell. This was not observed in cells which had not phagocytosed any of the zymosan particles (figure 7.3.1.1). In order to visualise this process in real time, the process of phagocytosis in lucifer yellow containing medium was monitored and the appearance of fluid filled inclusions was recorded in real time. The first fluid filled inclusion appeared immediately after phagosome closure and fluid filled inclusions continued to appear up to 5 minutes after phagosome closure (figure 7.3.1.2). In all but one experiment the fluid filled inclusions did not appear until after phagosome closure and in most cases the number of fluid filled inclusions steadily increased after phagosome closure until a plateau was reached (figure 7.3.1.3a). This plateau was most likely the result of an equilibrium between some of the fluid filled inclusions disappearing from the confocal plane while others replace them. The total number of inclusions formed and the time they took to form varied between cell and donor (figure 7.3.1.3a). The volume of fluid filled inclusions and their distance from the phagosome and the plasma membrane was analysed using IMARIS 3D reconstruction software. No relationship was found between the volume of the fluid filled inclusion and the distance of the inclusion from the phagosome (figure 7.3.1.3b), however there was some correlation between the volume of the inclusion and the distance from the plasma membrane (figure 7.3.1.3c). This correlation suggests that the further away from the plasma membrane the inclusions travel, the smaller they became.

7.3.2 Phagocytosis in the presence of rhodamine dextran and Lucifer yellow.

In order to establish whether the fluid filled inclusions were forming from transient binding of cytoplasmic granules to the phagosome where the binding time is so transient that there is only enough time for small molecules to diffuse from the phagosome into the granules cells were allowed to phagocytose in the presence of the larger fluorescent marker, rhodamine dextran (MWt 10,000) as well as the smaller fluorescent marker Lucifer yellow. Both of these markers were added to the extracellular fluid. Fluid phase inclusions containing Lucifer yellow were visible but there were no visible fluid filled inclusions containing rhodamine dextran (figure 7.3.2.1)

7.3.3 Phagocytosis in the presence of fluorescein conjugated BSA

In order to confirm the results observed when cells were allowed to phagocytose in the presence of rhodamine dextran, the experiments were repeated using a difference molecule, BSA conjugated to fluorescein. As the BSA is conjugated to fluorescein which is more highly fluorescent than rhodamine this would indicate whether or not the lack of visible fluid phase inclusions observed when using the rhodamine dextran was true or whether it was due to the molecule not being highly fluorescent enough to be detected in the small quantities found in the inclusions. Fluid filled inclusions were clearly visible in cells allowed to phagocytose with fluorescein-BSA in the extracellular fluid with the first inclusion forming just after phagosome closure and inclusions continuing to form for more than 5 minutes after the phagosome had closed (figure 7.3.3.1). In order to try and establish whether the fluid filled inclusions were forming from the plasma membrane or from existing vesicles in the cell, the cell membrane was stained with PKH26 red membrane marker and fluorescein-BSA was added to the extracellular fluid and phagocytosis was recorded. No correlation was found between the location of the fluid filled

inclusions and the membrane marker although the membrane marker could clearly be seen around the phagosome (figure 7.3.3.2)

7.3.4 Establishing the location of fluid uptake

In order to establish whether the fluorescent marker found in the fluid filled inclusions after phagosome closure was coming from the phagosome or from the extracellular fluid fluorescein-BSA was added to the cells either before or after phagosome closure. When fluorescein-BSA was added after phagosome closure no fluid filled inclusions were observed, even more than 7 minutes after phagosome closure (figure 7.3.4.1a). However, when the fluorescein-BSA was added to the cells before phagosome closure and therefore was taken up into the phagosome, fluid filled inclusions were observed consistently (figure 7.3.4.1b). This suggests that the fluid filled inclusions observed after phagocytosis are derived from the phagosome rather than the extracellular fluid.

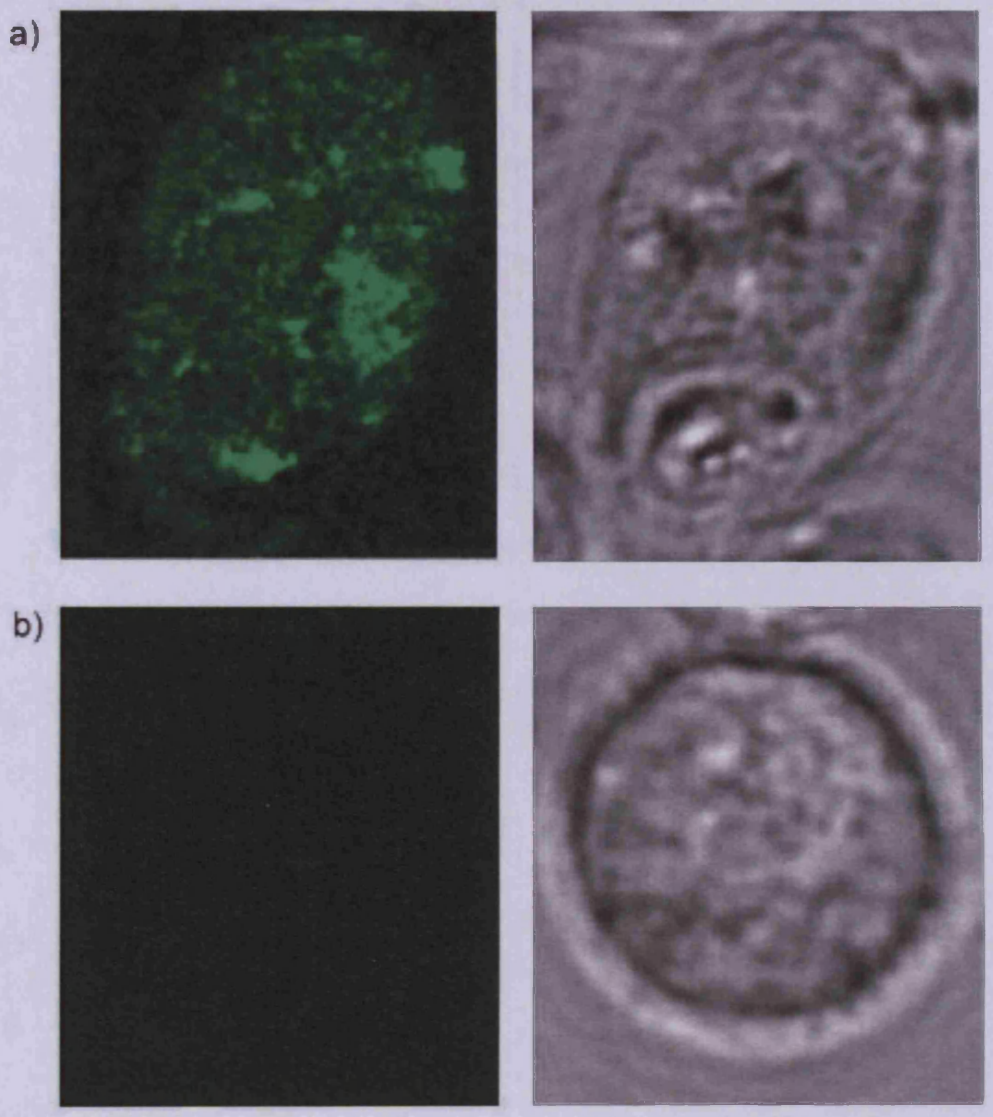


Figure 7.3.1.1: An example of neutrophils that had been incubated with opsonised zymosan with Lucifer yellow for 30 minutes *(n>10)*. a) Fluid phase inclusions were found in neutrophils that had phagocytosed zymosan b) but were not found in neutrophils that had not undergone phagocytosis.

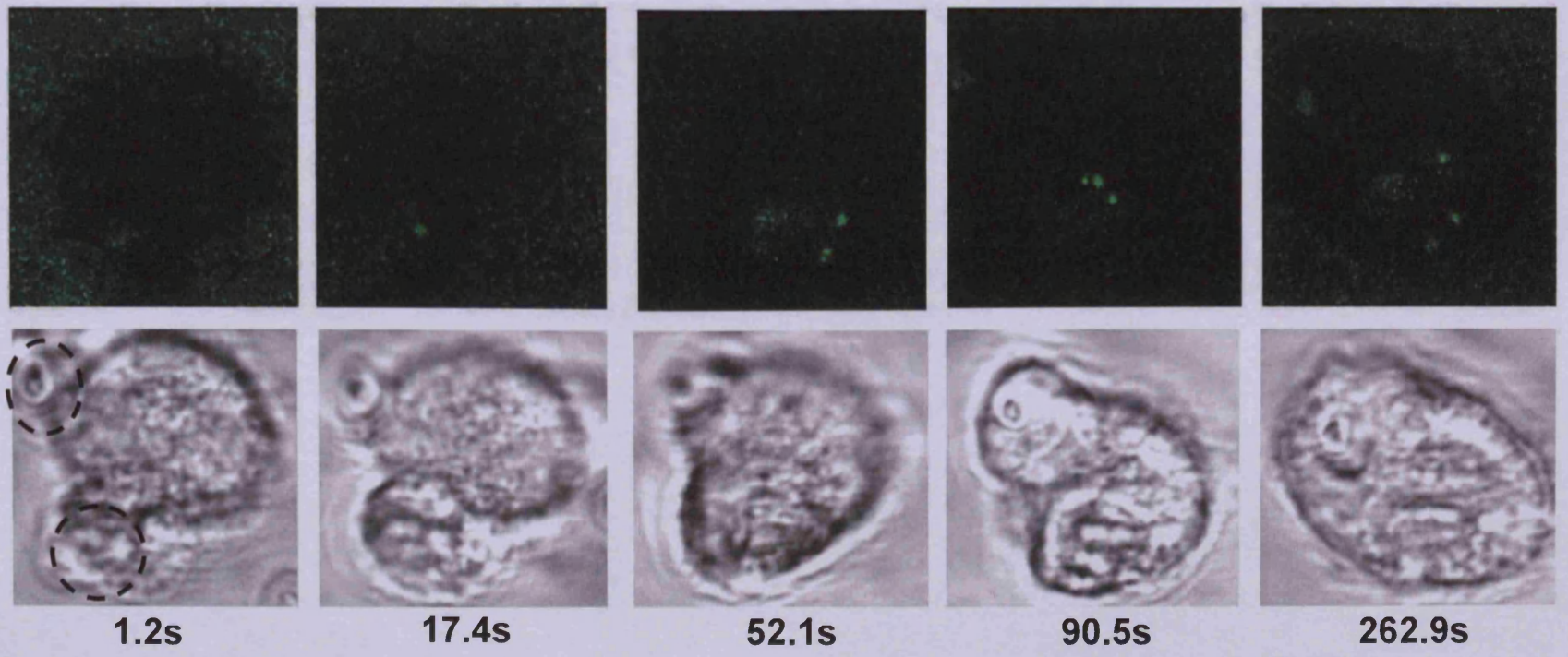


Figure 7.3.1.2: Fluid phase inclusions appearing in real time as the neutrophil engulfs 2 zymosan particles *(n>5)*. Lucifer yellow was added to the solution containing the neutrophils before zymosan was added and cells were allowed to phagocytose (zymosan highlighted by circles). The first fluid phase inclusion appears at 17.4 seconds, just after the phagosome has closed and fluid phase inclusion continue to appear up to 5 minutes after phagosome closure.

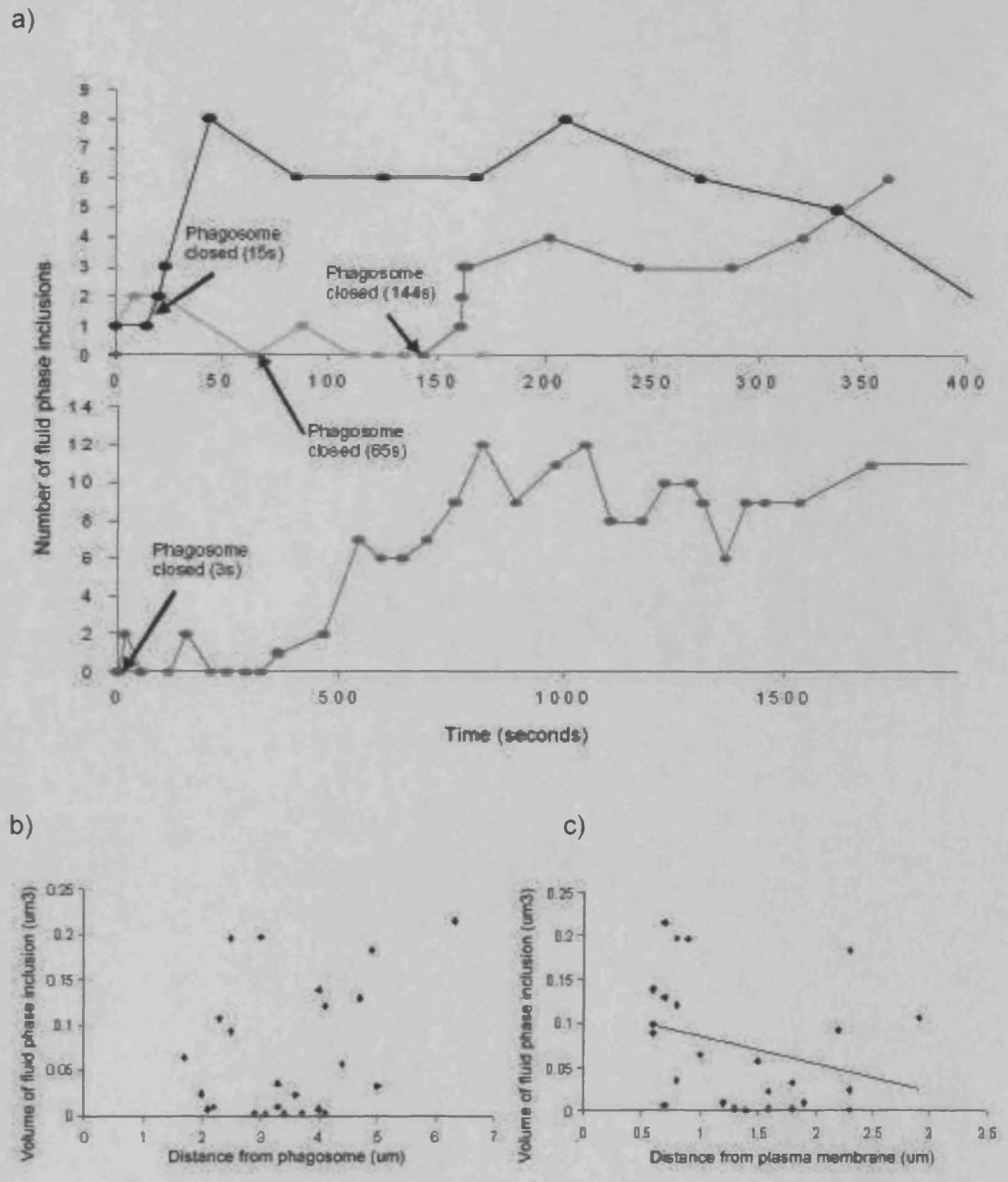


Figure 7.3.1.3: Analysis of the characteristics of fluid phase inclusions. (with IMARIS software) a) In all but one case the fluid phase inclusions did not appear until after the phagosome had closed. In most cases the number of fluid phase inclusions in the cell increased steadily after phagosome closure before reaching a plateau. The time this took varied depending on the cell. b) There was no relationship between the volume of the fluid phase inclusion and the distance from the phagosome *(Spearmans correlation coefficient did not exceed the critical value at p=0.05)* but c) there was some relationship between the volume of the fluid phase inclusion and the distance from the plasma membrane *(Spearmans correlation coefficient exceeded the critical value of 0.358 at p=0.05)*.

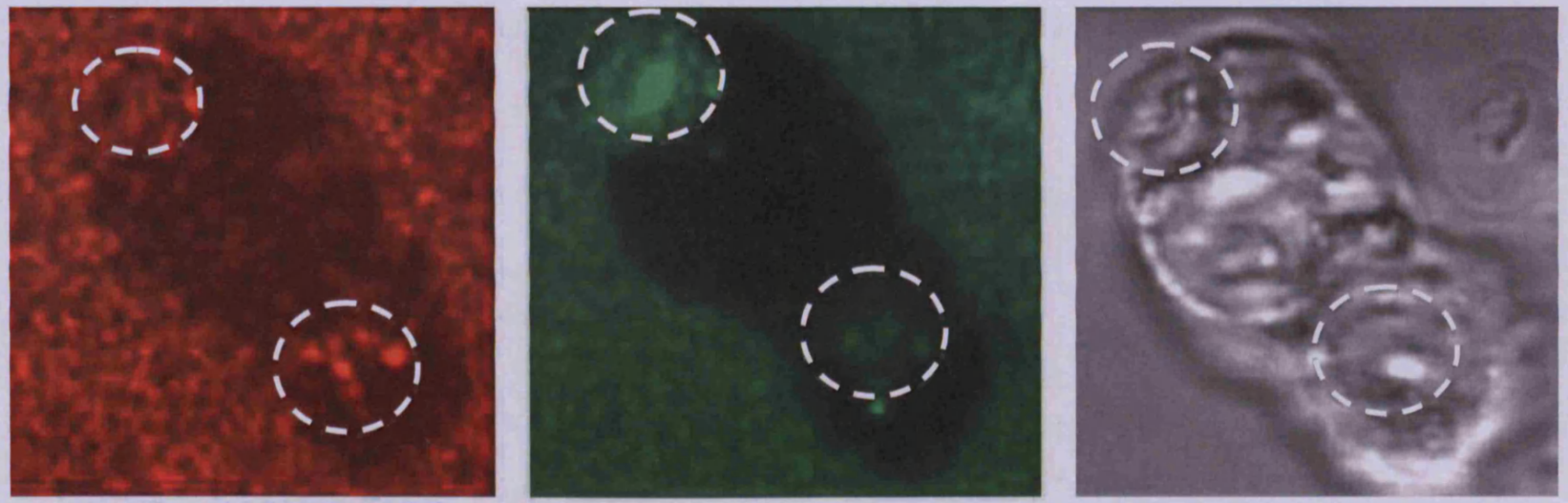


Figure 7.3.2.1: Example of fluid phase inclusions in cells which had been incubated with zymosan in Lucifer yellow and rhodamine dextran *(n>10)*. Fluid phase inclusions containing Lucifer yellow were visible but fluid phase inclusions containing rhodamine dextran could not be found. This may be due to the differing strength of the lasers used to detect the Lucifer yellow fluorescence and the rhodamine dextran fluorescence.

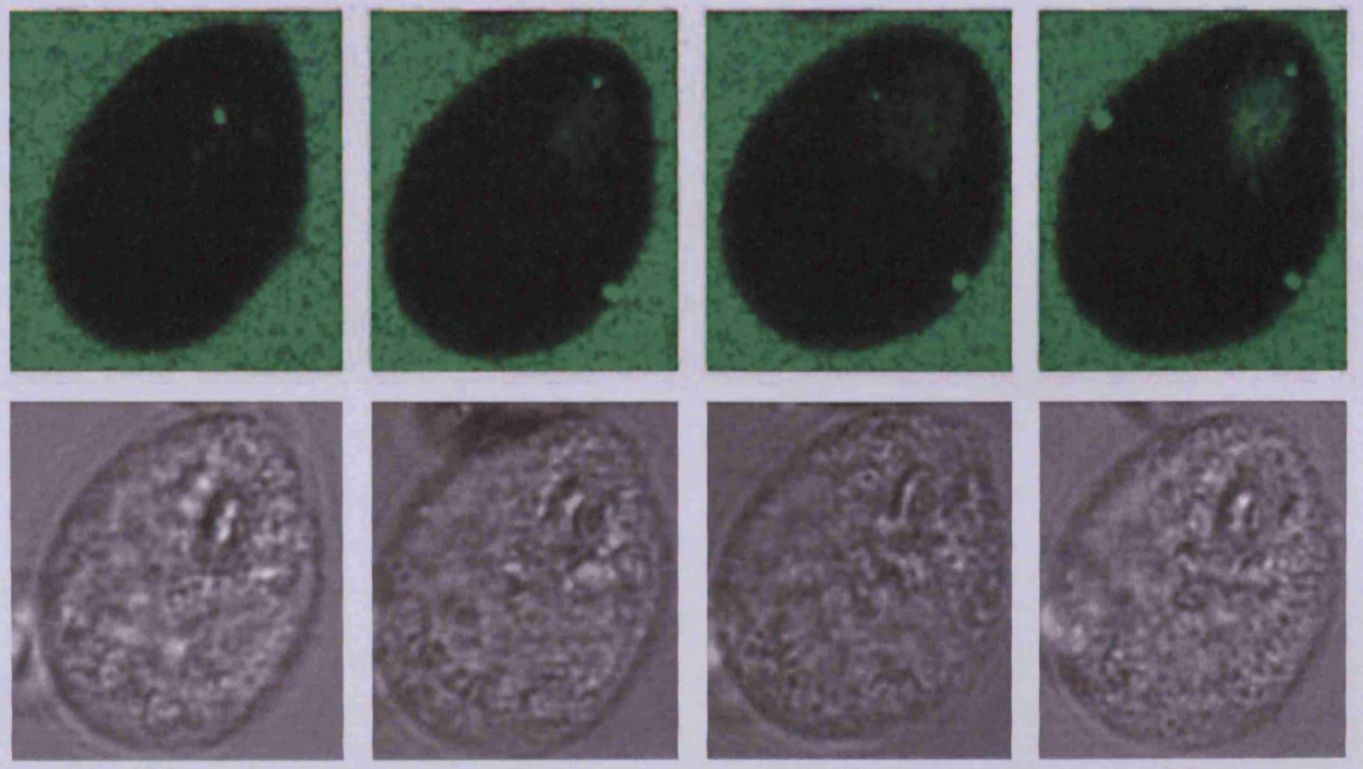


Figure 7.3.3.1: An example of fluid phase inclusions occurring in real time after the phagosome has closed around a zymosan particle *(n=3)*. Fluoroscein tagged BSA (FI-BSA) was added to the solution containing the neutrophils before zymosan was added and cells were allowed to phagocytose. FI-BSA was used instead of Lucifer yellow as it has a higher molecular weight in order to rule out the possibility that the reason no inclusions were observed when the cells were incubated in rhodamine dextran was that high molecular weight molecules could not pass into the pinosomes. The first pinosome appears at 22 seconds, just after the phagosome had closed and pinosomes were still appearing 5 minutes after the phagosome had closed. The pinosomes were clearly visible suggesting that there was no problem with large molecular weight molecule getting into them.

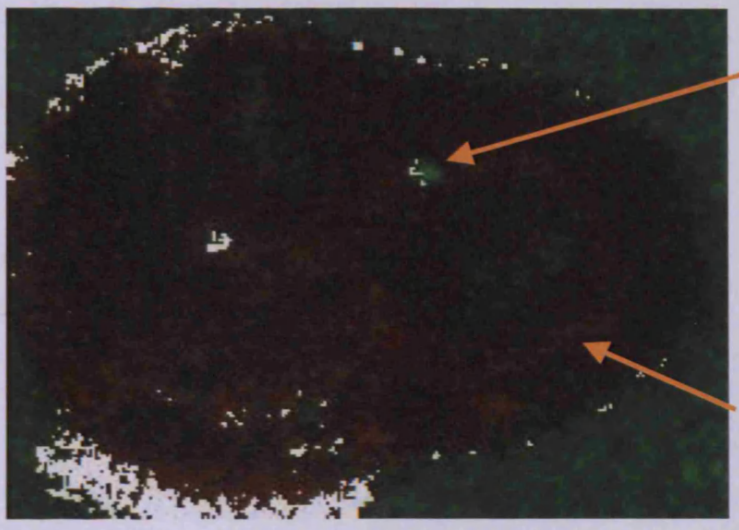


Figure 7.3.3.2: An example of a neutrophil stained with PkH26 red membrane stain and then incubated in FI-BSA and zymosan particles *(n=6)*. Co localisation analysis was performed using ImageJ in order to see whether any of the red fluorescent membrane co localised with any of the green fluorescent fluid phase inclusions. The white areas on the image show the areas where there is co localisation. *Pearsons R value was -0.2 indicating that there was no correlation and in fact a small degree of inverse correlation*, indicating that there was no association with the plasma membrane and the fluid filled inclusions.

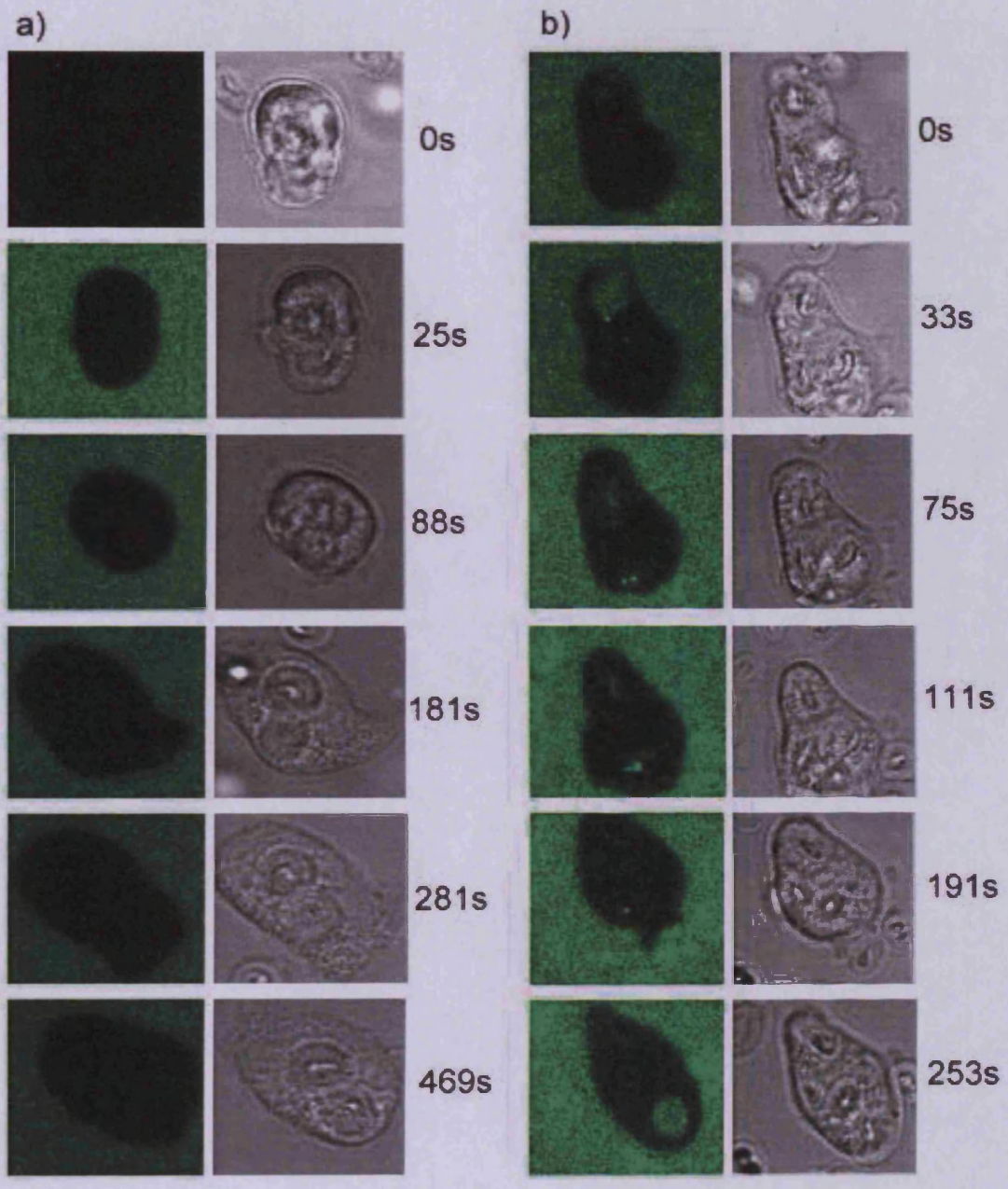


Figure 7.3.4.1: Neutrophils incubated with zymosan and allowed to phagocytose. Fluorescein tagged BSA added to the neutrophil solution either before or immediately after the phagosome closed *(n=4)*. a) When fluorescein BSA was added after the phagosome had closed (at 25s in picture a)) no fluid phase inclusions were observed, even 7 minutes after phagosome closure by which time earlier experiments showed that several fluid phase inclusions had formed. b) When fluorescein BSA was added before the phagosome closed or before phagocytosis had started fluid phase inclusions were observed to form in the usual way. This suggests that the fluid phase inclusions form from the membrane around the phagosome and not the rest of the plasma membrane.

7.4 Discussion

7.4.1 The formation of fluid filled inclusions during phagocytosis.

The results presented in this chapter confirm the appearance of fluid filled inclusions in the cytoplasm during phagocytosis and confirm the findings of a previous report which stated that phagocytosis was accompanied by the appearance of pinosomal structures (Bauer and Tapper, 2004). These results show that the fluid filled inclusions do not begin to appear in the cytoplasm until after phagosome closure and that the number of inclusions present increases over time until a plateau is reached. No correlation was found between the volume of the fluid filled inclusion and the distance of the inclusion from the phagosome that would suggest a relationship between the inclusion and the phagosome. However some correlation was found between the volume of the inclusion and the distance of the inclusion from the plasma membrane which suggested that the closer to the plasma membrane the inclusion was the greater the volume of the inclusion was likely to be. This raised the possibilities that the inclusions were coming from the plasma membrane and splitting off into smaller vesicles as they moved into the cell or that smaller vesicles were fusing together as the moved towards the plasma membrane. As the results discussed later in this section will suggest, both situations may be occurring.

7.4.2 Granule fusion and membrane retrieval

During the phagosome maturation stage of phagocytosis neutrophil granules fuse with the phagosomal membrane in a process known as 'kiss and run' fusion. During 'kiss and run' fusion the granule partly fuses with the phagosomal membrane and the contents of the granule and the phagosome are exchanged (Burgoyne and Morgan, 2003). It is therefore possible that the fluid filled inclusions observed here are a result of granules fusing with the phagosome in a 'kiss and run' fashion and in doing so taking up some of the fluorescent extracellular fluid marker from the phagosome. In order to answer this question two approaches were adopted. During 'kiss and run' fusion

smaller molecules would pass between the granule and the phagosome more readily than larger molecules as the rate of diffusion depends of inverse square root of the molecular weight (Berg, 1993). Therefore the experiments from earlier in the chapter were repeated using larger fluorescent molecules as markers of the extracellular fluid. BSA (68kDa) with a fluorescein tag was taken up just as readily into the fluid phase inclusions as the smaller Lucifer yellow marker was suggesting that larger size did not inhibit uptake into the fluid filled inclusions and that if these inclusions were the result of granule fusion with the phagosome then the granule had to remain bound to the phagosome for long enough to allow the diffusion of a 68kDa protein from the phagosome into the granule. This probably rules out transfer of material during the "flickering " of the prefusion pore, whose diameter is about the same size as a single gap junction (2nm) which permit diffusion of molecules less than 1kDa (Almers, 1990). The second approach adopted to verify if the fluid filled inclusions were a result of granule fusions was to stain the plasma membrane with PKH26 membrane marker. The marker clearly stained the plasma membrane and the membrane around the phagosome but could not be found around the fluid filled inclusions. This suggests that the membrane for these inclusions did not come from the plasma membrane or the phagosomal membrane and therefore must be from existing vesicles or granules within the cell.

7.4.3 Confirming the location of fluid uptake

The results discussed in the previous section suggest that the location of fluid uptake is likely to be from the phagosome rather than the extracellular fluid. The location of fluid uptake was confirmed to be from the phagosome by adding the extracellular fluid marker before or after phagosome closure. The location of fluid uptake fits with the hypothesis of membrane retrieval from the phagosome and with the hypothesis that the fluid is being taken up from the phagosome by granule fusion.

However, it should be noted that in neutrophils which had assumed a round shape, larger inclusion formed at the cell edge away from the phagosome (see e.g. fig 7.3.3.1). This opens the possibility that a second route for the formation of inclusion was directly from the plasma membrane and that this occurred as part of the restoration of cell surface area after completion of phagocytosis.

7.4.4 General discussion

The aim of this chapter was to investigate whether or not fluid filled inclusions observed in the neutrophil cytoplasm after phagocytosis are a result of active membrane retrieval after phagocytosis. There is evidence presented here to suggest that is the case; the source of the fluid uptake is the phagosome, large proteins are readily taken up suggesting a slow process to allow time for these proteins to diffuse and the size of the fluid filled inclusions increases as they move closer to the plasma membrane suggesting that the inclusions fuse together before their membrane is reinserted into the plasma membrane. There are contradictions however. If the inclusions were coming from the phagosome and fusing before being inserted into the plasma membrane then the size of the inclusion should decrease as you move closer to the phagosome. No correlation between volume of inclusion and distance from phagosome was found though. Also when the plasma membrane was stained using PKH26 membrane marker, no marker of plasma membrane was found around the inclusions despite the marker being clearly visible around the phagosome. This points to an alternative hypothesis that the fluid filled inclusions are the result of 'kiss and run' fusion of neutrophil granules with the phagosome during which the granules deposit some of their contents into the phagosome and in turn take up some of the phagosomes contents. It does remain possible however that the reason that no membrane marker was seen around the inclusions is simply that they are so small and perhaps the membrane marker was not concentrated enough that it is not visible on such small quantities of plasma membrane. In order to fully solve these doubts it would be useful in the future to repeat some of the experiments with a different

membrane marker to see if the same outcome was observed and conduct further experiments into the relationship between the distances of the inclusion from the phagosome to see if any relationship can be found.

CHAPTER 8

General Discussion

8.1 Introduction

Neutrophils are the most predominant type of white blood cell in the body and they are the first leukocytes to enter an area of inflammation, where they phagocytose microorganisms and kill them by generating toxic free radicals. However when inappropriately activated in inflammatory conditions such as Chronic Obstructive Pulmonary Disease they can cause extensive damage to tissues. When activated neutrophils first need to leave the blood stream in order to move to the area of inflammation. They do this by rolling along the blood vessel endothelium binding transiently via selectins to facilitate rolling and then integrins on the neutrophil bind ICAM-1 on the blood vessel endothelium where they adhere and spread out before they extravasate from the blood vessel into the tissue. Despite much being known about the molecules that trigger neutrophil extravasation, very little is known about the mechanism by which the neutrophil leaves the blood stream including the route that the neutrophil takes through the endothelium and how the neutrophil is able to spread onto the endothelium. Spreading onto the endothelium before leaving the bloodstream is a key step in the process of extravasation. If the key molecule(s) involved in this spreading step could be inhibited then this would provide potential therapeutic targets for the treatment of inflammatory diseases.

When a neutrophil spreads on the blood vessel endothelium the surface area of the cell increases by two fold (Dewitt and Hallett, 2007). It is most likely that the reservoir of extra membrane required for neutrophil spreading comes from the 'un-wrinkling' of the neutrophil membrane as it spreads out.

Biophysical experiments have shown that there is only a limited amount of slack in the wrinkles in the neutrophil membrane (Herant et al., 2005, Herant et al., 2006). L-selectins and β2 integrins, involved in neutrophil rolling and adhesion are found on the neutrophil membrane wrinkles. L-selectin is found on the apical surface of neutrophil membrane wrinkles (Bruehl et al., 1996, Erlandsen

et al., 1993) and β2 integrins are found on the cell body between the membrane wrinkles (Erlandsen et al., 1993). This would be a logical position if the neutrophil did unwrinkle its membrane during spreading as during extravasation neutrophils first roll along the blood vessel endothelium binding transiently via selectins before they spread and make firm adhesions via β2 integrins. L-selectin is bound to the actin cytoskeleton by the linker molecule ezrin (Ivetic et al., 2002) and β2 integrin is anchored between the plasma membrane and the cytoskeleton via talin (Sampath et al., 1998), both members of the ERM family of proteins. Cleavage of these molecules would release the membrane when the neutrophil is activated and it is proposed that these hold the membrane wrinkles in place in resting neutrophils like a 'molecular velcro' (Dewitt and Hallett, 2007).

It is known that neutrophil spreading is directly linked to an increase in cytosolic calcium concentration as a large rise in cytosolic calcium accompanies neutrophil spreading and un-caging cytosolic Ca^{2+} or IP_3 provides an increase in Ca^{2+} large enough to trigger neutrophil spreading (Pettit and Hallett, 1998a). This raises the possibility that an enzyme or second messenger activated by the cytosolic calcium signal which triggers neutrophil spreading and phagocytosis is actively increasing the amount of membrane available.

Calpain is a Ca^{2+} activated protease which has been shown to cleave the linker molecule talin in vitro. Calpain 1 or μ-calpain is the predominant calpain found in neutrophils (Goll et al., 2003). It is made up of a large proteolytic subunit (80kDa) and a small regulatory subunit (30kDa). Each subunit is then made up of several different domains. Domains 1 – 4 make up the large subunit and domains 5 and 6 make up the small subunit (Goll et al., 2003). The C2 like domain in found in domain 3 on the large subunit enables the protein to translocate to the plasma membrane and bind phosphatidylserine in areas of high calcium.

μ Calpain (calpain 1) requires unusually high calcium concentrations to be activated, around 30 μM (Michetti et al., 1997). During calcium influx there are areas of calcium concentrations high enough to activate calpain just below the plasma membrane, especially within the membrane wrinkles (Brasen et al., 2010, Davies and Hallett, 1998). It was postulated that calpain's C2 like domain may enable it to translocate from the cytoplasm and bind to the phosphatidylserine on the membrane where calcium is highest. These factors would ensure that calpain is activated specifically at the plasma membrane. Therefore calpain inhibition poses a potential therapeutic target for the treatment of inflammatory disease as calpain inhibition has the potential to inhibit neutrophil spreading.

The aims of this thesis were to show that calpain translocation and activation occurs during morphological change, including spreading and phagocytosis.

A number of challenges were encountered in achieving these aims. Neutrophils are terminally differentiated cells with a lifespan of only 3-4 days and as such widely used methods of transfecting these cells are not viable in neutrophils. Even myeloid cell lines are not easily transfected using conventional methods. There are just two stably transfected HL60 cell lines generated (Servant et al., 2000, van Bruggen et al., 2004). In addition to this, some success has been reported in transiently expressing proteins in HL60s (Lacalle et al., 2007, Nuzzi et al., 2007). As such considerable effort was spent in establishing an efficient method for stably transfecting myeloid cell lines for use in this study and a phagocytic macrophage cell line (RAW264) which was easy to transfect had to be used as a model cell line for the majority of experiments. Although these cells were easier to transfect than the myeloid cell lines they were not as efficient at phagocytosis as primary neutrophils and as such studying phagocytosis as part of membrane expansion was difficult in these cells. Despite these challenges a method of stably transfecting myeloid

cell lines was established as well as further evidence of the role of calpain in neutrophil shape change.

8.2 Results presented in this thesis

8.2a C2 domain translocation during phagocytosis

The calcium influx triggered by phagocytosis is sufficient to trigger C2 domain translocation. C2 domain translocation occurred before phagosome closure suggesting that calcium influx starts before closure of the phagosome with some suggestion that C2 domain proteins translocate first to the membrane around the phagosome before the rest of the plasma membrane. This suggests that the initial source of calcium influx is from the calcium channels in the membrane around the phagosome.

8.2b Activity of C2 domains in apoptotic cells

Total C2 domain translocation is diminished but not completely abolished in apoptotic cells. The rate of translocation however remained the same. The decrease in the amount of C2 domain translocation is most likely due to there simply being less phosphatidyl serine on the inner leaflet of the plasma membrane for the C2 domain to bind and as such the rate of translocation remains the same.

8.2c Location of calpain in apoptotic neutrophils

Despite translocation of classical C2 domains being diminished but not completely abolished in apoptotic cells μ-calpain (which contains a 'C2 like' domain) is completely absent from the membrane in apoptotic neutrophils. This suggests that calpains 'C2 like' domain does not act in the same manner as a

typical C2 domain and has a lower calcium affinity than the classical C2 domain from protein kinase C used in these studies. This suggested that if the calcium affinity of calpains 'C2 like' domain was lower than that of classical C2 domains then calpain would only be present at the membrane where calcium concentration was at its highest such as in the membrane wrinkles during calcium influx (Brasen et al., 2010, Davies and Hallett, 1998) where it could specifically cleave its substrates and allow membrane 'unwrinkling' and cell spreading.

8.2d Calpain is located at the plasma membrane in activated human neutrophils

Calpain was found to be located uniformly at the plasma membrane in human neutrophils spread on glass and at the leading edge in neutrophils which were spread and undergoing chemotaxis. Calpain was also found to be located around the phagocytic cup during and immediately after phagocytosis but was not found around the phagosome when phagocytosis was complete. This suggested that calpain is present in the right place and at the right time to play a role in neutrophil shape change.

8.2e Calpain4-YFP activity in living cells during phagocytosis and shape change.

Calpain4-YFP over expressed in RAW264 cells was found to be located in the cytoplasm in resting cells. Calpain4-YFP did not translocate in response to increases in cytosolic calcium concentration. Potential reasons for this were discussed in detail in section 4.4. But briefly, as it has been established that calpains 'C2 like' domain probably has a low calcium affinity it is possible that only the calpain already within the membrane wrinkles at the time of calcium influx encounters calcium concentrations high enough to bind phosphatidyl serine in the plasma membrane.

8.2f Lentiviral transduction is the most efficient method for the stable transfection of myeloid cell lines.

A number of widely-used methods are unsuitable for transfecting neutrophils and myeloid cells. Lentiviral transduction was established to be the best method of transfection as it gave a highly efficient transfection of the growing precursor cells (cell line) such that sufficient expressed protein remained when protein synthesis was shut-down during terminal differentiation.

8.2g Possible mechanism of membrane retrieval after neutrophil shape change

Fluid filled inclusions appear in the cytoplasm after phagosome closure during phagocytosis. Large proteins are readily taken up into the inclusions and they are derived from the phagosome and appear to coalesce as they approach the plasma membrane. This suggests that the cell is potentially retrieving the excess membrane from the phagosome for reinsertion into the plasma membrane. There is another potential explanation for these results though. It is possible that the inclusions are the results of 'kiss and run' fusion of granules with the phagosome as when the plasma membrane was marked with a membrane marker none of this marker was found around the inclusions despite it being clearly visible around the phagosome.

8.3 Future Prospects

The work presented in this thesis has raised some interesting new questions as well as some old questions which are yet to be answered.

- The location of calcium influx during phagocytosis.
- The effect of phosphatidyl serine externalisation on C2 domain translocation was only investigated in one C2 domain with a high calcium affinity in this thesis, further work should be done to investigate this effect in different C2 domain proteins with different calcium affinities.
- Here it has only been established that the calcium affinity of calpains 'C2 like' domain is likely to be lower than that of the C2 domain from PKCγ, a more definitive answer would be useful.
- It is yet to be established how calpain translocates to the plasma membrane in real time.
- If the fluid filled inclusions observed after phagosome closure are a method of membrane retrieval then the exact mechanism of this remains unknown.

8.3.1 Calcium influx during phagocytosis

Phagocytosis involves extensive membrane expansion and is a calcium dependent process (Edberg et al., 1995, Kobayashi et al., 1995). The results presented in this thesis show C2 domain translocation occurs during phagocytosis. C2 domain (C2γ) binding to the phosphatidyl serine in the plasma membrane means that the localised calcium concentration which results from Ca^{2+} influx must be high (probably in excess of at least 7μM for maximum effect) (Kohout et al., 2002) at the plasma membrane indicating that the plasma membrane calcium channels must be open so that the calcium at the plasma membrane is high enough to stimulate C2 domain binding. The C2 domain translocation occurred before phagosome closure suggesting that the plasma membrane calcium channels were open before phagosome closure. Not all of the cells displayed C2 domain translocation during phagocytosis but it

is widely accepted that calcium influx is not obligatory for the internalisation phase of phagocytosis (Dewitt et al., 2003, Jaconi et al., 1990) so this is not surprising. There is evidence to suggest that the initial increase in calcium concentration responsible for particle internalisation is caused by release of calcium from intracellular stores and not from calcium influx (Steinckwich et al.). However, the released Ca^{2+} would need to elevate Ca^{2+} near the plasma membrane to at least 7μM to be responsible for the C2 domain translocation. As bulk cytosolic Ca^{2+} rarely exceeds 1μM, and the Ca^{2+} release sites are usually within the cell rather than at the membrane, this is unlikely.

Previous studies have reported a localised increase in cytosolic calcium concentration around the phagosome during phagocytosis (Jaconi et al., 1990). There has been controversy over the contribution of calcium influx and store depletion to this localised calcium increase. It has been shown that intracellular calcium stores translocate to the phagosome during phagocytosis and it has been postulated that release of calcium from these stores is responsible for the local calcium increase around the phagosome (Stendahl et al., 1994). However, more recently, evidence has shown a decrease in calcium concentration in the phagosome after phagosome closure (Lundqvist-Gustafsson et al., 2000). This calcium concentration decrease was inhibited by blocking plasma membrane calcium channels. This suggests that the increase in calcium concentration around the phagosome could be the result of calcium influx from the phagosome. Further experiments investigating C2 domain translocation during phagocytosis could provide further insight into this. Preliminary evidence presented in this thesis suggests that C2 domains can translocate first to the plasma membrane around the phagocytic cup before the plasma membrane around the rest of the cell. This was however before phagosome closure and the C2 domain quickly translocated to the rest of the plasma membrane. Further experiments using C2 domains with differing calcium affinities would provide more evidence as to the first plasma membrane calcium channels to open during phagocytosis. This will provide evidence as to the source of the increase in calcium concentration around the phagosome as

well as further insight into what calcium channels may play a role in the calcium influx during phagocytosis.

8.3.2 Effect of phosphatidyl serine concentration on C2 domain translocation

C2 domains most likely bind to phosphatidyl serine in the plasma membrane in a calcium dependent manner through co-operation between calcium binding and phosphatidyl serine binding (Nalefski and Falke, 1996). During apoptosis ~50% of the total phosphatidyl serine is externalised to the outer leaflet of the plasma membrane (Martin et al., 1995) and is therefore unavailable for C2 domain proteins to bind to. In this study translocation of a C2 domain with a high calcium affinity (from protein kinase C γ) was diminished but not abolished in apoptotic cells. The rate of translocation remained the same but the total amount of C2 domain bound to the plasma membrane decreased. The decrease in the total amount of C2 domain bound to the plasma membrane was most likely due to there simply being less phosphatidyl serine available for the C2 domains to bind. However, the fact that the rate of translocation remains unchanged in apoptotic cells suggests that calcium may be the limiting factor in C2 domain-phosphatidyl serine binding. Therefore further experiments investigating the translocation of C2 domains with lower calcium affinities would provide further insight into the role of calcium in C2 domain-phosphatidyl serine binding.

8.3.3 Calpains 'C2 like' domain

Domain III of calpain is known as a 'C2 like domain as it bears amino acid sequence homology to classical C2 domains (Goll et al., 2003). However, there is yet to be any significant evidence of the functional similarity of calpains 'C2 like' domain to classical C2 domains. It has been shown in this thesis that despite classical C2 domains with high calcium affinities translocating in

apoptotic cells in which phosphatidyl serine on the inner leaflet of the plasma membrane is decreased, calpain is completely absent from the membrane in apoptotic neutrophils. This suggests that calpains 'C2 like' domain does not function in the same way as the classical C2 domain used in this study. The $[Ca2+]^{1/2}$ of the C2 domain used in this study was 0.7μM, it follows that as calpain was not at the plasma membrane in apoptotic neutrophils but the classical C2 domain as still able to translocate in apoptotic cells that the $[Ca2+]^{1/2}$ of calpains 'C2 like' domain must be higher than 0.7μM. It would be useful to further pinpoint the calcium affinity of calpains 'C2 like' domain, either by comparison with other classical C2 domains or through expression of just the domain III from μ-calpain as attempted in this thesis, to gain an idea of under what circumstances it would translocate to the plasma membrane. It is highly likely that calpains 'C2 like' domain has a very low calcium affinity as calpain also requires a very high calcium concentration to be activated. Therefore it would be logical for calpain to only be found at the plasma membrane where calcium concentrations are high enough for it to be activated (Brasen et al., 2010, Davies and Hallett, 1998). It would therefore be in the right place to cleave its substrates, talin and ezrin (Ivetic et al., 2002, Shcherbina et al., 1999) which hold the plasma membrane wrinkles in place (Ivetic et al., 2002, Sampath et al., 1998) and cause the membrane to unwrinkle and allow the neutrophil to spread.

8.3.4 Calpain translocation

Translocation of μ calpain from the cytosol where it is present in resting cells to the plasma membrane is a key step if it is involved in neutrophil shape change. Results presented in this thesis show that calpain is present at the plasma membrane where shape change is taking place in human neutrophils. This means that calpain is present in the right place and at the right time to be activated by the high calcium concentrations in the plasma membrane wrinkles during calcium influx (Brasen et al., 2010, Davies and Hallett, 1998) and able to cleave its substrates allowing the membrane to 'unwrinkle' and the neutrophil to

spread. This translocation has yet to be observed in real time however. Calpain 4-YFP over expressed in the RAW264 cell line failed to translocate upon cytosolic calcium increase. The potential reasons for this are discussed fully in section 4.4.1. Over expression of calpain 1 with a fluorescent tag in a myeloid cell line would provide a more definitive answer. This approach was addressed in this thesis but a considerable amount of time had to be spent developing the most appropriate method of transfecting myeloid cell lines. Although a suitable method was eventually established the time constraints of this project meant that this final step could not be seen through to completion. Once translocation has been established the exact role that calpain plays in neutrophil spreading would need to be addressed. It has been shown that calpain inhibition disrupts neutrophil shape change (Parr C, unpublished observation). Next, evidence would need to be sought that calpains target substrates are undergoing cleavage at the plasma membrane upon cytosolic calcium increase. Preliminary data suggests that the calpain substrates talin and ezrin are being cleaved during cytosolic calcium influx in human neutrophils (Elumalai G, unpublished observation). Finally it will need to be confirmed that calpain is indeed activated and causing the cleavage of these substrates (talin and ezrin) when it is at the plasma membrane under the very specific calcium conditions involved in neutrophil spreading in order to confirm the role of calpain in neutrophil spreading.

8.3.5 Membrane retrieval

If neutrophils undergo extensive membrane expansion during processes which involve shape change such as spreading and phagocytosis then there must be a mechanism for retrieving the excess membrane after the process is complete. Pinosome like structures have been reported to accompany phagocytosis in human neutrophils (Bauer and Tapper, 2004, Botelho et al., 2002) and it has been postulated that these may play a role in membrane retrieval. This has been investigated in this thesis. Although an alternative hypothesis for the existence of these pinosome like structures has been proposed, that they come

about as a result of 'kiss and run' fusion of granules with the phagosome, evidence still remains that they may be a sign that membrane recycling is occurring. This thesis has focused on the mechanism by which neutrophil membrane wrinkles are released to expand the plasma membrane and allow shape change to occur. If the excess membrane produced for neutrophil shape change is indeed retrieved and recycled back into the plasma membrane then further research needs to be carried out into a mechanism by which the excess membrane is reinserted into the plasma membrane and how the plasma membrane wrinkles are reintroduced, if they are at all.

8.4 Conclusion

Neutrophil spreading and membrane expansion is a complicated process and studying any process in neutrophils is always challenging as neutrophils are terminally differentiated cells which are not amenable to the genetic manipulations that can be used to study various processes in many other cell types. Even myeloid cell lines which can be differentiated into phagocytic neutrophil like cells and can be of use in modelling neutrophil behaviour are notoriously problematic when it comes to common genetic manipulation techniques such as transfection. Despite these many challenges an effective method of establishing stable expression of a transfected gene in myeloid cell lines has been established. It has been shown that μ calpain is present in the right place and at the right time to play a critical role in the control of neutrophil membrane expansion. It is postulated that calpain is able to move from the cytoplasm to the plasma membrane where it is present during membrane expansion by virtue of its 'C2 like' domain. It has been shown that this 'C2 like' domain has a much lower calcium affinity than classical C2 domains and as such calpain would only be present at the plasma membrane in areas where calcium concentration is at its highest i.e. within the membrane wrinkles. Here the calcium concentration is also high enough to activate the protease and enable calpain to cleave its substrate, talin and ezrin, which hold the membrane wrinkles in place. This would lead to 'unwrinkling' of the plasma membrane

allowing for the membrane expansion that is needed for the neutrophil to spread.

References

ADJOBO-HERMANS, M. J., GOEDHART, J. & GADELLA, T. W., JR. (2008) Regulation of PLCbeta1a membrane anchoring by its substrate phosphatidylinositol (4,5)-bisphosphate. *J Cell Sci,* 121, 3770-7.
ALLINGHAM, M. J., VAN BUUL, J. D. & BURRIDGE, K. (2007) ICAM-1-mediated, Src- and Pyk2-dependent vascular endothelial cadherin tyrosine phosphorylation is required for leukocyte transendothelial migration. *J Immunol,* 179, 4053-64.
ALMERS, W. (1990) Exocytosis. *Annu Rev Physiol,* 52, 607-24.
ALUIGI, M., FOGLI, M., CURTI, A., ISIDORI, A., GRUPPIONI, E., CHIODONI, C., COLOMBO, M. P., VERSURA, P., D'ERRICO-GRIGIONI, A., FERRI, E., BACCARANI, M. & LEMOLI, R. M. (2006) Nucleofection is an efficient nonviral transfection technique for human bone marrow-derived mesenchymal stem cells. *Stem Cells,* 24, 454-61.
AOKI, K., IMAJOH, S., OHNO, S., EMORI, Y., KOIKE, M., KOSAKI, G. & SUZUKI, K. (1986) Complete amino acid sequence of the large subunit of the low-Ca2+-requiring form of human Ca2+-activated neutral protease (muCANP) deduced from its cDNA sequence. *FEBS Lett,* 205, 313-7.
ARTHUR, J. S., ELCE, J. S., HEGADORN, C., WILLIAMS, K. & GREER, P. A. (2000) Disruption of the murine calpain small subunit gene, Capn4: calpain is essential for embryonic development but not for cell growth and division. *Mol Cell Biol,* 20, 4474-81.
AURRAND-LIONS, M., LAMAGNA, C., DANGERFIELD, J. P., WANG, S., HERRERA, P., NOURSHARGH, S. & IMHOF, B. A. (2005) Junctional adhesion molecule-C regulates the early influx of leukocytes into tissues during inflammation. *J Immunol,* 174, 6406-15.
AZAM, M., ANDRABI, S. S., SAHR, K. E., KAMATH, L., KULIOPULOS, A. & CHISHTI, A. H. (2001) Disruption of the mouse mu-calpain gene reveals an essential role in platelet function. *Mol Cell Biol,* 21, 2213-20.
BAUER, S. & TAPPER, H. (2004) Membrane retrieval in neutrophils during phagocytosis: inhibition by M protein-expressing S. pyogenes bacteria. *J Leukoc Biol,* 76, 1142-50.
BAZZI, M. D. & NELSESTUEN, G. L. (1987) Association of protein kinase C with phospholipid vesicles. *Biochemistry,* 26, 115-22.
BERG (1993) *Random Walks in Biology*, Princeton Press.
BESSIS, M. (1973) *Living blood cells and their ultrastructure*, Springer, Berlin.
BLANCHARD, H., GROCHULSKI, P., LI, Y., ARTHUR, J. S., DAVIES, P. L., ELCE, J. S. & CYGLER, M. (1997) Structure of a calpain Ca(2+)-binding domain reveals a novel EF-hand and Ca(2+)-induced conformational changes. *Nat Struct Biol,* 4, 532-8.
BOGEN, S., PAK, J., GARIFALLOU, M., DENG, X. & MULLER, W. A. (1994) Monoclonal antibody to murine PECAM-1 (CD31) blocks acute inflammation in vivo. *J Exp Med,* 179, 1059-64.
BORREGAARD, N., KJELDSEN, L., SENGELOV, H., DIAMOND, M. S., SPRINGER, T. A., ANDERSON, H. C., KISHIMOTO, T. K. & BAINTON, D. F. (1994) Changes in subcellular localization and surface expression of L-selectin, alkaline phosphatase, and Mac-1 in human neutrophils during stimulation with inflammatory mediators. *J Leukoc Biol,* 56, 80-7.

BOTELHO, R. J., TAPPER, H., FURUYA, W., MOJDAMI, D. & GRINSTEIN, S. (2002) Fc gamma R-mediated phagocytosis stimulates localized pinocytosis in human neutrophils. *J Immunol,* 169, 4423-9.
BRADFIELD, P. F., NOURSHARGH, S., AURRAND-LIONS, M. & IMHOF, B. A. (2007) JAM family and related proteins in leukocyte migration (Vestweber series). *Arterioscler Thromb Vasc Biol,* 27, 2104-12.
BRASEN, J. C., OLSEN, L. F. & HALLETT, M. B. (2010) Cell surface topology creates high Ca2+ signalling microdomains. *Cell Calcium,* 47, 339-49.
BRECHARD, S. & TSCHIRHART, E. J. (2008) Regulation of superoxide production in neutrophils: role of calcium influx. *J Leukoc Biol,* 84, 1223-37.
BRUEHL, R. E., SPRINGER, T. A. & BAINTON, D. F. (1996) Quantitation of L-selectin distribution on human leukocyte microvilli by immunogold labeling and electron microscopy. *J Histochem Cytochem,* 44, 835-44.
BURGOYNE, R. D. & MORGAN, A. (2003) Secretory granule exocytosis. *Physiol Rev,* 83, 581-632.
CHATTERJEE, P. K., TODOROVIC, Z., SIVARAJAH, A., MOTA-FILIPE, H., BROWN, P. A., STEWART, K. N., MAZZON, E., CUZZOCREA, S. & THIEMERMANN, C. (2005) Inhibitors of calpain activation (PD150606 and E-64) and renal ischemia-reperfusion injury. *Biochem Pharmacol,* 69, 1121-31.
CHAVAKIS, T., KEIPER, T., MATZ-WESTPHAL, R., HERSEMEYER, K., SACHS, U. J., NAWROTH, P. P., PREISSNER, K. T. & SANTOSO, S. (2004) The junctional adhesion molecule-C promotes neutrophil transendothelial migration in vitro and in vivo. *J Biol Chem,* 279, 55602-8.
CHEUNG, T. Y., FAIRCHILD, M. J., ZARIVACH, R., TANENTZAPF, G. & VAN PETEGEM, F. (2009) Crystal structure of the talin integrin binding domain 2. *J Mol Biol,* 387, 787-93.
CLARK, J. D., LIN, L. L., KRIZ, R. W., RAMESHA, C. S., SULTZMAN, L. A., LIN, A. Y., MILONA, N. & KNOPF, J. L. (1991) A novel arachidonic acid-selective cytosolic PLA2 contains a Ca(2+)-dependent translocation domain with homology to PKC and GAP. *Cell,* 65, 1043-51.
COCKCROFT, S. & GOMPERTS, B. D. (1985) Role of guanine nucleotide binding protein in the activation of polyphosphoinositide phosphodiesterase. *Nature,* 314, 534-6.
COFFIN, J. M., HUGHES, S. H. & VARMUS, H. E. (1997) The Interactions of Retroviruses and their Hosts.
COHNHEIM (1867) Entendung und Eitung. *Virchow's Arch Pathol Anat,* 40, 1-25.
COHNHEIM (1967) Inflammation in Lectures in General Pathology. *Syndenham Society of London,* 1, 242-254.
COLLINS, S. J., GALLO, R. C. & GALLAGHER, R. E. (1977) Continuous growth and differentiation of human myeloid leukaemic cells in suspension culture. *Nature,* 270, 347-9.
COLLINS, S. J., RUSCETTI, F. W., GALLAGHER, R. E. & GALLO, R. C. (1978) Terminal differentiation of human promyelocytic leukemia cells

induced by dimethyl sulfoxide and other polar compounds. *Proc Natl Acad Sci U S A,* 75, 2458-62.
CONG, J., THOMPSON, V. F. & GOLL, D. E. (1993) Effect of monoclonal antibodies specific for the 28-kDa subunit on catalytic properties of the calpains. *J Biol Chem,* 268, 25740-7.
CRITCHLEY, D. R. & GINGRAS, A. R. (2008) Talin at a glance. *J Cell Sci,* 121, 1345-7.
CROCE, K., FLAUMENHAFT, R., RIVERS, M., FURIE, B., FURIE, B. C., HERMAN, I. M. & POTTER, D. A. (1999) Inhibition of calpain blocks platelet secretion, aggregation, and spreading. *J Biol Chem,* 274, 36321-7.
CUZZOCREA, S., MCDONALD, M. C., MAZZON, E., SIRIWARDENA, D., SERRAINO, I., DUGO, L., BRITTI, D., MAZZULLO, G., CAPUTI, A. P. & THIEMERMANN, C. (2000) Calpain inhibitor I reduces the development of acute and chronic inflammation. *Am J Pathol,* 157, 2065-79.
DARLEY, R. L., PEARN, L., OMIDVAR, N., SWEENEY, M., FISHER, J., PHILLIPS, S., HOY, T. & BURNETT, A. K. (2002) Protein kinase C mediates mutant N-Ras-induced developmental abnormalities in normal human erythroid cells. *Blood,* 100, 4185-92.
DAVIES-COX, E. V., LAFFAFIAN, I. & HALLETT, M. B. (2001) Control of Ca2+ influx in human neutrophils by inositol 1,4,5-trisphosphate (IP3) binding: differential effects of micro-injected IP3 receptor antagonists. *Biochem J,* 355, 139-43.
DAVIES, E. V. & HALLETT, M. B. (1995) A novel pathway for Ca2+ signalling in neutrophils by immune complexes. *Immunology,* 85, 538-43.
DAVIES, E. V. & HALLETT, M. B. (1998) High micromolar Ca2+ beneath the plasma membrane in stimulated neutrophils. *Biochem Biophys Res Commun,* 248, 679-83.
DAVIES, E. V., HALLETT, M. B. & CAMPBELL, A. K. (1991) Localized superoxide release by neutrophils can be provoked by a cytosolic calcium 'cloud'. *Immunology,* 73, 228-34.
DAVLETOV, B. A. & SUDHOF, T. C. (1993) A single C2 domain from synaptotagmin I is sufficient for high affinity Ca2+/phospholipid binding. *J Biol Chem,* 268, 26386-90.
DAVLETOV, B. A. & SUDHOF, T. C. (1994) Ca(2+)-dependent conformational change in synaptotagmin I. *J Biol Chem,* 269, 28547-50.
DEMAUREX, N., LEW, D. P. & KRAUSE, K. H. (1992) Cyclopiazonic acid depletes intracellular Ca2+ stores and activates an influx pathway for divalent cations in HL-60 cells. *J Biol Chem,* 267, 2318-24.
DEMAUREX, N., MONOD, A., LEW, D. P. & KRAUSE, K. H. (1994) Characterization of receptor-mediated and store-regulated Ca2+ influx in human neutrophils. *Biochem J,* 297 (Pt 3), 595-601.
DEWITT, S. & HALLETT, M. (2007) Leukocyte membrane "expansion": a central mechanism for leukocyte extravasation. *J Leukoc Biol,* 81, 1160-4.
DEWITT, S. & HALLETT, M. B. (2002) Cytosolic free Ca(2+) changes and calpain activation are required for beta integrin-accelerated phagocytosis by human neutrophils. *J Cell Biol,* 159, 181-9.

DEWITT, S., LAFFAFIAN, I. & HALLETT, M. B. (2003) Phagosomal oxidative activity during beta2 integrin (CR3)-mediated phagocytosis by neutrophils is triggered by a non-restricted Ca2+ signal: Ca2+ controls time not space. *J Cell Sci,* 116, 2857-65.

DICK, E. P., PRINCE, L. R., PRESTWICH, E. C., RENSHAW, S. A., WHYTE, M. K. & SABROE, I. (2009) Pathways regulating lipopolysaccharide-induced neutrophil survival revealed by lentiviral transduction of primary human neutrophils. *Immunology,* 127, 249-55.

DICK, E. P., PRINCE, L. R. & SABROE, I. (2008) Ex vivo-expanded bone marrow CD34+ derived neutrophils have limited bactericidal ability. *Stem Cells,* 26, 2552-63.

DINARELLO, C. A. (1996) Biologic basis for interleukin-1 in disease. *Blood,* 87, 2095-147.

DING, Z. M., BABENSEE, J. E., SIMON, S. I., LU, H., PERRARD, J. L., BULLARD, D. C., DAI, X. Y., BROMLEY, S. K., DUSTIN, M. L., ENTMAN, M. L., SMITH, C. W. & BALLANTYNE, C. M. (1999) Relative contribution of LFA-1 and Mac-1 to neutrophil adhesion and migration. *J Immunol,* 163, 5029-38.

DOURDIN, N., BHATT, A. K., DUTT, P., GREER, P. A., ARTHUR, J. S., ELCE, J. S. & HUTTENLOCHER, A. (2001) Reduced cell migration and disruption of the actin cytoskeleton in calpain-deficient embryonic fibroblasts. *J Biol Chem,* 276, 48382-8.

DREXLER, H. G., DIRKS, W. G., MATSUO, Y. & MACLEOD, R. A. (2003) False leukemia-lymphoma cell lines: an update on over 500 cell lines. *Leukemia,* 17, 416-26.

DU, J., XIE, J. & YUE, L. (2009) Intracellular calcium activates TRPM2 and its alternative spliced isoforms. *Proc Natl Acad Sci U S A,* 106, 7239-44.

DUNNE, J. L., BALLANTYNE, C. M., BEAUDET, A. L. & LEY, K. (2002) Control of leukocyte rolling velocity in TNF-alpha-induced inflammation by LFA-1 and Mac-1. *Blood,* 99, 336-41.

DWIR, O., KANSAS, G. S. & ALON, R. (2001) Cytoplasmic anchorage of L-selectin controls leukocyte capture and rolling by increasing the mechanical stability of the selectin tether. *J Cell Biol,* 155, 145-56.

EDBERG, J. C., LIN, C. T., LAU, D., UNKELESS, J. C. & KIMBERLY, R. P. (1995) The Ca2+ dependence of human Fc gamma receptor-initiated phagocytosis. *J Biol Chem,* 270, 22301-7.

EHRLICH P, L. E. (1898) Die Anaemie. Wein. Kessinger Publishing Company.

ERLANDSEN, S. L., HASSLEN, S. R. & NELSON, R. D. (1993) Detection and spatial distribution of the beta 2 integrin (Mac-1) and L-selectin (LECAM-1) adherence receptors on human neutrophils by high-resolution field emission SEM. *J Histochem Cytochem,* 41, 327-33.

FAVRE, C. J., JERSTROM, P., FOTI, M., STENDHAL, O., HUGGLER, E., LEW, D. P. & KRAUSE, K. H. (1996) Organization of Ca2+ stores in myeloid cells: association of SERCA2b and the type-1 inositol-1,4,5-trisphosphate receptor. *Biochem J,* 316 (Pt 1), 137-42.

FENG, D., NAGY, J. A., PYNE, K., DVORAK, H. F. & DVORAK, A. M. (1998) Neutrophils emigrate from venules by a transendothelial cell pathway in response to FMLP. *J Exp Med,* 187, 903-15.

FRANCO, S. J., RODGERS, M. A., PERRIN, B. J., HAN, J., BENNIN, D. A., CRITCHLEY, D. R. & HUTTENLOCHER, A. (2004) Calpain-mediated proteolysis of talin regulates adhesion dynamics. *Nat Cell Biol,* 6, 977-83.

GALLIN, J. I. & SNYDERMAN, R. (1999) *Inflammation: Basic Principles and Clinical Correlates*, Lippincott Williams and Wilkins.

GARRIDO-URBANI, S., BRADFIELD, P. F., LEE, B. P. & IMHOF, B. A. (2008) Vascular and epithelial junctions: a barrier for leucocyte migration. *Biochem Soc Trans,* 36, 203-11.

GEE, K. R., BROWN, K. A., CHEN, W. N., BISHOP-STEWART, J., GRAY, D. & JOHNSON, I. (2000) Chemical and physiological characterization of fluo-4 Ca(2+)-indicator dyes. *Cell Calcium,* 27, 97-106.

GENNARO, R., POZZAN, T. & ROMEO, D. (1984) Monitoring of cytosolic free Ca2+ in C5a-stimulated neutrophils: loss of receptor-modulated Ca2+ stores and Ca2+ uptake in granule-free cytoplasts. *Proc Natl Acad Sci U S A,* 81, 1416-20.

GIL-PARRADO, S., POPP, O., KNOCH, T. A., ZAHLER, S., BESTVATER, F., FELGENTRAGER, M., HOLLOSCHI, A., FERNANDEZ-MONTALVAN, A., AUERSWALD, E. A., FRITZ, H., FUENTES-PRIOR, P., MACHLEIDT, W. & SPIESS, E. (2003) Subcellular localization and in vivo subunit interactions of ubiquitous mu-calpain. *J Biol Chem,* 278, 16336-46.

GINGRAS, A. R., ZIEGLER, W. H., BOBKOV, A. A., JOYCE, M. G., FASCI, D., HIMMEL, M., ROTHEMUND, S., RITTER, A., GROSSMANN, J. G., PATEL, B., BATE, N., GOULT, B. T., EMSLEY, J., BARSUKOV, I. L., ROBERTS, G. C., LIDDINGTON, R. C., GINSBERG, M. H. & CRITCHLEY, D. R. (2009) Structural determinants of integrin binding to the talin rod. *J Biol Chem,* 284, 8866-76.

GOLL, D. E., THOMPSON, V. F., LI, H., WEI, W. & CONG, J. (2003) The calpain system. *Physiol Rev,* 83, 731-801.

GORSKA, K., MASKEY-WARZECHOWSKA, M. & KRENKE, R. (2010) Airway inflammation in chronic obstructive pulmonary disease. *Curr Opin Pulm Med,* 16, 89-96.

GOTSCH, U., BORGES, E., BOSSE, R., BOGGEMEYER, E., SIMON, M., MOSSMANN, H. & VESTWEBER, D. (1997) VE-cadherin antibody accelerates neutrophil recruitment in vivo. *J Cell Sci,* 110 (Pt 5), 583-8.

GRIFFIN, ARIF & MUFTI (2003) *Immunology and Haematology: Crash Course*, Elsevier Science.

GUTTMANN, R. P., ELCE, J. S., BELL, P. D., ISBELL, J. C. & JOHNSON, G. V. (1997) Oxidation inhibits substrate proteolysis by calpain I but not autolysis. *J Biol Chem,* 272, 2005-12.

HAAS, K., SIN, W. C., JAVAHERIAN, A., LI, Z. & CLINE, H. T. (2001) Single-cell electroporation for gene transfer in vivo. *Neuron,* 29, 583-91.

HALLETT & LLOYDS (1997) *The Molecular and Ionic Signalling of Neutrophils*, Landes Bioscience.

HALLETT, M. B. & CAMPBELL, A. K. (1984) Is intracellular Ca2+ the trigger for oxygen radical production by polymorphonuclear leucocytes? *Cell Calcium,* 5, 1-19.

HALLETT, M. B., DAVIES, E. V. & CAMPBELL, A. K. (1990) Oxidase activation in individual neutrophils is dependent on the onset and magnitude of the Ca2+ signal. *Cell Calcium,* 11, 655-63.
HALLETT, M. B. & DEWITT, S. (2007) Ironing out the wrinkles of neutrophil phagocytosis. *Trends Cell Biol,* 17, 209-14.
HALLETT, M. B. & LLOYDS, D. (1995) Neutrophil priming: the cellular signals that say 'amber' but not 'green'. *Immunol Today,* 16, 264-8.
HALLETT, M. B. & PETTIT, E. J. (1997) Stochastic events underlie Ca2+ signalling in neutrophils. *J Theor Biol,* 186, 1-6.
HAMILL, O. P. & MARTINAC, B. (2001) Molecular basis of mechanotransduction in living cells. *Physiol Rev,* 81, 685-740.
HAMM, A., KROTT, N., BREIBACH, I., BLINDT, R. & BOSSERHOFF, A. K. (2002) Efficient transfection method for primary cells. *Tissue Eng,* 8, 235-45.
HEINER, I., RADUKINA, N., EISFELD, J., KUHN, F. & LUCKHOFF, A. (2005) Regulation of TRPM2 channels in neutrophil granulocytes by ADP-ribose: a promising pharmacological target. *Naunyn Schmiedebergs Arch Pharmacol,* 371, 325-33.
HELLBERG, C., MOLONY, L., ZHENG, L. & ANDERSSON, T. (1996) Ca2+ signalling mechanisms of the beta 2 integrin on neutrophils: involvement of phospholipase C gamma 2 and Ins(1,4,5)P3. *Biochem J,* 317 (Pt 2), 403-9.
HEMMINGS, L., REES, D. J., OHANIAN, V., BOLTON, S. J., GILMORE, A. P., PATEL, B., PRIDDLE, H., TREVITHICK, J. E., HYNES, R. O. & CRITCHLEY, D. R. (1996) Talin contains three actin-binding sites each of which is adjacent to a vinculin-binding site. *J Cell Sci,* 109 (Pt 11), 2715-26.
HERANT, M., HEINRICH, V. & DEMBO, M. (2005) Mechanics of neutrophil phagocytosis: behavior of the cortical tension. *J Cell Sci,* 118, 1789-97.
HERANT, M., HEINRICH, V. & DEMBO, M. (2006) Mechanics of neutrophil phagocytosis: experiments and quantitative models. *J Cell Sci,* 119, 1903-13.
HIRSCH, J. G. & COHN, Z. A. (1960) Degranulation of polymorphonuclear leucocytes following phagocytosis of microorganisms. *J Exp Med,* 112, 1005-14.
HOFFSTEIN, S. T. (1979) Ultrastructural demonstration of calcium loss from local regions of the plasma membrane of surface-stimulated human granulocytes. *J Immunol,* 123, 1395-402.
HOFMANN, T., OBUKHOV, A. G., SCHAEFER, M., HARTENECK, C., GUDERMANN, T. & SCHULTZ, G. (1999) Direct activation of human TRPC6 and TRPC3 channels by diacylglycerol. *Nature,* 397, 259-63.
HOGG, J. C. (1987) Neutrophil kinetics and lung injury. *Physiol Rev,* 67, 1249-95.
HOSFIELD, C. M., ELCE, J. S., DAVIES, P. L. & JIA, Z. (1999) Crystal structure of calpain reveals the structural basis for Ca(2+)-dependent protease activity and a novel mode of enzyme activation. *Embo J,* 18, 6880-9.

HUANG, M. T., LARBI, K. Y., SCHEIERMANN, C., WOODFIN, A., GERWIN, N., HASKARD, D. O. & NOURSHARGH, S. (2006) ICAM-2 mediates neutrophil transmigration in vivo: evidence for stimulus specificity and a role in PECAM-1-independent transmigration. *Blood,* 107, 4721-7.
IDRES, N., BENOIT, G., FLEXOR, M. A., LANOTTE, M. & CHABOT, G. G. (2001) Granulocytic differentiation of human NB4 promyelocytic leukemia cells induced by all-trans retinoic acid metabolites. *Cancer Res,* 61, 700-5.
IMAJOH, S., AOKI, K., OHNO, S., EMORI, Y., KAWASAKI, H., SUGIHARA, H. & SUZUKI, K. (1988) Molecular cloning of the cDNA for the large subunit of the high-Ca2+-requiring form of human Ca2+-activated neutral protease. *Biochemistry,* 27, 8122-8.
IRVINE, R. F. (1990) 'Quantal' Ca2+ release and the control of Ca2+ entry by inositol phosphates--a possible mechanism. *FEBS Lett,* 263, 5-9.
ITAGAKI, K., KANNAN, K. B., SINGH, B. B. & HAUSER, C. J. (2004) Cytoskeletal reorganization internalizes multiple transient receptor potential channels and blocks calcium entry into human neutrophils. *J Immunol,* 172, 601-7.
IVETIC, A., DEKA, J., RIDLEY, A. & AGER, A. (2002) The cytoplasmic tail of L-selectin interacts with members of the Ezrin-Radixin-Moesin (ERM) family of proteins: cell activation-dependent binding of Moesin but not Ezrin. *J Biol Chem,* 277, 2321-9.
IVETIC, A. & RIDLEY, A. J. (2004) Ezrin/radixin/moesin proteins and Rho GTPase signalling in leucocytes. *Immunology,* 112, 165-76.
JACOB, C., LEPORT, M., SZILAGYI, C., ALLEN, J. M., BERTRAND, C. & LAGENTE, V. (2002) DMSO-treated HL60 cells: a model of neutrophil-like cells mainly expressing PDE4B subtype. *Int Immunopharmacol,* 2, 1647-56.
JACONI, M. E., LEW, D. P., CARPENTIER, J. L., MAGNUSSON, K. E., SJOGREN, M. & STENDAHL, O. (1990) Cytosolic free calcium elevation mediates the phagosome-lysosome fusion during phagocytosis in human neutrophils. *J Cell Biol,* 110, 1555-64.
JACONI, M. E., THELER, J. M., SCHLEGEL, W., APPEL, R. D., WRIGHT, S. D. & LEW, P. D. (1991) Multiple elevations of cytosolic-free Ca2+ in human neutrophils: initiation by adherence receptors of the integrin family. *J Cell Biol,* 112, 1249-57.
JOHNSON, J. L., ELLIS, B. A., MUNAFO, D. B., BRZEZINSKA, A. A. & CATZ, S. D. (2006) Gene transfer and expression in human neutrophils. The phox homology domain of p47phox translocates to the plasma membrane but not to the membrane of mature phagosomes. *BMC Immunol,* 7, 28.
JORDAN, M., SCHALLHORN, A. & WURM, F. M. (1996) Transfecting mammalian cells: optimization of critical parameters affecting calcium-phosphate precipitate formation. *Nucleic Acids Res,* 24, 596-601.
JORDAN, M. & WURM, F. (2004) Transfection of adherent and suspended cells by calcium phosphate. *Methods,* 33, 136-43.
JOSHI, R. P. & SCHOENBACH, K. H. (2000) Electroporation dynamics in biological cells subjected to ultrafast electrical pulses: a numerical

simulation study. *Phys Rev E Stat Phys Plasmas Fluids Relat Interdiscip Topics,* 62, 1025-33.
JUTRAS, I. & DESJARDINS, M. (2005) Phagocytosis: at the crossroads of innate and adaptive immunity. *Annu Rev Cell Dev Biol,* 21, 511-27.
KANSAS, G. S., LEY, K., MUNRO, J. M. & TEDDER, T. F. (1993) Regulation of leukocyte rolling and adhesion to high endothelial venules through the cytoplasmic domain of L-selectin. *J Exp Med,* 177, 833-8.
KOBAYASHI, K., TAKAHASHI, K. & NAGASAWA, S. (1995) The role of tyrosine phosphorylation and Ca2+ accumulation in Fc gamma-receptor-mediated phagocytosis of human neutrophils. *J Biochem,* 117, 1156-61.
KOHOUT, S. C., CORBALAN-GARCIA, S., TORRECILLAS, A., GOMEZ-FERNANDEZ, J. C. & FALKE, J. J. (2002) C2 domains of protein kinase C isoforms alpha, beta, and gamma: activation parameters and calcium stoichiometries of the membrane-bound state. *Biochemistry,* 41, 11411-24.
KOYNOVA, R., WANG, L., TARAHOVSKY, Y. & MACDONALD, R. C. (2005) Lipid phase control of DNA delivery. *Bioconjug Chem,* 16, 1335-9.
KRAUSE, K. H., PITTET, D., VOLPE, P., POZZAN, T., MELDOLESI, J. & LEW, D. P. (1989) Calciosome, a sarcoplasmic reticulum-like organelle involved in intracellular Ca2+-handling by non-muscle cells: studies in human neutrophils and HL-60 cells. *Cell Calcium,* 10, 351-61.
KRUSKAL, B. A., SHAK, S. & MAXFIELD, F. R. (1986) Spreading of human neutrophils is immediately preceded by a large increase in cytoplasmic free calcium. *Proc Natl Acad Sci U S A,* 83, 2919-23.
LACALLE, R. A., PEREGIL, R. M., ALBAR, J. P., MERINO, E., MARTINEZ, A. C., MERIDA, I. & MANES, S. (2007) Type I phosphatidylinositol 4-phosphate 5-kinase controls neutrophil polarity and directional movement. *J Cell Biol,* 179, 1539-53.
LANOTTE, M., MARTIN-THOUVENIN, V., NAJMAN, S., BALERINI, P., VALENSI, F. & BERGER, R. (1991) NB4, a maturation inducible cell line with t(15;17) marker isolated from a human acute promyelocytic leukemia (M3). *Blood,* 77, 1080-6.
LAWRENCE, M. B. & SPRINGER, T. A. (1991) Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins. *Cell,* 65, 859-73.
LEBART, M. C. & BENYAMIN, Y. (2006) Calpain involvement in the remodeling of cytoskeletal anchorage complexes. *Febs J,* 273, 3415-26.
LEE, W. L., HARRISON, R. E. & GRINSTEIN, S. (2003) Phagocytosis by neutrophils. *Microbes Infect,* 5, 1299-306.
LEITINGER, B., MCDOWALL, A., STANLEY, P. & HOGG, N. (2000) The regulation of integrin function by Ca(2+). *Biochim Biophys Acta,* 1498, 91-8.
LEWIS, BAIN & BATES (2006) *Dacie and Lewis Practical Haematology,* Churchill Livingstone Elsevier.
LINDEMANN, D. & SCHNITTLER, H. (2009) Genetic manipulation of endothelial cells by viral vectors. *Thromb Haemost,* 102, 1135-43.

LLOYDS, D., BRINDLE, N. P. & HALLETT, M. B. (1995) Priming of human neutrophils by tumour necrosis factor-alpha and substance P is associated with tyrosine phosphorylation. *Immunology,* 84, 220-6.
LOKUTA, M. A., NUZZI, P. A. & HUTTENLOCHER, A. (2003) Calpain regulates neutrophil chemotaxis. *Proc Natl Acad Sci U S A,* 100, 4006-11.
LORANT, D. E., PATEL, K. D., MCINTYRE, T. M., MCEVER, R. P., PRESCOTT, S. M. & ZIMMERMAN, G. A. (1991) Coexpression of GMP-140 and PAF by endothelium stimulated by histamine or thrombin: a juxtacrine system for adhesion and activation of neutrophils. *J Cell Biol,* 115, 223-34.
LUNDQVIST-GUSTAFSSON, H., GUSTAFSSON, M. & DAHLGREN, C. (2000) Dynamic ca(2+)changes in neutrophil phagosomes A source for intracellular ca(2+)during phagolysosome formation? *Cell Calcium,* 27, 353-62.
MAASHO, K., MARUSINA, A., REYNOLDS, N. M., COLIGAN, J. E. & BORREGO, F. (2004) Efficient gene transfer into the human natural killer cell line, NKL, using the Amaxa nucleofection system. *J Immunol Methods,* 284, 133-40.
MARKS, P. W. & MAXFIELD, F. R. (1990) Local and global changes in cytosolic free calcium in neutrophils during chemotaxis and phagocytosis. *Cell Calcium,* 11, 181-90.
MARTIN, S. J., REUTELINGSPERGER, C. P., MCGAHON, A. J., RADER, J. A., VAN SCHIE, R. C., LAFACE, D. M. & GREEN, D. R. (1995) Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J Exp Med,* 182, 1545-56.
MARZOCCO, S., DI PAOLA, R., AUTORE, G., MAZZON, E., PINTO, A., CAPUTI, A. P., THIEMERMANN, C. & CUZZOCREA, S. (2004) Calpain inhibitor I reduces intestinal ischemia-reperfusion injury in the rat. *Shock,* 21, 38-44.
MCDONALD, J. U., CORTINI, A., ROSAS, M., FOSSATI-JIMACK, L., LING, G. S., LEWIS, K. J., DEWITT, S., LIDDIARD, K., BROWN, G. D., JONES, S. A., HALLETT, M. B., BOTTO, M. & TAYLOR, P. R. In vivo functional analysis and genetic modification of in vitro-derived mouse neutrophils. *FASEB J.*
MCEVER, R. P. & CUMMINGS, R. D. (1997) Perspectives series: cell adhesion in vascular biology. Role of PSGL-1 binding to selectins in leukocyte recruitment. *J Clin Invest,* 100, 485-91.
METCALF, D. (1985) The granulocyte-macrophage colony-stimulating factors. *Science,* 229, 16-22.
METCHNIKOFF (1893) Lecon sur la pathologie comparee de inflammation. *Ann l'Institure Pasteur,* 7, 348-357.
METCHNIKOFF (1901) *Lectures on the Comparative Physiology of Inflammation*, Kegan Paul: London.
METCHNIKOFF (1905) *Immunity in Infectious Disease*, Cambridge University: London.

MICHETTI, M., SALAMINO, F., MINAFRA, R., MELLONI, E. & PONTREMOLI, S. (1997) Calcium-binding properties of human erythrocyte calpain. *Biochem J,* 325 (Pt 3), 721-6.
MILLAN, J., HEWLETT, L., GLYN, M., TOOMRE, D., CLARK, P. & RIDLEY, A. J. (2006) Lymphocyte transcellular migration occurs through recruitment of endothelial ICAM-1 to caveola- and F-actin-rich domains. *Nat Cell Biol,* 8, 113-23.
MOLINARI, M. & CARAFOLI, E. (1997) Calpain: a cytosolic proteinase active at the membranes. *J Membr Biol,* 156, 1-8.
MOLLINEDO, F., LOPEZ-PEREZ, R. & GAJATE, C. (2008) Differential gene expression patterns coupled to commitment and acquisition of phenotypic hallmarks during neutrophil differentiation of human leukaemia HL-60 cells. *Gene,* 419, 16-26.
MORGAN, B. P., VAN DEN BERG, C. W., DAVIES, E. V., HALLETT, M. B. & HOREJSI, V. (1993) Cross-linking of CD59 and of other glycosyl phosphatidylinositol-anchored molecules on neutrophils triggers cell activation via tyrosine kinase. *Eur J Immunol,* 23, 2841-50.
MOSER, M., LEGATE, K. R., ZENT, R. & FASSLER, R. (2009) The tail of integrins, talin, and kindlins. *Science,* 324, 895-9.
NAKADA, M. T., AMIN, K., CHRISTOFIDOU-SOLOMIDOU, M., O'BRIEN, C. D., SUN, J., GURUBHAGAVATULA, I., HEAVNER, G. A., TAYLOR, A. H., PADDOCK, C., SUN, Q. H., ZEHNDER, J. L., NEWMAN, P. J., ALBELDA, S. M. & DELISSER, H. M. (2000) Antibodies against the first Ig-like domain of human platelet endothelial cell adhesion molecule-1 (PECAM-1) that inhibit PECAM-1-dependent homophilic adhesion block in vivo neutrophil recruitment. *J Immunol,* 164, 452-62.
NALDINI, L., BLOMER, U., GALLAY, P., ORY, D., MULLIGAN, R., GAGE, F. H., VERMA, I. M. & TRONO, D. (1996) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science,* 272, 263-7.
NALEFSKI, E. A. & FALKE, J. J. (1996) The C2 domain calcium-binding motif: structural and functional diversity. *Protein Sci,* 5, 2375-90.
NEUMANN, E., SCHAEFER-RIDDER, M., WANG, Y. & HOFSCHNEIDER, P. H. (1982) Gene transfer into mouse lyoma cells by electroporation in high electric fields. *EMBO J,* 1, 841-5.
NORMAN, K. E., MOORE, K. L., MCEVER, R. P. & LEY, K. (1995) Leukocyte rolling in vivo is mediated by P-selectin glycoprotein ligand-1. *Blood,* 86, 4417-21.
NUSSE, O. & LINDAU, M. (1988) The dynamics of exocytosis in human neutrophils. *J Cell Biol,* 107, 2117-23.
NUZZI, P. A., SENETAR, M. A. & HUTTENLOCHER, A. (2007) Asymmetric localization of calpain 2 during neutrophil chemotaxis. *Mol Biol Cell,* 18, 795-805.
OHNO, S., EMORI, Y. & SUZUKI, K. (1986) Nucleotide sequence of a cDNA coding for the small subunit of human calcium-dependent protease. *Nucleic Acids Res,* 14, 5559.
OKUDA, K., SANGHERA, J. S., PELECH, S. L., KANAKURA, Y., HALLEK, M., GRIFFIN, J. D. & DRUKER, B. J. (1992) Granulocyte-macrophage

colony-stimulating factor, interleukin-3, and steel factor induce rapid tyrosine phosphorylation of p42 and p44 MAP kinase. *Blood,* 79, 2880-7.
OTSUKA, Y. & GOLL, D. E. (1987) Purification of the Ca2+-dependent proteinase inhibitor from bovine cardiac muscle and its interaction with the millimolar Ca2+-dependent proteinase. *J Biol Chem,* 262, 5839-51.
PALMERI, D., VAN ZANTE, A., HUANG, C. C., HEMMERICH, S. & ROSEN, S. D. (2000) Vascular endothelial junction-associated molecule, a novel member of the immunoglobulin superfamily, is localized to intercellular boundaries of endothelial cells. *J Biol Chem,* 275, 19139-45.
PATTERSON, R. L., VAN ROSSUM, D. B. & GILL, D. L. (1999) Store-operated Ca2+ entry: evidence for a secretion-like coupling model. *Cell,* 98, 487-99.
PAVALKO, F. M., WALKER, D. M., GRAHAM, L., GOHEEN, M., DOERSCHUK, C. M. & KANSAS, G. S. (1995) The cytoplasmic domain of L-selectin interacts with cytoskeletal proteins via alpha-actinin: receptor positioning in microvilli does not require interaction with alpha-actinin. *J Cell Biol,* 129, 1155-64.
PERRAUD, A. L., FLEIG, A., DUNN, C. A., BAGLEY, L. A., LAUNAY, P., SCHMITZ, C., STOKES, A. J., ZHU, Q., BESSMAN, M. J., PENNER, R., KINET, J. P. & SCHARENBERG, A. M. (2001) ADP-ribose gating of the calcium-permeable LTRPC2 channel revealed by Nudix motif homology. *Nature,* 411, 595-9.
PETERSEN, M., WILLIAMS, J. D. & HALLETT, M. B. (1993) Cross-linking of CD11b or CD18 signals release of localized Ca2+ from intracellular stores in neutrophils. *Immunology,* 80, 157-9.
PETTIT, E. J. & HALLETT, M. B. (1995) Early Ca2+ signalling events in neutrophils detected by rapid confocal laser scanning. *Biochem J,* 310 (Pt 2), 445-8.
PETTIT, E. J. & HALLETT, M. B. (1996) Localised and global cytosolic Ca2+ changes in neutrophils during engagement of Cd11b/CD18 integrin visualised using confocal laser scanning reconstruction. *J Cell Sci,* 109 (Pt 7), 1689-94.
PETTIT, E. J. & HALLETT, M. B. (1997) Pulsatile Ca2+ influx in human neutrophils undergoing CD11b/CD18 integrin engagement. *Biochem Biophys Res Commun,* 230, 258-61.
PETTIT, E. J. & HALLETT, M. B. (1998a) Release of 'caged' cytosolic Ca2+ triggers rapid spreading of human neutrophils adherent via integrin engagement. *J Cell Sci,* 111 (Pt 15), 2209-15.
PETTIT, E. J. & HALLETT, M. B. (1998b) Two distinct Ca2+ storage and release sites in human neutrophils. *J Leukoc Biol,* 63, 225-32.
PETTY, H. R., HAFEMAN, D. G. & MCCONNELL, H. M. (1981) Disappearance of macrophage surface folds after antibody-dependent phagocytosis. *J Cell Biol,* 89, 223-9.
PRYZWANSKY, K. B., MACRAE, E. K., SPITZNAGEL, J. K. & COONEY, M. H. (1979) Early degranulation of human neutrophils: immunocytochemical studies of surface and intracellular phagocytic events. *Cell,* 18, 1025-33.
RAE, J. L. & LEVIS, R. A. (2002) Single-cell electroporation. *Pflugers Arch,* 443, 664-70.

ROBERTS, G. M., DAVIES, E. V., PETTIT, E. J. & HALLETT, M. B. (1997) The timing and magnitude of Ca2+ signaling by CD32 depends on its redistribution on the cell surface. *Exp Cell Res,* 230, 303-9.
ROSADO, J. A., BROWNLOW, S. L. & SAGE, S. O. (2002) Endogenously expressed Trp1 is involved in store-mediated Ca2+ entry by conformational coupling in human platelets. *J Biol Chem,* 277, 42157-63.
ROSALES, C., JONES, S. L., MCCOURT, D. & BROWN, E. J. (1994) Bromophenacyl bromide binding to the actin-bundling protein l-plastin inhibits inositol trisphosphate-independent increase in Ca2+ in human neutrophils. *Proc Natl Acad Sci U S A,* 91, 3534-8.
ROSENBERGER, G., GAL, A. & KUTSCHE, K. (2005) AlphaPIX associates with calpain 4, the small subunit of calpain, and has a dual role in integrin-mediated cell spreading. *J Biol Chem,* 280, 6879-89.
SALAMINO, F., DE TULLIO, R., MENGOTTI, P., VIOTTI, P. L., MELLONI, E. & PONTREMOLI, S. (1993) Site-directed activation of calpain is promoted by a membrane-associated natural activator protein. *Biochem J,* 290 (Pt 1), 191-7.
SAMPATH, R., GALLAGHER, P. J. & PAVALKO, F. M. (1998) Cytoskeletal interactions with the leukocyte integrin beta2 cytoplasmic tail. Activation-dependent regulation of associations with talin and alpha-actinin. *J Biol Chem,* 273, 33588-94.
SCHMID-SCHONBEIN, G. W., SHIH, Y. Y. & CHIEN, S. (1980) Morphometry of human leukocytes. *Blood,* 56, 866-75.
SEGAL, A. W. (1987) Absence of both cytochrome b-245 subunits from neutrophils in X-linked chronic granulomatous disease. *Nature,* 326, 88-91.
SEGAL, A. W. (1996) The NADPH oxidase and chronic granulomatous disease. *Mol Med Today,* 2, 129-35.
SELVATICI, R., FALZARANO, S., MOLLICA, A. & SPISANI, S. (2006) Signal transduction pathways triggered by selective formylpeptide analogues in human neutrophils. *Eur J Pharmacol,* 534, 1-11.
SENGELOV, H., KJELDSEN, L. & BORREGAARD, N. (1993) Control of exocytosis in early neutrophil activation. *J Immunol,* 150, 1535-43.
SERVANT, G., WEINER, O. D., HERZMARK, P., BALLA, T., SEDAT, J. W. & BOURNE, H. R. (2000) Polarization of chemoattractant receptor signaling during neutrophil chemotaxis. *Science,* 287, 1037-40.
SHAO, J. Y., TING-BEALL, H. P. & HOCHMUTH, R. M. (1998) Static and dynamic lengths of neutrophil microvilli. *Proc Natl Acad Sci U S A,* 95, 6797-802.
SHAO, X., DAVLETOV, B. A., SUTTON, R. B., SUDHOF, T. C. & RIZO, J. (1996) Bipartite Ca2+-binding motif in C2 domains of synaptotagmin and protein kinase C. *Science,* 273, 248-51.
SHAW, S. K., BAMBA, P. S., PERKINS, B. N. & LUSCINSKAS, F. W. (2001) Real-time imaging of vascular endothelial-cadherin during leukocyte transmigration across endothelium. *J Immunol,* 167, 2323-30.
SHAW, S. K., MA, S., KIM, M. B., RAO, R. M., HARTMAN, C. U., FROIO, R. M., YANG, L., JONES, T., LIU, Y., NUSRAT, A., PARKOS, C. A. & LUSCINSKAS, F. W. (2004) Coordinated redistribution of leukocyte LFA-

1 and endothelial cell ICAM-1 accompany neutrophil transmigration. *J Exp Med,* 200, 1571-80.

SHCHERBINA, A., BRETSCHER, A., KENNEY, D. M. & REMOLD-O'DONNELL, E. (1999) Moesin, the major ERM protein of lymphocytes and platelets, differs from ezrin in its insensitivity to calpain. *FEBS Lett,* 443, 31-6.

SIMON, H. U. (2003) Neutrophil apoptosis pathways and their modifications in inflammation. *Immunol Rev,* 193, 101-10.

SIMON, S. I., BURNS, A. R., TAYLOR, A. D., GOPALAN, P. K., LYNAM, E. B., SKLAR, L. A. & SMITH, C. W. (1995) L-selectin (CD62L) cross-linking signals neutrophil adhesive functions via the Mac-1 (CD11b/CD18) beta 2-integrin. *J Immunol,* 155, 1502-14.

SIMON, S. I., HU, Y., VESTWEBER, D. & SMITH, C. W. (2000) Neutrophil tethering on E-selectin activates beta 2 integrin binding to ICAM-1 through a mitogen-activated protein kinase signal transduction pathway. *J Immunol,* 164, 4348-58.

SMYTH, J. T., DEHAVEN, W. I., JONES, B. F., MERCER, J. C., TREBAK, M., VAZQUEZ, G. & PUTNEY, J. W., JR. (2006) Emerging perspectives in store-operated Ca2+ entry: roles of Orai, Stim and TRP. *Biochim Biophys Acta,* 1763, 1147-60.

SPASSOVA, M. A., SOBOLOFF, J., HE, L. P., XU, W., DZIADEK, M. A. & GILL, D. L. (2006) STIM1 has a plasma membrane role in the activation of store-operated Ca(2+) channels. *Proc Natl Acad Sci U S A,* 103, 4040-5.

SPERANDIO, M., SMITH, M. L., FORLOW, S. B., OLSON, T. S., XIA, L., MCEVER, R. P. & LEY, K. (2003) P-selectin glycoprotein ligand-1 mediates L-selectin-dependent leukocyte rolling in venules. *J Exp Med,* 197, 1355-63.

STEINCKWICH, N., SCHENTEN, V., MELCHIOR, C., BRECHARD, S. & TSCHIRHART, E. J. An essential role of STIM1, Orai1, and S100A8-A9 proteins for Ca2+ signaling and FcgammaR-mediated phagosomal oxidative activity. *J Immunol,* 186, 2182-91.

STENDAHL, O., KRAUSE, K. H., KRISCHER, J., JERSTROM, P., THELER, J. M., CLARK, R. A., CARPENTIER, J. L. & LEW, D. P. (1994) Redistribution of intracellular Ca2+ stores during phagocytosis in human neutrophils. *Science,* 265, 1439-41.

STEWART, M. P., MCDOWALL, A. & HOGG, N. (1998) LFA-1-mediated adhesion is regulated by cytoskeletal restraint and by a Ca2+-dependent protease, calpain. *J Cell Biol,* 140, 699-707.

STOSSEL, T. P. (1989) From signal to pseudopod. How cells control cytoplasmic actin assembly. *J Biol Chem,* 264, 18261-4.

SUGAR, I. P. & NEUMANN, E. (1984) Stochastic model for electric field-induced membrane pores. Electroporation. *Biophys Chem,* 19, 211-25.

TERUEL, M. N. & MEYER, T. (2002) Parallel single-cell monitoring of receptor-triggered membrane translocation of a calcium-sensing protein module. *Science,* 295, 1910-2.

TOMPA, P., EMORI, Y., SORIMACHI, H., SUZUKI, K. & FRIEDRICH, P. (2001) Domain III of calpain is a ca2+-regulated phospholipid-binding domain. *Biochem Biophys Res Commun,* 280, 1333-9.

TSEIN & POENIE (1986) Fluorescence ratio imaging: a new window into intracellular ionic signalling *Trends Biochem Sci,* 11, 450-452.

UNDYALA, V. V., DEMBO, M., CEMBROLA, K., PERRIN, B. J., HUTTENLOCHER, A., ELCE, J. S., GREER, P. A., WANG, Y. L. & BENINGO, K. A. (2008) The calpain small subunit regulates cell-substrate mechanical interactions during fibroblast migration. *J Cell Sci,* 121, 3581-8.

VAN BRUGGEN, R., ANTHONY, E., FERNANDEZ-BORJA, M. & ROOS, D. (2004) Continuous translocation of Rac2 and the NADPH oxidase component p67(phox) during phagocytosis. *J Biol Chem,* 279, 9097-102.

VOLPE, P., KRAUSE, K. H., HASHIMOTO, S., ZORZATO, F., POZZAN, T., MELDOLESI, J. & LEW, D. P. (1988) "Calciosome," a cytoplasmic organelle: the inositol 1,4,5-trisphosphate-sensitive Ca2+ store of nonmuscle cells? *Proc Natl Acad Sci U S A,* 85, 1091-5.

VON TSCHARNER, V., PROD'HOM, B., BAGGIOLINI, M. & REUTER, H. (1986) Ion channels in human neutrophils activated by a rise in free cytosolic calcium concentration. *Nature,* 324, 369-72.

WAKELIN, M. W., SANZ, M. J., DEWAR, A., ALBELDA, S. M., LARKIN, S. W., BOUGHTON-SMITH, N., WILLIAMS, T. J. & NOURSHARGH, S. (1996) An anti-platelet-endothelial cell adhesion molecule-1 antibody inhibits leukocyte extravasation from mesenteric microvessels in vivo by blocking the passage through the basement membrane. *J Exp Med,* 184, 229-39.

WALLER (1846) Microscopic observation on the perforation of capillaries by the corpuscles of blood and the origin of mucus and pus globules. *London Edinburgh Philos Mag J Sci,* 29, 271-280.

WANG, G. G., CALVO, K. R., PASILLAS, M. P., SYKES, D. B., HACKER, H. & KAMPS, M. P. (2006) Quantitative production of macrophages or neutrophils ex vivo using conditional Hoxb8. *Nat Methods,* 3, 287-93.

WEAVER & CHIZMADZHEV (1996) Theory of electroporation: A review. *Bioelectrochemistry and Bioenergetics,* 41, 135-160.

WIDMAIER, R., STRANG (2006) *Vander's Human Physiology The Mechanisms of Body Function*, McGraw-Hill.

WIEMER, A. J., LOKUTA, M. A., SURFUS, J. C., WERNIMONT, S. A. & HUTTENLOCHER, A. (2009) Calpain inhibition impairs TNF-alpha-mediated neutrophil adhesion, arrest and oxidative burst. *Mol Immunol.*

XU, Y. & SZOKA, F. C., JR. (1996) Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. *Biochemistry,* 35, 5616-23.

YAMAKI, K., HONG, J. J., HIRAIZUMI, K. H., AHN, J. W., ZEE, O. & OHUCHI, K. (2002) Participation of various kinases in staurosporine-induced apoptosis of RAW 264.7 cells. *Journal of Pharmacy and Pharmacology,* 54, 1535-1544.

YAN, B., CALDERWOOD, D. A., YASPAN, B. & GINSBERG, M. H. (2001) Calpain cleavage promotes talin binding to the beta 3 integrin cytoplasmic domain. *J Biol Chem,* 276, 28164-70.

YOSHIFUJI, H., UMEHARA, H., MARUYAMA, H., ITOH, M., TANAKA, M., KAWABATA, D., FUJII, T. & MIMORI, T. (2005) Amelioration of experimental arthritis by a calpain-inhibitory compound: regulation of cytokine production by E-64-d in vivo and in vitro. *Int Immunol,* 17, 1327-36.

ZARBOCK, A. & LEY, K. (2008) Mechanisms and consequences of neutrophil interaction with the endothelium. *Am J Pathol,* 172, 1-7.

ZHANG, X., JIANG, G., CAI, Y., MONKLEY, S. J., CRITCHLEY, D. R. & SHEETZ, M. P. (2008) Talin depletion reveals independence of initial cell spreading from integrin activation and traction. *Nat Cell Biol.*

Appendix I – General reagents

Reagent	*Source*
Acridine Orange	Sigma-Aldrich, Poole, U.K.
Agar	Sigma-Aldrich, Poole, U.K.
All Trans Retinoic Acid (ATRA)	Sigma-Aldrich, Poole, U.K..
Amaxa Nucleofector solution T	Lonza, Cologne, Germany
Amaxa Nucleofector solution V	Lonza, Cologne, Germany
Ampicillin	Sigma-Aldrich, Poole, U.K.
Annexin V FITC	Invitrogen, Paisly, U.K.
β – Mercaptoethanol	Sigma-Aldrich, Poole, U.K..
β – Estradiol	Sigma-Aldrich, Poole, U.K.
Boric acid	Duchefa Biochemie, Haarlem, Netherlands
Bovine Serum Albumin (BSA)	Sigma-Aldrich, Poole, U.K.
MACS CD34 micro bead kit	Miltenyi Biotec, Surrey, U.K.
$CaCl_2$	Sigma-Aldrich, Poole, U.K.
Chloroform	Sigma-Aldrich, Poole, U.K.
Chloroquine	Sigma-Aldrich, Poole, U.K.
Dextran (mol. Wt. 70,000)	Sigma-Aldrich, Poole, U.K..
Diethyl Pyrocarbonate (DEPC)	Sigma-Aldrich, Poole, U.K.
DiI	Invitrogen, Paisly, U.K.
Dimethyl sulphoxide (DMSO)	Sigma-Aldrich, Poole, U.K.
DMEM	Flow Laboratories, Paisley, U.K.
EDTA	Sigma-Aldrich, Poole, U.K.
Effectene transfection reagent	Qiagen, Crawley, U.K.
EGTA	Sigma-Aldrich, Poole, U.K.
Ethanol	Fisher Scientific, Leicester, U.K.
Ethidium bromide	Sigma-Aldrich, Poole, U.K.
Fetal Calf Serum (FCS)	Sigma-Aldrich, Poole, U.K.
Fibronectin	Sigma-Aldrich, Poole, U.K.
Ficoll-Paque	Amersham Biosciences, Uppsala, Sweden

Fluo-4 salt	Invitrogen, Carlsbad, California, U.S.A.
Fluo-4-AM	Invitrogen, Carlsbad, California, U.S.A.
Flt3	Peprotech, London, UK
fMLP	Sigma-Aldrich, Poole, U.K.
Fugene 6	Promega UK, Southampton, U.K.
Fura-2 dextran	Molecular Probes, Eugene, Oregon, U.S.A.
Fura-red	Molecular Probes, Eugene, Oregon, U.S.A.
Glutamine	Sigma-Aldrich, Poole, U.K.
Glycine	Sigma-Aldrich, Poole, U.K.
Glycerol	Sigma-Aldrich, Poole, U.K.
Granulocyte-Colony Stimulating Factor (G-CSF)	Peprotech, London, UK
Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF)	Peprotech, London, UK
Heparin	CP Pharmaceuticals Ltd, Wrexham, U.K.
Hepes	Fisher Scientific, Leicester, U.K.
Horse Serum	Sigma-Aldrich, Poole, U.K.
Infusion Advantage PCR cloning kit	Takara Bio Europe/Clontech, France
Interleukin-3 (IL-3)	Peprotech, London, UK
Interleukin-6 (IL-6)	Peprotech, London, UK
Ionomycin	Calbiochem, Nottingham, U.K.
Isopropanol	Sigma-Aldrich, Poole, U.K.
Kanamycin	Sigma-Aldrich, Poole, U.K.
KCl	Sigma-Aldrich, Poole, U.K.
KH_2PO_4	Sigma-Aldrich, Poole, U.K.
Lucifer yellow	Sigma-Aldrich, Poole, U.K.
Machery-Nagel Nucleobond® Extra Maxi kit	Abgene, Surrey, U.K.
Methanol	Fisher Scientific, Leicester, U.K.
$MgCl_2$	Sigma-Aldrich, Poole, U.K.
$MgSO_4$	Sigma-Aldrich, Poole, U.K.
Na_2CO_3	BDH Laboratory Supplies, Poole, U.K.
NaCl	Sigma-Aldrich, Poole, U.K.

$NaHC0_3$	Fisher Scientific, Leicester, U.K.
Na_2HPO_4	Sigma-Aldrich, Poole, U.K.
NaOH	Sigma-Aldrich, Poole, U.K.
$NiCl_2$	Sigma-Aldrich, Poole, U.K.
OneShot®Top10 e.coli	Invitrogen, Paisly, U.K.
Opti-Mem	Invitrogen, Paisly, U.K.
Para formaldehyde	Sigma-Aldrich, Poole, U.K.
Penicillin	Sigma-Aldrich, Poole, U.K.
PkH 26 red membrane marker	Sigma-Aldrich, Poole, U.K.
Polybrene	Sigma-Aldrich, Poole, U.K.
Protease Inhibitor Cocktail	Sigma-Aldrich, Poole, U.K.
Propidium Iodide	Sigma-Aldrich, Poole, U.K.
RetroNectin®	Takara Bio Europe, France
Phusion Hot Start high fidelity DNA polymerase	New England Biolabs, Hitchin, U.K.
Precision qScript™ reverse transcription kit	PrimerDesign Ltd, Southampton, U.K.
Reducing agent	Invitrogen, Paisly, U.K.
Rhodamine dextran	Invitrogen, Paisly, U.K.
Running buffer	Invitrogen, Paisly, U.K.
RPMI	Flow Laboratories, Paisley, U.K.
Sample buffer	Invitrogen, Paisly, U.K.
Sodium acetate	Sigma-Aldrich, Poole, U.K.
Stem cell factor (SCF)	Peprotech, London, UK
Streptomycin	Sigma-Aldrich, Poole, U.K.
Staurosporine (STS)	Sigma-Aldrich, Poole, U.K.
Thapsigargin	Sigma-Aldrich, Poole, U.K.
TRI reagent	Sigma-Aldrich, Poole, U.K.
TRIS acetate pre-cast 3-11% gel	Invitrogen, Paisly, U.K.
TRIS-Cl	Duchefa Biochemie, Haarlem, Netherlands
Triton-X100	Sigma-Aldrich, Poole, U.K.
Trypsin	Sigma-Aldrich, Poole, U.K.

Tryptone	Duchefa Biochemie, Haarlem, Netherlands
Tween-20	Sigma-Aldrich, Poole, U.K.
Yeast Extract	Duchefa Biochemie, Haarlem, Netherlands
Zymosan A	Sigma-Aldrich, Poole, U.K.

Appendix II – Cell lines

Cell Line	*Source*
HL60 (human promyelocytic leukaemia)	European Cell Culture Collection
NB4 (human promyelocytic leukaemia)	European Cell Culture Collection
PLB-985 (human promyelocytic leukaemia)	DSMZ
NIH 3T3 (mouse fibroblast)	European Cell Culture Collection
RAW 264 (mouse monocyte macrophage)	European Cell Culture Collection
MyPH8B6	Dr P Taylor and Dr J McDonald
Phoenix ampotrophic packaging cells	Dr P Taylor
Phoenix ecotropic packaging cells	Dr R Darley

Appendix III – Antibodies

Antibody	*Source*
Anti-calpain1 (6C-12)	Santa Cruz, Calne, UK
Goat anti-mouse IgG FITC	Santa Cruz, Calne, UK
goat anti-mouse IgG Rhodamine	Santa Cruz, Calne, UK
Anti-GFP-HRP	Santa Cruz, Calne, UK

Appendix IV – Primers

Primer	Sequence	Tm (°C)	Source
T7F	TAATACGACTCACTATAGGG	52	Sigma Chemicals, Poole, U.K.
BGHR	CCTCGACTGTGCCTTCTAG	62	Sigma Chemicals, Poole, U.K.
B Actin F	ATGATATCGCCGCGCTCA	68	Sigma Chemicals, Poole, U.K.
B Actin R	CGCTCGGTGAGGATCTTCA	67	Sigma Chemicals, Poole, U.K.
Capn1EXF1	ATGTCGGAGGAGATCATCA	61	Sigma Chemicals, Poole, U.K.
Capn1EXF2	ATGTCGGAGGAGATCATCAC	62	Sigma Chemicals, Poole, U.K.
Capn1EXR1	TGCAAACATGGTCAGCT	59	Sigma Chemicals, Poole, U.K.
Capn1ExR2	TGCAAACATGGTCAGCTG	63	Sigma Chemicals, Poole, U.K.
C2EXF1	CCCGACGCCCTCAAGA	67	Sigma Chemicals, Poole, U.K.
C2EXF2	CCCGACGCCCTCAAG	65	Sigma Chemicals, Poole, U.K.
C2EXR1	GTCATCCAGCTCCACAGTC	61	Sigma Chemicals, Poole, U.K.
C2EXR2	GTCATCCAGCTCCACAGTCC	65	Sigma Chemicals, Poole, U.K.
Calp4EXF1	ATGTTCCTGGTTAACTCGTTC	60	Sigma Chemicals, Poole, U.K.
Calp4EXF2	ATGTTCCTGGTTAACTCG	55	Sigma Chemicals, Poole, U.K.
Calp4EXR1	GGAATACATAGTCAGCTGC	55	Sigma Chemicals, Poole, U.K.
Calp4EXR2	GGAATACATAGTCAGCTGCAG	59	Sigma Chemicals, Poole, U.K.
CAPN1/C2F1	CAGTTCAAGATCCGGCTG	62	Sigma Chemicals, Poole, U.K.
CAPN1/C2R1	GAGGGCACCACCACATACTC	65	Sigma Chemicals, Poole, U.K.
Calp4F1	TGACACGACACCCTGA	58	Sigma Chemicals, Poole, U.K.
Calp4R1	CCACTCCTGGATGTTCA	58	Sigma Chemicals, Poole, U.K.
SxWF	ACGCGTGCGGCCGC	73	Dr P Brennan
SxWR	GGATCCCGGGCTCGAG	68	Dr P Brennan

Appendix V – Plasmids

Plasmid	*Source*
C2-gamma-YFP	T.Gadella, University of Amsterdam, Amsterdam, The Netherlands.
C2-beta-RFP	T.Gadella, University of Amsterdam, Amsterdam, The Netherlands
pEYFP-C1-Calpain 4	S.Gil-Parrado Klinikum der Universitat, Muchen, Germany
pMX-μCalpain-EGFP construct	A.Huttenlocher, University of Wisconson, Wisconsin, U.S.A.
Vivid Colours™ pcDNA™ 6.2/EmGFP-GW/TOPO®	Invitrogen, Paisly, U.K.
LentiSxW	Paul Brennan, Cardiff University, Cardiff, UK
LentiGFP	Paul Brennan, Cardiff University, Cardiff, UK
P8.91	Paul Brennan, Cardiff University, Cardiff, UK
pMD2G	Paul Brennan, Cardiff University, Cardiff, UK

Appendix VI – Equipment

Equipment	*Source*
Amaxa nulceofector device	Lonza, Cologne, Germany
Eppendorf Injectman micromanipulator	Eppendorf, Hamburg, Germany
Eppendorf 5629 micromanipulator	Eppendorf, Hamburg, Germany
WPI micromanipulator	WPI, Florida, U.S.A.
Eppendorf Femtojet pressure controller	Eppendorf, Hamburg, Germany
Grass SD9 Stimulator	
Micropipette Puller P2000	Sutter Instruments, Novato, California, U.S.A.
Micropipette capillaries	Sutter Instruments, Novato, California, U.S.A.
Rapid monochromator - Delta RAM	PTI, Surbiton, U.K.
Nikon Eclipse inverted microscope	Nikon, Kingston-upon-Thames, U.K.
IC100 Intensified CCD camera	PTI, Surbiton, U.K.
Red sensitive camera	Watec, Tsuruoka-shi, Japan
LPS-220b lamp power supply	PTI, Surbiton, U.K.
Filters, dichroic mirrors	Omega Optical Inc Filters, , Stanmore, U.K.
Beam Splitter	Cairn Research Instruments, Faversham, U.K.
Microscope stage heater	Linkam Scientific Instruments, Tadworth, U.K.
CLSM Confocal microscope SP2	Leica, Milton Keynes, U.K.
CLSM Confocal microscope SP5	Leica, Milton Keynes, U.K.
Single 488nm line laser	Laser Physics, Milton Green, U.K.
ISIS Intensified CCD camera	Photonic Science Ltd, Robertsbridge, U.K.
Fluorimeter Fluolog 3	SPEX CertiPrep, Metuchen, Watford, U.K.
UV filter	Chroma Filters, Stanmore, UK
BD Accuri C6 Flow cytometer	Accuri Cytometers Lyd, St Ives, U.K.

Appendix VII – Software

Software	*Source*
Image Master 1.4b8	PTI, Surbiton, U.K.
Microsoft Excel 2010	Microsoft, Redmond, Washington, U.S.A.
Paintshop Pro Version 4.15 SE	Jasc Software Inc., Minnesota, U.S.A.Eden Prairie,
Microsoft PhotoEditor 3.0	Microsoft, Redmond, Washington, U.S.A.
Gif Animator (32-bit)	Alchemy Mindworks Inc., Ontario, Canada
HyperCam Version 1.34.00	Shareware
Microsoft Paint Version 5.1	Microsoft, Redmond, Washington, U.S.A.
Adobe Photoshop 7.0	Adobe Systems Inc, San Jose, California, U.S.A.
Leica Applications Suite Advanced Fluorescence Lite	Leica Microsystems
Microsoft Word 2010	Microsoft, Redmond, Washington, U.S.A.
Image J	NIH, Bethesda USA
IMARIS imaging software	Bitplane Scientific Software
DNA Baser v3	Heracle Biosoft HCL
Accuri Cflow software	Accuri Cytometers Ltd, St Ives, U.K.

Appendix VIII – Buffers and Solutions

Balanced Salt Solution (BSS)

0.13M NaCl

2.6mM-KCl

8.0mM-Na_2HPO_4

1.83mM-KH_2PO_4

DEPC water

5% Diethyl Pyrocarbonate (DEPC) dissolved in distilled water and autoclaved before use.

Dextran

6% of 70kDa dextran dissolved in BSS (6g in 100ml), aliquoted and stored at 4°C until use

0.05M EDTA

1g KCl

5.72g Na_2HPO_4

1g KH_2PO_4

40g NaCl

1.4g EDTA

All dissolved in 5 litres distilled water and pH adjusted to 7.4 and autoclaved before use.

Ethidium Bromide

One hundred milligrams of ethidium bromide powder was dissolved in 10ml of distilled water. Container was wrapped in aluminium foil to protect solution from sunlight and stored safely before use.

Hepes buffered Krebs

120mM-NaCl

25mM-Hepes

4.8mM-KCl

1.2mM-KH_2PO_4

1.2mM-$MgSO_4.7H_20$

1.3mM-$CaCl_2.2H_20$

0.1% Bovine Serum Albumin (BSA)

LB agar

10g Tryptone

5g Yeast extract

10g NaCl

15g Agar

Dissolved in 1 litre of distilled water and pH adjusted to 7.0 and autoclaved. Before use the solution was heated to a liquid and cooled slightly before adding selective antibiotic. The solution was then poured into 10cm^2 petri dish plates

and allowed to cool and solidify before being inverted and stored at 4°C until needed.

LB broth

10g Tryptone

5g Yeast extract

10g NaCl

Dissolved in 1 litre distilled water and pH adjusted to 7.0, autoclaved and cooled before adding selective antibiotic and stored at room temperature.

Neutrophil lysis buffer

50mM Hepes

150mM NaCl

10% glycerol

1% Triton X-100

1.5mM $MgCl_2$

5mM EGTA

5mM EDTA

1mM Na_3PO_4

0.5% w/v SDS

10x TBS

121g TRIS

400.3g NaCl

Dissolved in 5 litres distilled water and pH adjusted to 7.4

Transfer buffer

0.03M TRIS base

0.2M glycine

10% methanol

0.2% w/v SDS

Tris-Boric acid-EDTA (TBE) buffer (5x conc)

1.1M Tris-Cl

900mM Boric acid

25mM EDTA

pH8.3

Trypsin (25mg/ml)

500mg trypsin dissolved in 20ml 0.05mM EDTA and filtered through a 0.2µm filter. For use in cell culture one 250µl aliquot was diluted in a further 10ml 0.05M EDTA.

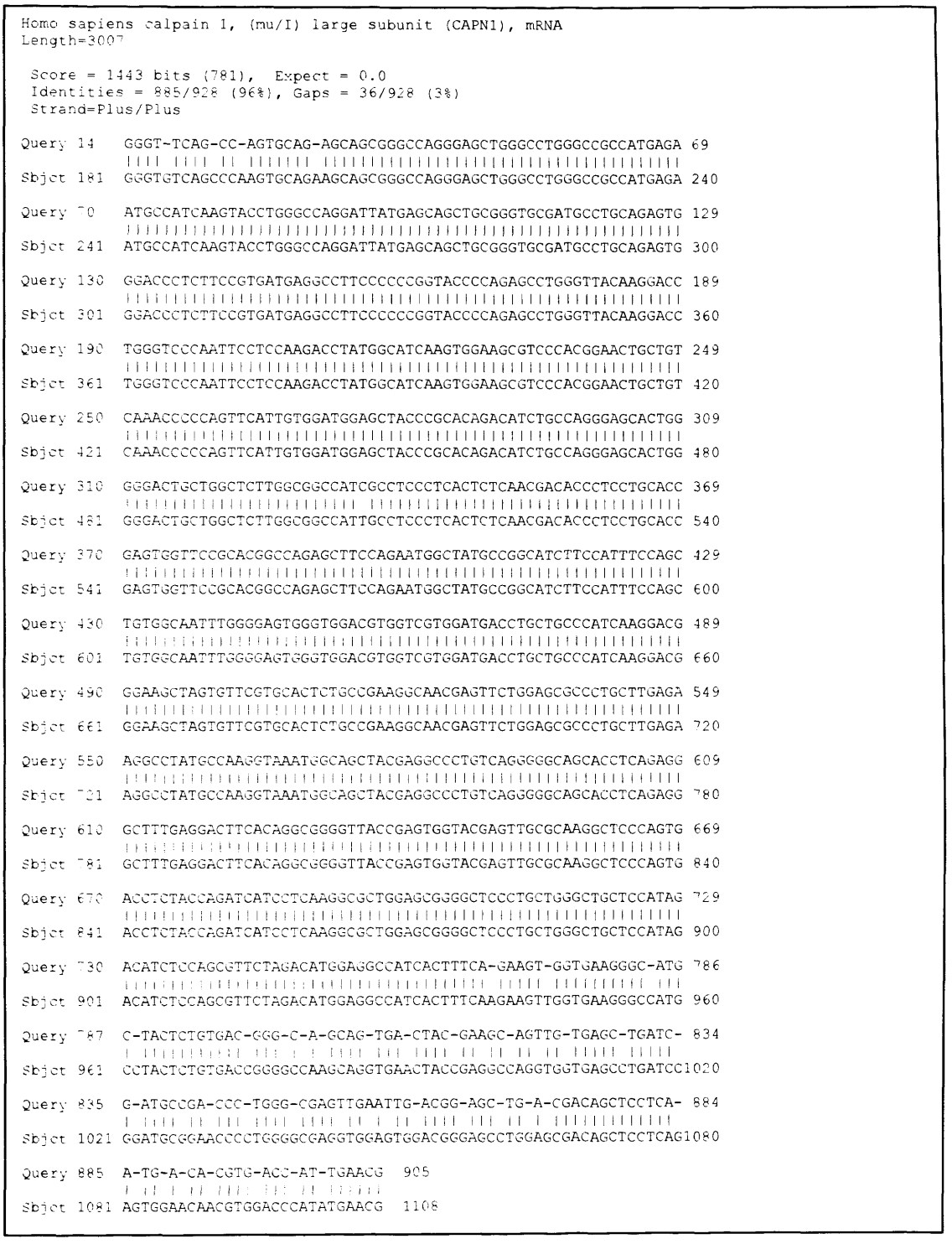
Appendix IV – Plasmid sequences

```
 calpain, small subunit 1 [Homo sapiens]
 Score =  699 bits (378),  Expect = 0.0
 Identities = 381/382 (99%), Gaps = 1/382 (0%)
 Strand=Plus/Plus

Query  19   ACATGATCGCAGCATGGTGGCCGTGATGGATAGCGACACCACAGGCAAGCTGGGCTTTGA 78
            ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  584  ACATG-TCGCAGCATGGTGGCCGTGATGGATAGCGACACCACAGGCAAGCTGGGCTTTGA642

Query  79   GGAATTCAAGTACTTGTGGAACAACATCAAAAGGTGGCAGGCCATATACAAACAGTTCGA138
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  643  GGAATTCAAGTACTTGTGGAACAACATCAAAAGGTGGCAGGCCATATACAAACAGTTCGA702

Query  139  CACTGACCGATCAGGGACCATTTGCAGTAGTGAACTCCCAGGTGCCTTTGAGGCAGCAGG198
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  703  CACTGACCGATCAGGGACCATTTGCAGTAGTGAACTCCCAGGTGCCTTTGAGGCAGCAGG762

Query  199  GTTCCACCTGAATGAGCATCTCTATAACATGATCATCCGACGCTACTCAGATGAAAGTGG258
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  763  GTTCCACCTGAATGAGCATCTCTATAACATGATCATCCGACGCTACTCAGATGAAAGTGG822

Query  259  GAACATGGATTTTGACAACTTCATCAGCTGCTTGGTCAGGCTGGACGCCATGTTCCGTGC318
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  823  GAACATGGATTTTGACAACTTCATCAGCTGCTTGGTCAGGCTGGACGCCATGTTCCGTGC882

Query  319  CTTCAAATCTCTTGACAAAGATGGCACTGGACAAATCCAGGTGAACATCCAGGAGTGGCT378
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  883  CTTCAAATCTCTTGACAAAGATGGCACTGGACAAATCCAGGTGAACATCCAGGAGTGGCT942

Query  379  GCAGCTGACTATGTATTCCTGA  400
            |||||||||||||||||||||
Sbjct  943  GCAGCTGACTATGTATTCCTGA  964
```

BLAST search results using sequence from pEYFP-C1-calpain 4 plasmid sequenced with calp4F1 forward primer

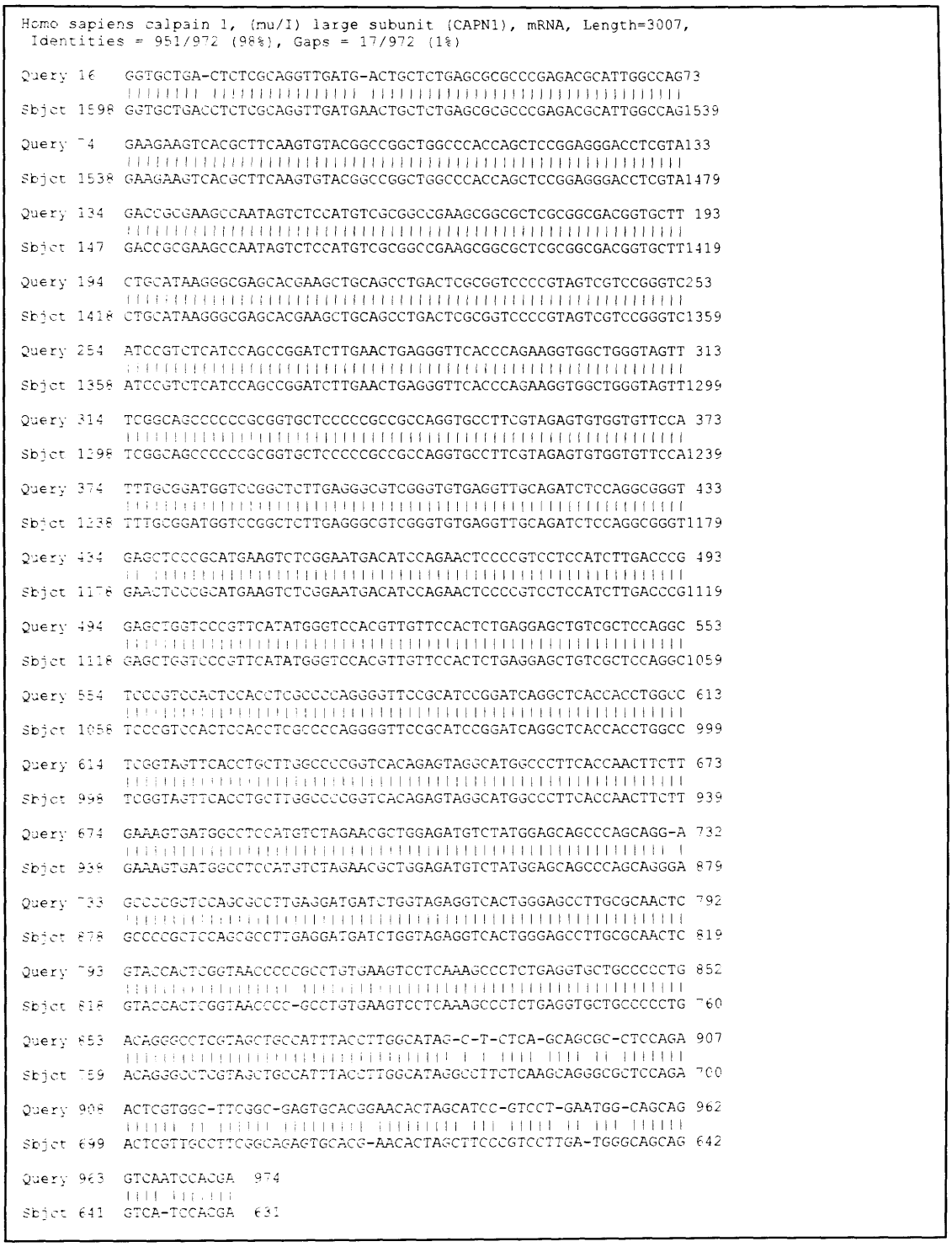
```
calpain, small subunit 1 [Homo sapiens]

 Score = 1354 bits (733),  Expect = 0.0
 Identities = 736/737 (99%), Gaps = 1/737 (0%)
 Strand=Plus/Minus

Query  20   AGAGATTTG-AGGCACGGAACATGGCGTCCAGCCTGACCAAGCAGCTGATGAAGTTGTCA 78
            ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  894  AGAGATTTGAAGGCACGGAACATGGCGTCCAGCCTGACCAAGCAGCTGATGAAGTTGTCA835

Query  79   AAATCCATGTTCCCACTTTCATCTGAGTAGCGTCGGATGATCATGTTATAGAGATGCTCA138
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  834  AAATCCATGTTCCCACTTTCATCTGAGTAGCGTCGGATGATCATGTTATAGAGATGCTCA775

Query  139  TTCAGGTGGAACCCTGCTGCCTCAAAGGCACCTGGGAGTTCACTACTGCAAATGGTCCCT198
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  774  TTCAGGTGGAACCCTGCTGCCTCAAAGGCACCTGGGAGTTCACTACTGCAAATGGTCCCT715

Query  199  GATCGGTCAGTGTCGAACTGTTTGTATATGGCCTGCCACCTTTTGATGTTGTTCCACAAG258
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  714  GATCGGTCAGTGTCGAACTGTTTGTATATGGCCTGCCACCTTTTGATGTTGTTCCACAAG655

Query  259  TACTTGAATTCCTCAAAGCCCAGCTTGCCTGTGGTGTCGCTATCCATCACGGCCACCATG318
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  654  TACTTGAATTCCTCAAAGCCCAGCTTGCCTGTGGTGTCGCTATCCATCACGGCCACCATG595

Query  319  CTGCGACATGTGTCAATGCCAAAACCATCAGTCTTCAGATCAGGGTGTCGTGTCACAACC378
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  594  CTGCGACATGTGTCAATGCCAAAACCATCAGTCTTCAGATCAGGGTGTCGTGTCACAACC535

Query  379  TTATTGAGAATGTTCATGAGTTCTGTGGCGCTGACCTCCATGTCATCTCCAGCCAGCTGG438
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  534  TTATTGAGAATGTTCATGAGTTCTGTGGCGCTGACCTCCATGTCATCTCCAGCCAGCTGG475

Query  439  GCAAAGAGTCTCCGGAACTGCCGGACCTCCTCACTCTCGTTGGCCTCAATGTTGGAGTAA498
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  474  GCAAAGAGTCTCCGGAACTGCCGGACCTCCTCACTCTCGTTGGCCTCAATGTTGGAGTAA415

Query  499  TGTGTGCGTGGGGGCGGGGGCTCCGGGTTGTACTGCGCAGCCGCCTCGCTGATGGCGCTG558
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  414  TGTGTGCGTGGGGGCGGGGGCTCCGGGTTGTACTGCGCAGCCGCCTCGCTGATGGCGCTG355

Query  559  ATGACTCCGCCTAGGATGCGCATGGCCGTTCCACCGCCACCGCCGCCTCCACCACCGCCG618
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  354  ATGACTCCGCCTAGGATGCGCATGGCCGTTCCACCGCCACCGCCGCCTCCACCACCGCCG295

Query  619  CCGCCGCCGCCGCCGCCGCCGCCGCCCCCGGCCCCGCTGATCAGGCCTCCAAGCACATTT678
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  294  CCGCCGCCGCCGCCGCCGCCGCCCCCGGCCCCGCTGATCAGGCCTCCAAGCACATTT235

Query  679  CCCAGGCCCCCACCCAGGCCCCCGCCTCCCCCGCCGCCGCCGCCGCCGCCCTTCAAGAAC738
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  234  CCCAGGCCCCCACCCAGGCCCCCGCCTCCCCCGCCGCCGCCGCCGCCGCCCTTCAAGAAC175

Query  739  GAGTTAACCAGGAACAT  755
            |||||||||||||||||
Sbjct  174  GAGTTAACCAGGAACAT  158
```

BLAST search results using sequence from pEYFP-C1-calpain 4 plasmid sequenced with calp4R1 reverse primer



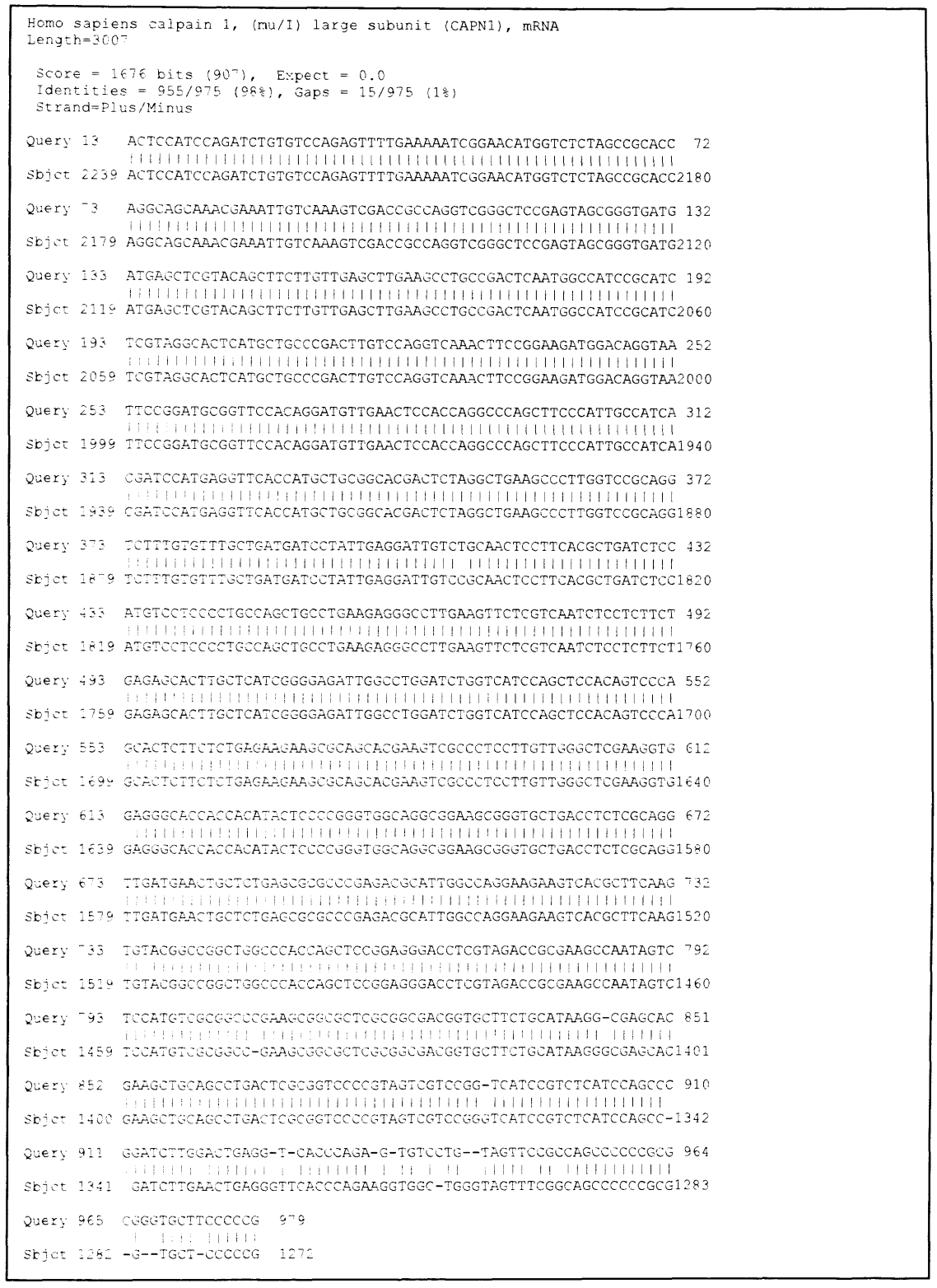
```
Homo sapiens calpain 1, (mu/I) large subunit (CAPN1), mRNA
Length=3007

 Score = 1443 bits (781),  Expect = 0.0
 Identities = 885/928 (96%), Gaps = 36/928 (3%)
 Strand=Plus/Plus

Query 14    GGGT-TCAG-CC-AGTGCAG-AGCAGCGGGCCAGGGAGCTGGGCCTGGGCCGCCATGAGA 69
            |||| |||| || ||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct 181   GGGTGTCAGCCCAAGTGCAGAAGCAGCGGGCCAGGGAGCTGGGCCTGGGCCGCCATGAGA 240

Query 70    ATGCCATCAAGTACCTGGGCCAGGATTATGAGCAGCTGCGGGTGCGATGCCTGCAGAGTG 129
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 241   ATGCCATCAAGTACCTGGGCCAGGATTATGAGCAGCTGCGGGTGCGATGCCTGCAGAGTG 300

Query 130   GGACCCTCTTCCGTGATGAGGCCTTCCCCCCGGTACCCCAGAGCCTGGGTTACAAGGACC 189
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 301   GGACCCTCTTCCGTGATGAGGCCTTCCCCCCGGTACCCCAGAGCCTGGGTTACAAGGACC 360

Query 190   TGGGTCCCAATTCCTCCAAGACCTATGGCATCAAGTGGAAGCGTCCCACGGAACTGCTGT 249
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 361   TGGGTCCCAATTCCTCCAAGACCTATGGCATCAAGTGGAAGCGTCCCACGGAACTGCTGT 420

Query 250   CAAACCCCCAGTTCATTGTGGATGGAGCTACCCGCACAGACATCTGCCAGGGAGCACTGG 309
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 421   CAAACCCCCAGTTCATTGTGGATGGAGCTACCCGCACAGACATCTGCCAGGGAGCACTGG 480

Query 310   GGGACTGCTGGCTCTTGGCGGCCATCGCCTCCCTCACTCTCAACGACACCCTCCTGCACC 369
            ||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct 481   GGGACTGCTGGCTCTTGGCGGCCATTGCCTCCCTCACTCTCAACGACACCCTCCTGCACC 540

Query 370   GAGTGGTTCCGCACGGCCAGAGCTTCCAGAATGGCTATGCCGGCATCTTCCATTTCCAGC 429
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 541   GAGTGGTTCCGCACGGCCAGAGCTTCCAGAATGGCTATGCCGGCATCTTCCATTTCCAGC 600

Query 430   TGTGGCAATTTGGGGAGTGGGTGGACGTGGTCGTGGATGACCTGCTGCCCATCAAGGACG 489
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 601   TGTGGCAATTTGGGGAGTGGGTGGACGTGGTCGTGGATGACCTGCTGCCCATCAAGGACG 660

Query 490   GGAAGCTAGTGTTCGTGCACTCTGCCGAAGGCAACGAGTTCTGGAGCGCCCTGCTTGAGA 549
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 661   GGAAGCTAGTGTTCGTGCACTCTGCCGAAGGCAACGAGTTCTGGAGCGCCCTGCTTGAGA 720

Query 550   AGGCCTATGCCAAGGTAAATGGCAGCTACGAGGCCCTGTCAGGGGGCAGCACCTCAGAGG 609
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 721   AGGCCTATGCCAAGGTAAATGGCAGCTACGAGGCCCTGTCAGGGGGCAGCACCTCAGAGG 780

Query 610   GCTTTGAGGACTTCACAGGCGGGGTTACCGAGTGGTACGAGTTGCGCAAGGCTCCCAGTG 669
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 781   GCTTTGAGGACTTCACAGGCGGGGTTACCGAGTGGTACGAGTTGCGCAAGGCTCCCAGTG 840

Query 670   ACCTCTACCAGATCATCCTCAAGGCGCTGGAGCGGGGCTCCCTGCTGGGCTGCTCCATAG 729
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 841   ACCTCTACCAGATCATCCTCAAGGCGCTGGAGCGGGGCTCCCTGCTGGGCTGCTCCATAG 900

Query 730   ACATCTCCAGCGTTCTAGACATGGAGGCCATCACTTTCA-GAAGT-GGTGAAGGGC-ATG 786
            |||||||||||||||||||||||||||||||||||||||| ||||| |||||||||| |||
Sbjct 901   ACATCTCCAGCGTTCTAGACATGGAGGCCATCACTTTCAAGAAGTTGGTGAAGGGCCATG 960

Query 787   C-TACTCTGTGAC-GGG-C-A-GCAG-TGA-CTAC-GAAGC-AGTTG-TGAGC-TGATC- 834
            | |||||||||||| ||| | | |||| ||| |||| || || || || ||||| |||||
Sbjct 961   CCTACTCTGTGACCGGGGCCAAGCAGGTGAACTACCGAGGCCAGGTGGTGAGCCTGATCC1020

Query 835   G-ATGCCGA-CCC-TGGG-CGAGTTGAATTG-ACGG-AGC-TG-A-CGACAGCTCCTCA- 884
            | |||| || ||| |||| |||| || | || |||| ||| || | ||||||||||||| 
Sbjct 1021  GGATGCGGAACCCCTGGGGCGAGGTGGAGTGGACGGGAGCCTGGAGCGACAGCTCCTCAG1080

Query 885   A-TG-A-CA-CGTG-ACC-AT-TGAACG  905
            | || | || ||| ||| || ||||||
Sbjct 1081  AGTGGAACAACGTGGACCCATATGAACG  1108
```

BLAST search results using sequence from calpain1-GFP retroviral plasmid sequenced with capn1ExF1 forward primer



```
Homo sapiens calpain 1, (mu/I) large subunit (CAPN1), mRNA, Length=3007,
 Identities = 951/972 (98%), Gaps = 17/972 (1%)

Query 16   GGTGCTGA-CTCTCGCAGGTTGATG-ACTGCTCTGAGCGCGCCCGAGACGCATTGGCCAG73
           |||||||| |||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct 1598 GGTGCTGACCTCTCGCAGGTTGATGAACTGCTCTGAGCGCGCCCGAGACGCATTGGCCAG1539

Query 74   GAAGAAGTCACGCTTCAAGTGTACGGCCGGCTGGCCCACCAGCTCCGGAGGGACCTCGTA133
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1538 GAAGAAGTCACGCTTCAAGTGTACGGCCGGCTGGCCCACCAGCTCCGGAGGGACCTCGTA1479

Query 134  GACCGCGAAGCCAATAGTCTCCATGTCGCGGCCGAAGCGGCGCTCGCGGCGACGGTGCTT 193
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 147  GACCGCGAAGCCAATAGTCTCCATGTCGCGGCCGAAGCGGCGCTCGCGGCGACGGTGCTT1419

Query 194  CTGCATAAGGGCGAGCACGAAGCTGCAGCCTGACTCGCGGTCCCCGTAGTCGTCCGGGTC253
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1418 CTGCATAAGGGCGAGCACGAAGCTGCAGCCTGACTCGCGGTCCCCGTAGTCGTCCGGGTC1359

Query 254  ATCCGTCTCATCCAGCCGGATCTTGAACTGAGGGTTCACCCAGAAGGTGGCTGGGTAGTT 313
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1358 ATCCGTCTCATCCAGCCGGATCTTGAACTGAGGGTTCACCCAGAAGGTGGCTGGGTAGTT1299

Query 314  TCGGCAGCCCCCCCGCGGTGCTCCCCCGCCGCCAGGTGCCTTCGTAGAGTGTGGTGTTCCA 373
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1298 TCGGCAGCCCCCCCGCGGTGCTCCCCCGCCGCCAGGTGCCTTCGTAGAGTGTGGTGTTCCA1239

Query 374  TTTGCGGATGGTCCGGCTCTTGAGGGCGTCGGGTGTGAGGTTGCAGATCTCCAGGCGGGT 433
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1238 TTTGCGGATGGTCCGGCTCTTGAGGGCGTCGGGTGTGAGGTTGCAGATCTCCAGGCGGGT1179

Query 434  GAGCTCCCGCATGAAGTCTCGGAATGACATCCAGAACTCCCCGTCCTCCATCTTGACCCG 493
           || ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1178 GAACTCCCGCATGAAGTCTCGGAATGACATCCAGAACTCCCCGTCCTCCATCTTGACCCG1119

Query 494  GAGCTGGTCCCGTTCATATGGGTCCACGTTGTTCCACTCTGAGGAGCTGTCGCTCCAGGC 553
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1118 GAGCTGGTCCCGTTCATATGGGTCCACGTTGTTCCACTCTGAGGAGCTGTCGCTCCAGGC1059

Query 554  TCCCGTCCACTCCACCTCGCCCCAGGGGTTCCGCATCCGGATCAGGCTCACCACCTGGCC 613
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1058 TCCCGTCCACTCCACCTCGCCCCAGGGGTTCCGCATCCGGATCAGGCTCACCACCTGGCC 999

Query 614  TCGGTAGTTCACCTGCTTGGCCCCGGTCACAGAGTAGGCATGGCCCTTCACCAACTTCTT 673
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 998  TCGGTAGTTCACCTGCTTGGCCCCGGTCACAGAGTAGGCATGGCCCTTCACCAACTTCTT 939

Query 674  GAAAGTGATGGCCTCCATGTCTAGAACGCTGGAGATGTCTATGGAGCAGCCCAGCAGG-A 732
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |
Sbjct 938  GAAAGTGATGGCCTCCATGTCTAGAACGCTGGAGATGTCTATGGAGCAGCCCAGCAGGGA 879

Query 733  GCCCCGCTCCAGCGCCTTGAGGATGATCTGGTAGAGGTCACTGGGAGCCTTGCGCAACTC 792
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 878  GCCCCGCTCCAGCGCCTTGAGGATGATCTGGTAGAGGTCACTGGGAGCCTTGCGCAACTC 819

Query 793  GTACCACTCGGTAACCCCCGCCTGTGAAGTCCTCAAAGCCCTCTGAGGTGCTGCCCCCTG 852
           |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct 818  GTACCACTCGGTAACCCC-GCCTGTGAAGTCCTCAAAGCCCTCTGAGGTGCTGCCCCCTG 760

Query 853  ACAGGGCCTCGTAGCTGCCATTTACCTTGGCATAG-C-T-CTCA-GCAGCGC-CTCCAGA 907
           ||||||||||||||||||||||||||||||||||| | | |||| |||| || |||||||
Sbjct 759  ACAGGGCCTCGTAGCTGCCATTTACCTTGGCATAGGCCTTCTCAAGCAGGGCGCTCCAGA 700

Query 908  ACTCGTGGC-TTCGGC-GAGTGCACGGAACACTAGCATCC-GTCCT-GAATGG-CAGCAG 962
           |||||| || |||||| |||||||| |||||||| ||| ||||| || ||| ||||||
Sbjct 699  ACTCGTTGCCTTCGGCAGAGTGCACG-AACACTAGCTTCCCGTCCTTGA-TGGGCAGCAG 642

Query 963  GTCAATCCACGA  974
           |||| ||||.|||
Sbjct 641  GTCA-TCCACGA  631
```

BLAST search results using sequence from calpain1-GFP retroviral plasmid sequenced with capn1R1 reverse primer

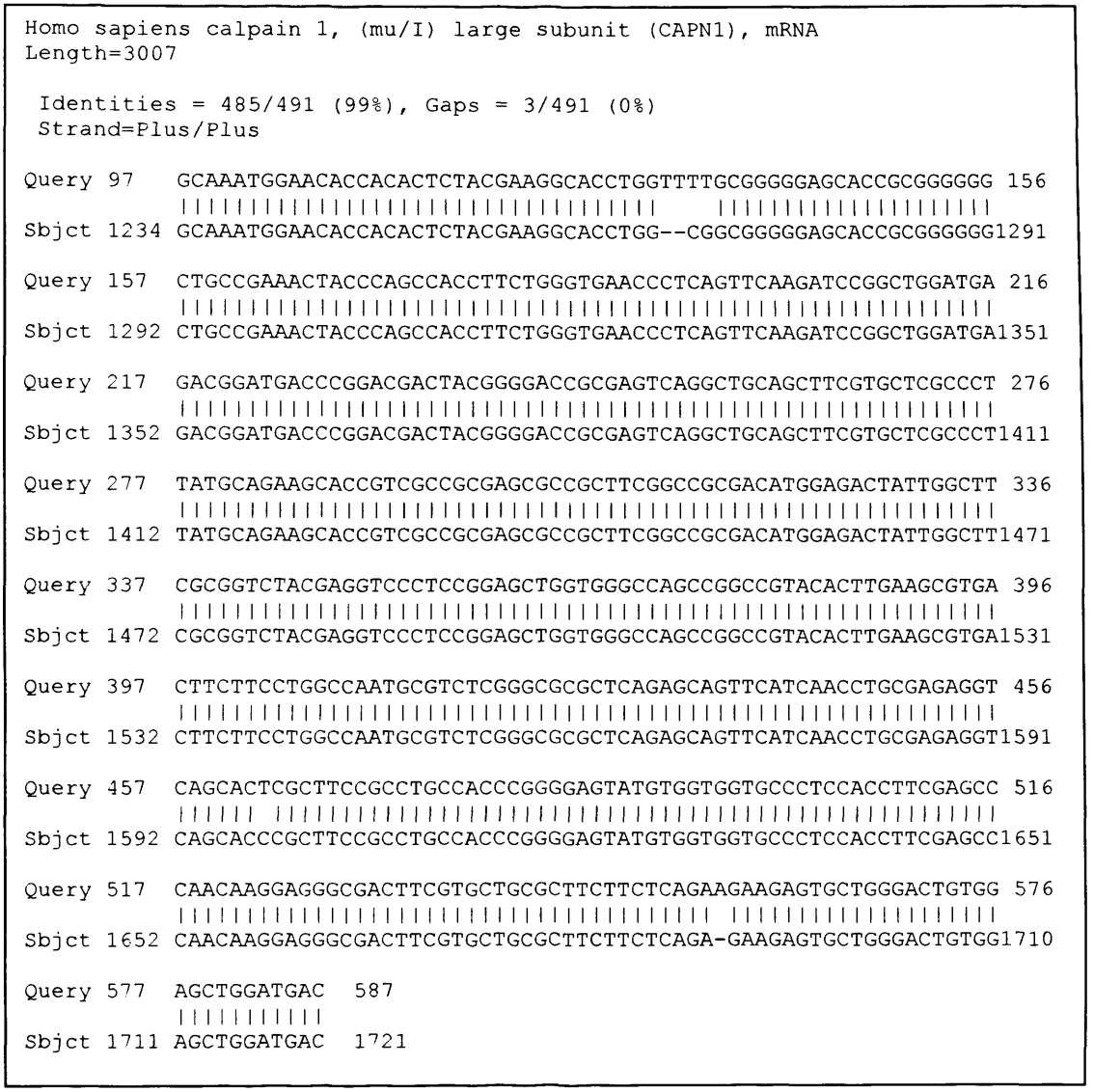


```
Homo sapiens calpain 1, (mu/I) large subunit (CAPN1), mRNA
Length=3007

 Score = 1676 bits (907),  Expect = 0.0
 Identities = 955/975 (98%), Gaps = 15/975 (1%)
 Strand=Plus/Minus

Query 13   ACTCCATCCAGATCTGTGTCCAGAGTTTTGAAAAATCGGAACATGGTCTCTAGCCGCACC  72
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 2239 ACTCCATCCAGATCTGTGTCCAGAGTTTTGAAAAATCGGAACATGGTCTCTAGCCGCACC2180

Query 73   AGGCAGCAAACGAAATTGTCAAAGTCGACCGCCAGGTCGGGCTCCGAGTAGCGGGTGATG 132
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 2179 AGGCAGCAAACGAAATTGTCAAAGTCGACCGCCAGGTCGGGCTCCGAGTAGCGGGTGATG2120

Query 133  ATGAGCTCGTACAGCTTCTTGTTGAGCTTGAAGCCTGCCGACTCAATGGCCATCCGCATC 192
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 2119 ATGAGCTCGTACAGCTTCTTGTTGAGCTTGAAGCCTGCCGACTCAATGGCCATCCGCATC2060

Query 193  TCGTAGGCACTCATGCTGCCCGACTTGTCCAGGTCAAACTTCCGGAAGATGGACAGGTAA 252
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 2059 TCGTAGGCACTCATGCTGCCCGACTTGTCCAGGTCAAACTTCCGGAAGATGGACAGGTAA2000

Query 253  TTCCGGATGCGGTTCCACAGGATGTTGAACTCCACCAGGCCCAGCTTCCCATTGCCATCA 312
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1999 TTCCGGATGCGGTTCCACAGGATGTTGAACTCCACCAGGCCCAGCTTCCCATTGCCATCA1940

Query 313  CGATCCATGAGGTTCACCATGCTGCGGCACGACTCTAGGCTGAAGCCCTTGGTCCGCAGG 372
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1939 CGATCCATGAGGTTCACCATGCTGCGGCACGACTCTAGGCTGAAGCCCTTGGTCCGCAGG1880

Query 373  TCTTTGTGTTTGCTGATGATCCTATTGAGGATTGTCTGCAACTCCTTCACGCTGATCTCC 432
           ||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct 1879 TCTTTGTGTTTGCTGATGATCCTATTGAGGATTGTCCGCAACTCCTTCACGCTGATCTCC1820

Query 433  ATGTCCTCCCCTGCCAGCTGCCTGAAGAGGGCCTTGAAGTTCTCGTCAATCTCCTCTTCT 492
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1819 ATGTCCTCCCCTGCCAGCTGCCTGAAGAGGGCCTTGAAGTTCTCGTCAATCTCCTCTTCT1760

Query 493  GAGAGCACTTGCTCATCGGGGAGATTGGCCTGGATCTGGTCATCCAGCTCCACAGTCCCA 552
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1759 GAGAGCACTTGCTCATCGGGGAGATTGGCCTGGATCTGGTCATCCAGCTCCACAGTCCCA1700

Query 553  GCACTCTTCTCTGAGAAGAAGCGCAGCACGAAGTCGCCCTCCTTGTTGGGCTCGAAGGTG 612
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1699 GCACTCTTCTCTGAGAAGAAGCGCAGCACGAAGTCGCCCTCCTTGTTGGGCTCGAAGGTG1640

Query 613  GAGGGCACCACCACATACTCCCCGGGTGGCAGGCGGAAGCGGGTGCTGACCTCTCGCAGG 672
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1639 GAGGGCACCACCACATACTCCCCGGGTGGCAGGCGGAAGCGGGTGCTGACCTCTCGCAGG1580

Query 673  TTGATGAACTGCTCTGAGCGCGCCCGAGACGCATTGGCCAGGAAGAAGTCACGCTTCAAG 732
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1579 TTGATGAACTGCTCTGAGCGCGCCCGAGACGCATTGGCCAGGAAGAAGTCACGCTTCAAG1520

Query 733  TGTACGGCCGGCTGGCCCACCAGCTCCGGAGGGACCTCGTAGACCGCGAAGCCAATAGTC 792
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1519 TGTACGGCCGGCTGGCCCACCAGCTCCGGAGGGACCTCGTAGACCGCGAAGCCAATAGTC1460

Query 793  TCCATGTCGCGGCCCGAAGCGGCGCTCGCGGCGACGGTGCTTCTGCATAAGG-CGAGCAC 851
           |||||||||||||| |||||||||||||||||||||||||||||||||||| |||||||
Sbjct 1459 TCCATGTCGCGGCC-GAAGCGGCGCTCGCGGCGACGGTGCTTCTGCATAAGGGCGAGCAC1401

Query 852  GAAGCTGCAGCCTGACTCGCGGTCCCCGTAGTCGTCCGG-TCATCCGTCTCATCCAGCCC 910
           |||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct 1400 GAAGCTGCAGCCTGACTCGCGGTCCCCGTAGTCGTCCGGGTCATCCGTCTCATCCAGCC-1342

Query 911  GGATCTTGGACTGAGG-T-CACCCAGA-G-TGTCCTG--TAGTTCCGCCAGCCCCCCGCG 964
           ||||||| ||||||| | |||||||| | || | ||  |||| || ||||||||||||
Sbjct 1341  GATCTTGAACTGAGGGTTCACCCAGAAGGTGGC-TGGGTAGTTTCGGCAGCCCCCCGCG1283

Query 965  CGGGTGCTTCCCCCG  979
             |  |||| ||||||
Sbjct 1282 -G--TGCT-CCCCCG  1272
```

BLAST search results using sequence from calpain1-GFP retroviral plasmid sequenced with capn1ExR1 reverse primer

```
Homo sapiens calpain 1, (mu/I) large subunit (CAPN1), mRNA
Length=3007

 Identities = 914/933 (98%), Gaps = 10/933 (1%)
 Strand=Plus/Plus

Query 68    ATGTCGGAGGAGATCATCAAGCCGAATGTACTGCACTGGGGTGTCAGCCCAAGTGCAGAA 127
            |||||||||||||||||||| ||||  ||||||||||||||||||||||||||||||||||
Sbjct 144   ATGTCGGAGGAGATCATCACGCCG-GTGTACTGCACTGGGGTGTCAGCCCAAGTGCAGAA 202

Query 128   GCAGCGGGCCAGGGAGCTGGGCCTGGGCCGCCATGAGAATGCCATCAAGTACCTGGGCCA 187
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 203   GCAGCGGGCCAGGGAGCTGGGCCTGGGCCGCCATGAGAATGCCATCAAGTACCTGGGCCA 262

Query 188   GGATTATGAGCAGCTGCGGGTGCGATGCCTGCAGAGTGGGACCCTCTTCCGTGATGAGGC 247
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 263   GGATTATGAGCAGCTGCGGGTGCGATGCCTGCAGAGTGGGACCCTCTTCCGTGATGAGGC 322

Query 248   CTTCCCCCCGGTACCCCAGAGCCTGGGTTACAAGGACCTGGGTCCCAATTCCTCCAAGAC 307
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 323   CTTCCCCCCGGTACCCCAGAGCCTGGGTTACAAGGACCTGGGTCCCAATTCCTCCAAGAC 382

Query 308   CTATGGCATCAAGTGGAAGCGTCCCACGGAACTGCTGTCAAACCCCCAGTTCATTGTGGA 367
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 383   CTATGGCATCAAGTGGAAGCGTCCCACGGAACTGCTGTCAAACCCCCAGTTCATTGTGGA 442

Query 368   TGGAGCTACCCGCACAGACATCTGCCAGGGAGCACTGGGGGACTGCTGGCTCTTGGCGGC 427
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 443   TGGAGCTACCCGCACAGACATCTGCCAGGGAGCACTGGGGGACTGCTGGCTCTTGGCGGC 502

Query 428   CATTGCCTCCCTCACTCTCAACGACACCCTCCTGCACCGAGTGGTTCCGCACGGCCAGAG 487
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 503   CATTGCCTCCCTCACTCTCAACGACACCCTCCTGCACCGAGTGGTTCCGCACGGCCAGAG 562

Query 488   CTTCCAGAATGGCTATGCCGGCATCTTCCATTTCCAGCTGTGGCAATTTGGGGAGTGGGT 547
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 563   CTTCCAGAATGGCTATGCCGGCATCTTCCATTTCCAGCTGTGGCAATTTGGGGAGTGGGT 622

Query 548   GGACGTGGTCGTGGATGACCCGCTGCCCATCAAGGACGGGAAGCTAGTGTTCGTGCACTC 607
            |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct 623   GGACGTGGTCGTGGATGACCTGCTGCCCATCAAGGACGGGAAGCTAGTGTTCGTGCACTC 682

Query 608   TGCCGAAGGCAACGAGTTCTGGAGCGCCCTGCTTGAGAAGGCCTATGCCAAGGTAAATGG 667
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 683   TGCCGAAGGCAACGAGTTCTGGAGCGCCCTGCTTGAGAAGGCCTATGCCAAGGTAAATGG 742

Query 668   CAGCTACGAGGCCCTGTCAGGGGGCAGCACCTCAGAGGGCTTTGAGGACTTCACAGGCGG 727
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 743   CAGCTACGAGGCCCTGTCAGGGGGCAGCACCTCAGAGGGCTTTGAGGACTTCACAGGCGG 802

Query 728   GGTTACCGAGTGGTCCGAGTTGCGCAAGGCTCCCAGTGACCTCTACCAGATCATCCTCAA 787
            |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 803   GGTTACCGAGTGGTACGAGTTGCGCAAGGCTCCCAGTGACCTCTACCAGATCATCCTCAA 862

Query 788   GGCGCTGGAGCGGGGCTCCCTGCTGGGCTGCTCCATAGACATCTCCAGCGTTCTAGACAT 847
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 863   GGCGCTGGAGCGGGGCTCCCTGCTGGGCTGCTCCATAGACATCTCCAGCGTTCTAGACAT 922

Query 848   GGAGGCCATCACTTCCCAAGAAGTTGGTGAAGG-CCATGCCTACTCTGTGACCGGG-CCA 905
            |||||||||||||| | ||||||||||||||| |||||||||||||||||||||| |||
Sbjct 923   GGAGGCCATCACTTTC-AAGAAGTTGGTGAAGGGCCATGCCTACTCTGTGACCGGGGCCA 981

Query 906   AGCAAGTGAACTACCGAGGCCAGGTGGTGAGCCTGATC-G-ATGCGGAAACCCTGGG-CG 962
            |||| |||||||||||||||||||||||||||||||| | |||||||| ||||||| ||
Sbjct 982   AGCAGGTGAACTACCGAGGCCAGGTGGTGAGCCTGATCCGGATGCGGAACCCCTGGGGCG1041

Query 963   AAGGTTGGAATTGACGGGAGCCTGGGAGCGACA  995
            | ||| ||| | ||||||||||||| |||||||
Sbjct 1042  A-GGT-GGAGTGGACGGGAGCCTGG-AGCGACA  1071
```

BLAST search results using sequence from CAPN1-GFP TOPO plasmid sequenced with T7Foward primer

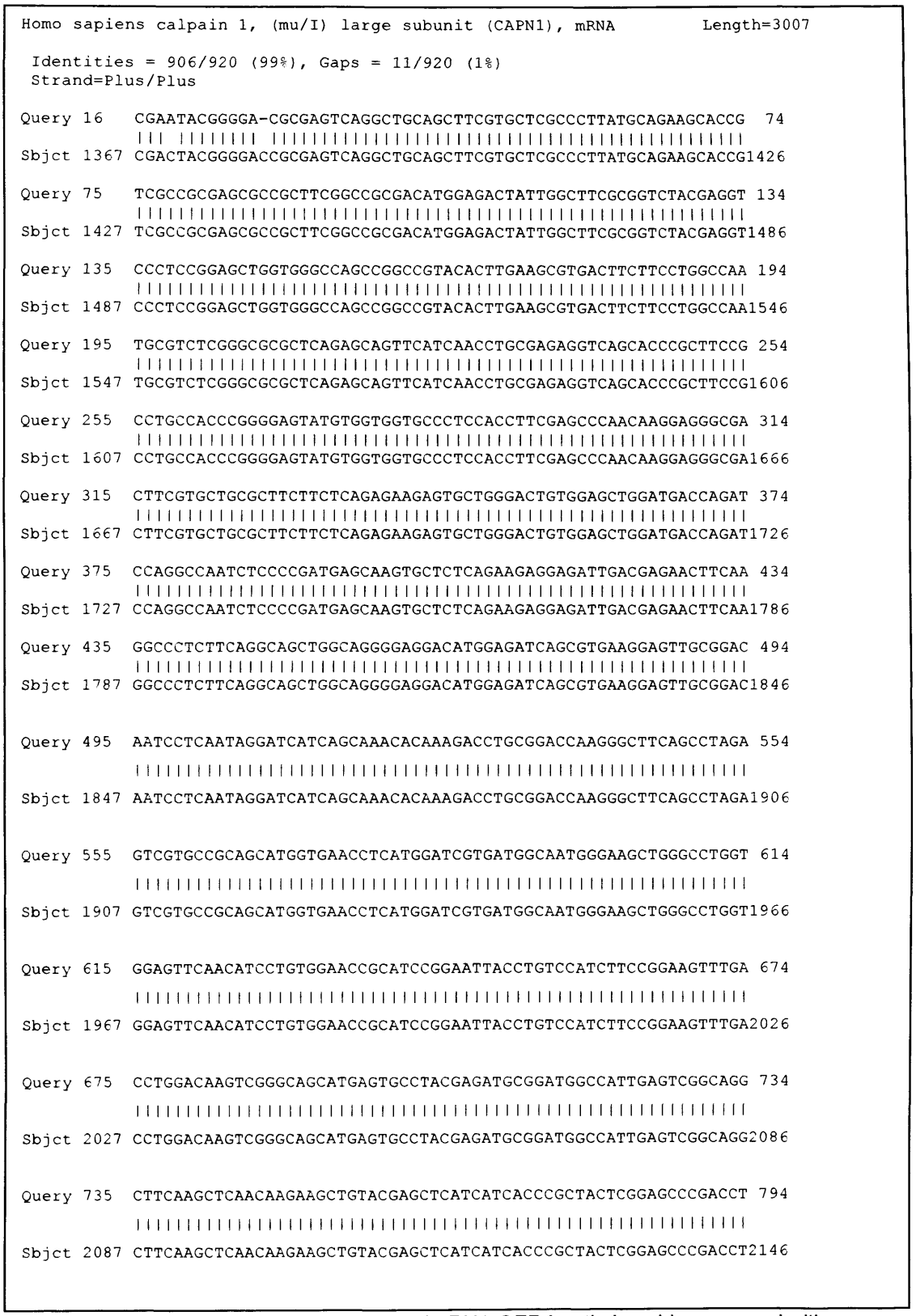


```
Homo sapiens calpain 1, (mu/I) large subunit (CAPN1), mRNA
Length=3007

 Identities = 485/491 (99%), Gaps = 3/491 (0%)
 Strand=Plus/Plus

Query 97   GCAAATGGAACACCACACTCTACGAAGGCACCTGGTTTTGCGGGGGAGCACCGCGGGGGG 156
           ||||||||||||||||||||||||||||||||||    ||||||||||||||||||||
Sbjct 1234 GCAAATGGAACACCACACTCTACGAAGGCACCTGG--CGGCGGGGGAGCACCGCGGGGGG1291

Query 157  CTGCCGAAACTACCCAGCCACCTTCTGGGTGAACCCTCAGTTCAAGATCCGGCTGGATGA 216
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1292 CTGCCGAAACTACCCAGCCACCTTCTGGGTGAACCCTCAGTTCAAGATCCGGCTGGATGA1351

Query 217  GACGGATGACCCGGACGACTACGGGGACCGCGAGTCAGGCTGCAGCTTCGTGCTCGCCCT 276
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1352 GACGGATGACCCGGACGACTACGGGGACCGCGAGTCAGGCTGCAGCTTCGTGCTCGCCCT1411

Query 277  TATGCAGAAGCACCGTCGCCGCGAGCGCCGCTTCGGCCGCGACATGGAGACTATTGGCTT 336
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1412 TATGCAGAAGCACCGTCGCCGCGAGCGCCGCTTCGGCCGCGACATGGAGACTATTGGCTT1471

Query 337  CGCGGTCTACGAGGTCCCTCCGGAGCTGGTGGGCCAGCCGGCCGTACACTTGAAGCGTGA 396
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1472 CGCGGTCTACGAGGTCCCTCCGGAGCTGGTGGGCCAGCCGGCCGTACACTTGAAGCGTGA1531

Query 397  CTTCTTCCTGGCCAATGCGTCTCGGGCGCGCTCAGAGCAGTTCATCAACCTGCGAGAGGT 456
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1532 CTTCTTCCTGGCCAATGCGTCTCGGGCGCGCTCAGAGCAGTTCATCAACCTGCGAGAGGT1591

Query 457  CAGCACTCGCTTCCGCCTGCCACCCGGGGAGTATGTGGTGGTGCCCTCCACCTTCGAGCC 516
           |||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1592 CAGCACCCGCTTCCGCCTGCCACCCGGGGAGTATGTGGTGGTGCCCTCCACCTTCGAGCC1651

Query 517  CAACAAGGAGGGCGACTTCGTGCTGCGCTTCTTCTCAGAAGAAGAGTGCTGGGACTGTGG 576
           |||||||||||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct 1652 CAACAAGGAGGGCGACTTCGTGCTGCGCTTCTTCTCAGA-GAAGAGTGCTGGGACTGTGG1710

Query 577  AGCTGGATGAC  587
           ||||||||||
Sbjct 1711 AGCTGGATGAC  1721
```

BLAST search results using sequence from CAPND3-GFP TOPO plasmid sequenced with T7Foward primer

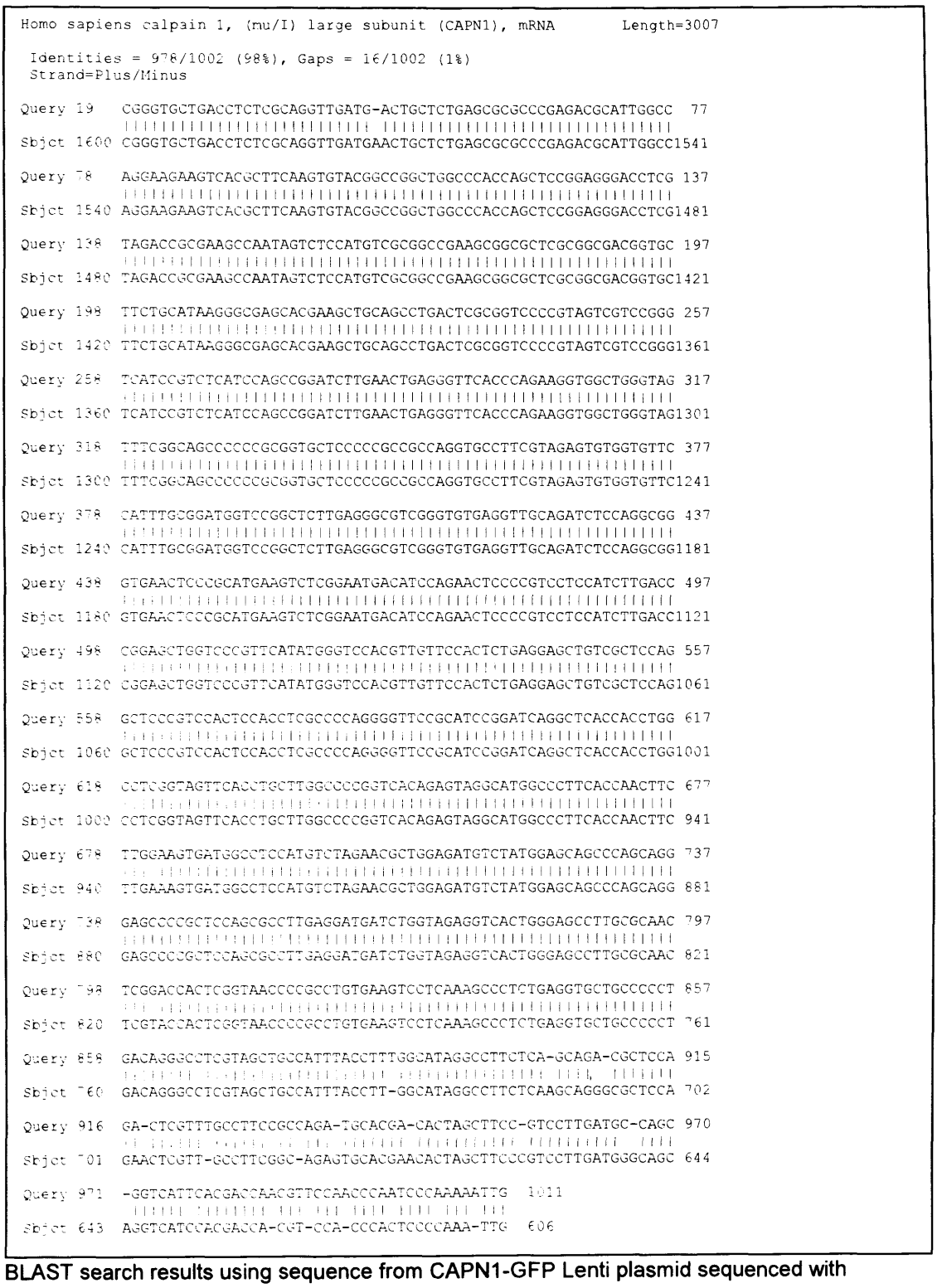
```
calpain, small subunit 1 [Homo sapiens]

 Score = 1242 bits (672),  Expect = 0.0
 Identities = 672/672 (100%), Gaps = 0/672 (0%)
 Strand=Plus/Plus

Query  90   GGCGGCGGCGGTGGTGGAGGCGGCGGTGGCGGTGGAACGGCCATGCGCATCCTAGGCGGA149
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  290  GGCGGCGGCGGTGGTGGAGGCGGCGGTGGCGGTGGAACGGCCATGCGCATCCTAGGCGGA349

Query  150  GTCATCAGCGCCATCAGCGAGGCGGCTGCGCAGTACAACCCGGAGCCCCCGCCCCCACGC209
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  350  GTCATCAGCGCCATCAGCGAGGCGGCTGCGCAGTACAACCCGGAGCCCCCGCCCCCACGC409

Query  210  ACACATTACTCCAACATTGAGGCCAACGAGAGTGAGGAGGTCCGGCAGTTCCGGAGACTC269
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  410  ACACATTACTCCAACATTGAGGCCAACGAGAGTGAGGAGGTCCGGCAGTTCCGGAGACTC469

Query  270  TTTGCCCAGCTGGCTGGAGATGACATGGAGGTCAGCGCCACAGAACTCATGAACATTCTC329
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  470  TTTGCCCAGCTGGCTGGAGATGACATGGAGGTCAGCGCCACAGAACTCATGAACATTCTC529

Query  330  AATAAGGTTGTGACACGACACCCTGATCTGAAGACTGATGGTTTTGGCATTGACACATGT389
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  530  AATAAGGTTGTGACACGACACCCTGATCTGAAGACTGATGGTTTTGGCATTGACACATGT589

Query  390  CGCAGCATGGTGGCCGTGATGGATAGCGACACCACAGGCAAGCTGGGCTTTGAGGAATTC449
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  590  CGCAGCATGGTGGCCGTGATGGATAGCGACACCACAGGCAAGCTGGGCTTTGAGGAATTC649

Query  450  AAGTACTTGTGGAACAACATCAAAAGGTGGCAGGCCATATACAAACAGTTCGACACTGAC509
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  650  AAGTACTTGTGGAACAACATCAAAAGGTGGCAGGCCATATACAAACAGTTCGACACTGAC709

Query  510  CGATCAGGGACCATTTGCAGTAGTGAACTCCCAGGTGCCTTTGAGGCAGCAGGGTTCCAC569
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  710  CGATCAGGGACCATTTGCAGTAGTGAACTCCCAGGTGCCTTTGAGGCAGCAGGGTTCCAC769

Query  570  CTGAATGAGCATCTCTATAACATGATCATCCGACGCTACTCAGATGAAAGTGGGAACATG629
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  770  CTGAATGAGCATCTCTATAACATGATCATCCGACGCTACTCAGATGAAAGTGGGAACATG829

Query  630  GATTTTGACAACTTCATCAGCTGCTTGGTCAGGCTGGACGCCATGTTCCGTGCCTTCAAA689
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  830  GATTTTGACAACTTCATCAGCTGCTTGGTCAGGCTGGACGCCATGTTCCGTGCCTTCAAA889

Query  690  TCTCTTGACAAAGATGGCACTGGACAAATCCAGGTGAACATCCAGGAGTGGCTGCAGCTG749
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  890  TCTCTTGACAAAGATGGCACTGGACAAATCCAGGTGAACATCCAGGAGTGGCTGCAGCTG949

Query  750  ACTATGTATTCC  761
            ||||||||||||
Sbjct  950  ACTATGTATTCC  961
```

BLAST search results using sequence from CAPNS1-GFP TOPO plasmid sequenced with T7Foward primer



```
Homo sapiens calpain 1, (mu/I) large subunit (CAPN1), mRNA          Length=3007

 Identities = 906/920 (99%), Gaps = 11/920 (1%)
 Strand=Plus/Plus

Query 16   CGAATACGGGGA-CGCGAGTCAGGCTGCAGCTTCGTGCTCGCCCTTATGCAGAAGCACCG  74
           ||| |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1367 CGACTACGGGGACCGCGAGTCAGGCTGCAGCTTCGTGCTCGCCCTTATGCAGAAGCACCG1426

Query 75   TCGCCGCGAGCGCCGCTTCGGCCGCGACATGGAGACTATTGGCTTCGCGGTCTACGAGGT 134
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1427 TCGCCGCGAGCGCCGCTTCGGCCGCGACATGGAGACTATTGGCTTCGCGGTCTACGAGGT1486

Query 135  CCCTCCGGAGCTGGTGGGCCAGCCGGCCGTACACTTGAAGCGTGACTTCTTCCTGGCCAA 194
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1487 CCCTCCGGAGCTGGTGGGCCAGCCGGCCGTACACTTGAAGCGTGACTTCTTCCTGGCCAA1546

Query 195  TGCGTCTCGGGCGCGCTCAGAGCAGTTCATCAACCTGCGAGAGGTCAGCACCCGCTTCCG 254
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1547 TGCGTCTCGGGCGCGCTCAGAGCAGTTCATCAACCTGCGAGAGGTCAGCACCCGCTTCCG1606

Query 255  CCTGCCACCCGGGGAGTATGTGGTGGTGCCCTCCACCTTCGAGCCCAACAAGGAGGGCGA 314
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1607 CCTGCCACCCGGGGAGTATGTGGTGGTGCCCTCCACCTTCGAGCCCAACAAGGAGGGCGA1666

Query 315  CTTCGTGCTGCGCTTCTTCTCAGAGAAGAGTGCTGGGACTGTGGAGCTGGATGACCAGAT 374
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1667 CTTCGTGCTGCGCTTCTTCTCAGAGAAGAGTGCTGGGACTGTGGAGCTGGATGACCAGAT1726

Query 375  CCAGGCCAATCTCCCCGATGAGCAAGTGCTCTCAGAAGAGGAGATTGACGAGAACTTCAA 434
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1727 CCAGGCCAATCTCCCCGATGAGCAAGTGCTCTCAGAAGAGGAGATTGACGAGAACTTCAA1786

Query 435  GGCCCTCTTCAGGCAGCTGGCAGGGGAGGACATGGAGATCAGCGTGAAGGAGTTGCGGAC 494
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1787 GGCCCTCTTCAGGCAGCTGGCAGGGGAGGACATGGAGATCAGCGTGAAGGAGTTGCGGAC1846

Query 495  AATCCTCAATAGGATCATCAGCAAACACAAAGACCTGCGGACCAAGGGCTTCAGCCTAGA 554
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1847 AATCCTCAATAGGATCATCAGCAAACACAAAGACCTGCGGACCAAGGGCTTCAGCCTAGA1906

Query 555  GTCGTGCCGCAGCATGGTGAACCTCATGGATCGTGATGGCAATGGGAAGCTGGGCCTGGT 614
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1907 GTCGTGCCGCAGCATGGTGAACCTCATGGATCGTGATGGCAATGGGAAGCTGGGCCTGGT1966

Query 615  GGAGTTCAACATCCTGTGGAACCGCATCCGGAATTACCTGTCCATCTTCCGGAAGTTTGA 674
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1967 GGAGTTCAACATCCTGTGGAACCGCATCCGGAATTACCTGTCCATCTTCCGGAAGTTTGA2026

Query 675  CCTGGACAAGTCGGGCAGCATGAGTGCCTACGAGATGCGGATGGCCATTGAGTCGGCAGG 734
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 2027 CCTGGACAAGTCGGGCAGCATGAGTGCCTACGAGATGCGGATGGCCATTGAGTCGGCAGG2086

Query 735  CTTCAAGCTCAACAAGAAGCTGTACGAGCTCATCATCACCCGCTACTCGGAGCCCGACCT 794
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 2087 CTTCAAGCTCAACAAGAAGCTGTACGAGCTCATCATCACCCGCTACTCGGAGCCCGACCT2146
```

BLAST search results using sequence from CAPN1-GFP Lenti plasmid sequenced with CAPN1/C2F1 forward (mid seq) primer



```
Homo sapiens calpain 1, (mu/I) large subunit (CAPN1), mRNA       Length=3007

 Identities = 978/1002 (98%), Gaps = 16/1002 (1%)
 Strand=Plus/Minus

Query 19   CGGGTGCTGACCTCTCGCAGGTTGATG-ACTGCTCTGAGCGCGCCCGAGACGCATTGGCC  77
           |||||||||||||||||||||||||| |||||||||||||||||||||||||||||||||
Sbjct 1600 CGGGTGCTGACCTCTCGCAGGTTGATGAACTGCTCTGAGCGCGCCCGAGACGCATTGGCC1541

Query 78   AGGAAGAAGTCACGCTTCAAGTGTACGGCCGGCTGGCCCACCAGCTCCGGAGGGACCTCG 137
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1540 AGGAAGAAGTCACGCTTCAAGTGTACGGCCGGCTGGCCCACCAGCTCCGGAGGGACCTCG1481

Query 138  TAGACCGCGAAGCCAATAGTCTCCATGTCGCGGCCGAAGCGGCGCTCGCGGCGACGGTGC 197
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1480 TAGACCGCGAAGCCAATAGTCTCCATGTCGCGGCCGAAGCGGCGCTCGCGGCGACGGTGC1421

Query 198  TTCTGCATAAGGGCGAGCACGAAGCTGCAGCCTGACTCGCGGTCCCCGTAGTCGTCCGGG 257
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1420 TTCTGCATAAGGGCGAGCACGAAGCTGCAGCCTGACTCGCGGTCCCCGTAGTCGTCCGGG1361

Query 258  TCATCCGTCTCATCCAGCCGGATCTTGAACTGAGGGTTCACCCAGAAGGTGGCTGGGTAG 317
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1360 TCATCCGTCTCATCCAGCCGGATCTTGAACTGAGGGTTCACCCAGAAGGTGGCTGGGTAG1301

Query 318  TTTCGGCAGCCCCCCGCGGTGCTCCCCCGCCGCCAGGTGCCTTCGTAGAGTGTGGTGTTC 377
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1300 TTTCGGCAGCCCCCCGCGGTGCTCCCCCGCCGCCAGGTGCCTTCGTAGAGTGTGGTGTTC1241

Query 378  CATTTGCGGATGGTCCGGCTCTTGAGGGCGTCGGGTGTGAGGTTGCAGATCTCCAGGCGG 437
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1240 CATTTGCGGATGGTCCGGCTCTTGAGGGCGTCGGGTGTGAGGTTGCAGATCTCCAGGCGG1181

Query 438  GTGAACTCCCGCATGAAGTCTCGGAATGACATCCAGAACTCCCCGTCCTCCATCTTGACC 497
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1180 GTGAACTCCCGCATGAAGTCTCGGAATGACATCCAGAACTCCCCGTCCTCCATCTTGACC1121

Query 498  CGGAGCTGGTCCCGTTCATATGGGTCCACGTTGTTCCACTCTGAGGAGCTGTCGCTCCAG 557
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1120 CGGAGCTGGTCCCGTTCATATGGGTCCACGTTGTTCCACTCTGAGGAGCTGTCGCTCCAG1061

Query 558  GCTCCCGTCCACTCCACCTCGCCCCAGGGGTTCCGCATCCGGATCAGGCTCACCACCTGG 617
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1060 GCTCCCGTCCACTCCACCTCGCCCCAGGGGTTCCGCATCCGGATCAGGCTCACCACCTGG1001

Query 618  CCTCGGTAGTTCACCTGCTTGGCCCCGGTCACAGAGTAGGCATGGCCCTTCACCAACTTC 677
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1000 CCTCGGTAGTTCACCTGCTTGGCCCCGGTCACAGAGTAGGCATGGCCCTTCACCAACTTC 941

Query 678  TTGGAAGTGATGGCCTCCATGTCTAGAACGCTGGAGATGTCTATGGAGCAGCCCAGCAGG 737
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 940  TTGAAAGTGATGGCCTCCATGTCTAGAACGCTGGAGATGTCTATGGAGCAGCCCAGCAGG 881

Query 738  GAGCCCCGCTCCAGCGCCTTGAGGATGATCTGGTAGAGGTCACTGGGAGCCTTGCGCAAC 797
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 880  GAGCCCCGCTCCAGCGCCTTGAGGATGATCTGGTAGAGGTCACTGGGAGCCTTGCGCAAC 821

Query 798  TCGGACCACTCGGTAACCCCGCCTGTGAAGTCCTCAAAGCCCTCTGAGGTGCTGCCCCCT 857
           ||| |||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 820  TCGTACCACTCGGTAACCCCGCCTGTGAAGTCCTCAAAGCCCTCTGAGGTGCTGCCCCCT 761

Query 858  GACAGGGCCTCGTAGCTGCCATTTACCTTTGGCATAGGCCTTCTCA-GCAGA-CGCTCCA 915
           ||||||| |||||||||||||||||||| |||||||||||||||| |||| |||||||||
Sbjct 760  GACAGGGCCTCGTAGCTGCCATTTACCTT-GGCATAGGCCTTCTCAAGCAGGGCGCTCCA 702

Query 916  GA-CTCGTTTGCCTTCCGCCAGA-TGCACGA-CACTAGCTTCC-GTCCTTGATGC-CAGC 970
           || |||||| |||||| || ||| |||||||| |||||||||| |||| |||||| ||||
Sbjct 701  GAACTCGTT-GCCTTCGGC-AGAGTGCACGAACACTAGCTTCCCGTCCTTGATGGGCAGC 644

Query 971  -GGTCATTCACGACCAACGTTCCAACCCAATCCCAAAAATTG  1011
            |||||| ||||||| ||| ||| |||| |||| ||| |||
Sbjct 643  AGGTCATCCACGACCA-CGT-CCA-CCCACTCCCCAAA-TTG  606
```

BLAST search results using sequence from CAPN1-GFP Lenti plasmid sequenced with CAPN1/C2R1 reverse (mid seq) primer

```
Homo sapiens calpain 1, (mu/I) large subunit (CAPN1), mRNA          Length=3007

 Identities = 901/923 (98%), Gaps = 15/923 (1%)
 Strand=Plus/Plus

Query 73    ATGTCGGAGGAGAACATCACGCCGGTGTACTGCACTGGGGTGTCAGCCCAAGTGCAGAAG 132
            |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct 144   ATGTCGGAGGAGATCATCACGCCGGTGTACTGCACTGGGGTGTCAGCCCAAGTGCAGAAG 203

Query 133   CAGCGGGCCAGGGAGCTGGGCCTGGGCCGCCATGAGAATGCCATCAAGTACCTGGGCCAG 192
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 204   CAGCGGGCCAGGGAGCTGGGCCTGGGCCGCCATGAGAATGCCATCAAGTACCTGGGCCAG 263

Query 193   GATTATGAGCAGCTGCGGGTGCGATGCCTGCAGAGTGGGACCCTCTTCCGTGATGAGGCC 252
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 264   GATTATGAGCAGCTGCGGGTGCGATGCCTGCAGAGTGGGACCCTCTTCCGTGATGAGGCC 323

Query 253   TTCCCCCCGGTACCCCAGAGCCTGGGTTACAAGGACCTGGGTCCCAATTCCTCCAAGACC 312
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 324   TTCCCCCCGGTACCCCAGAGCCTGGGTTACAAGGACCTGGGTCCCAATTCCTCCAAGACC 383

Query 313   TATGGCATCAAGTGGAAGCGTCCCACGGAACTGCTGTCAAACCCCCAGTTCATTGTGGAT 372
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 384   TATGGCATCAAGTGGAAGCGTCCCACGGAACTGCTGTCAAACCCCCAGTTCATTGTGGAT 443

Query 373   GGAGCTACCCGCACAGACATCTGCCAGGGAGCACTGGGGGACTGCTGGCTCTTGGCGGCC 432
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 444   GGAGCTACCCGCACAGACATCTGCCAGGGAGCACTGGGGGACTGCTGGCTCTTGGCGGCC 503

Query 433   ATTGCCTCCCTCACTCTCAACGACACCCTCCTGCACCGAGTGGTTCCGCACGGCCAGAGC 492
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 504   ATTGCCTCCCTCACTCTCAACGACACCCTCCTGCACCGAGTGGTTCCGCACGGCCAGAGC 563

Query 493   TTCCAGAATGGCTATGCCGGCATCTTCCATTTCCAGCTGTGGCAATTTGGGGAGTGGGTG 552
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 564   TTCCAGAATGGCTATGCCGGCATCTTCCATTTCCAGCTGTGGCAATTTGGGGAGTGGGTG 623

Query 553   GACGTGGTCGTGGATGACCCGCTGCCCATCAAGGACGGGAAGCTAGTGTTCGTGCACTCT 612
            |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct 624   GACGTGGTCGTGGATGACCTGCTGCCCATCAAGGACGGGAAGCTAGTGTTCGTGCACTCT 683

Query 613   GCCGAAGGCAACGAGTTCTGGAGCGCCCTGCTTGAGAAGGCCTATGCCAAGGTAAATGGC 672
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 684   GCCGAAGGCAACGAGTTCTGGAGCGCCCTGCTTGAGAAGGCCTATGCCAAGGTAAATGGC 743

Query 673   AGCTACGAGGCCCTGTCAGGGGGCAGCACCTCAGAGGGCTTTGAGGACTTCACAGGCGGG 732
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 744   AGCTACGAGGCCCTGTCAGGGGGCAGCACCTCAGAGGGCTTTGAGGACTTCACAGGCGGG 803

Query 733   GTTACCGAGTGGTCCGAGTTGCGCAAGGCTCCCAGTGACCTCTACCAGATCATCCTCAAG 792
            |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 804   GTTACCGAGTGGTACGAGTTGCGCAAGGCTCCCAGTGACCTCTACCAGATCATCCTCAAG 863

Query 793   GCGCTGGAGCGGGGCTCCCTGCTGGGCTGCTCCATAGACATCTCCAGCGTTCTAGACATG 852
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 864   GCGCTGGAGCGGGGCTCCCTGCTGGGCTGCTCCATAGACATCTCCAGCGTTCTAGACATG 923

Query 853   GAGGCCATCACTTCCAAGAAGTTGGTGAAGGGCCATGCCTACTCTGTGAC-GGG-CCA-G 909
            |||||||||||| |||||||||||||||||||||||||||||||||||| ||| ||| |
Sbjct 924   GAGGCCATCACTTTCAAGAAGTTGGTGAAGGGCCATGCCTACTCTGTGACCGGGGCCAAG 983

Query 910   CAAGTGAACTACCGAGGCCAGTTG-TGAGCCTGATC-G-ATGCG-A-CCC-TGG--CGAA 961
            || |||||||||||||||| || |||||||||| | ||||| | ||| ||| |||
Sbjct 984   CAGGTGAACTACCGAGGCCAGGTGGTGAGCCTGATCCGGATGCGGAACCCCTGGGGCGAG1043

Query 962   GTG-A-TGGACG--AGCCTGGAG  980
            ||| | ||||||  |||||||||
Sbjct 1044  GTGGAGTGGACGGGAGCCTGGAG  1066
```

BLAST search results using sequence from CAPN1-GFP Lenti plasmid sequenced with Lenti SxW Forward primer

```
Mus musculus transgenic enhanced green fluorescent protein (EGFP)
mRNA, complete cds
Length=1151

 Score = 1240 bits (671),  Expect = 0.0
 Identities = 706/721 (98%), Gaps = 9/721 (1%)
 Strand=Plus/Minus

Query 233 TTACTTGTACAGCTCGTCCATGCCGAGAGTGATCCCGGCGGCGGTCACGAACTCCAGCAG  292
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 852 TTACTTGTACAGCTCGTCCATGCCGAGAGTGATCCCGGCGGCGGTCACGAACTCCAGCAG  793

Query 293 GACCATGTGATCGCGCTTCTCGTTGGGGTCTTTGCTCAGGGCGGACTGGGTGCTCAGGTA  352
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 792 GACCATGTGATCGCGCTTCTCGTTGGGGTCTTTGCTCAGGGCGGACTGGGTGCTCAGGTA  733

Query 353 GTGGTTGTCGGGCAGCAGCACGGGGCCGTCGCCGATGGGGGTGTTCTGCTGGTAGTGGTC  412
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 732 GTGGTTGTCGGGCAGCAGCACGGGGCCGTCGCCGATGGGGGTGTTCTGCTGGTAGTGGTC  673

Query 413 GGCGAGCTGCACGCTGCCGTCCTCGATGTTGTGGCGGGTCTTGAAGTTCACCTTGATGCC  472
          |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct 672 GGCGAGCTGCACGCTGCCGTCCTCGATGTTGTGGCGGATCTTGAAGTTCACCTTGATGCC  613

Query 473 GTTCTTCTGCTTGTCGGCGGTGATATAGACCTTGTGGCTGTTGTAGTTGTACTCCAGCTT  532
          ||||||||||||||||  |||||||||| ||||||||||||||||||||||||||||||
Sbjct 612 GTTCTTCTGCTTGTCGGCCATGATATAGACGTTGTGGCTGTTGTAGTTGTACTCCAGCTT  553

Query 533 GTGCCCCAGGATGTTGCCGTCCTCCTTGAAGTCGATGCCCTTCAGCTCGATGCGGTTCAC  592
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 552 GTGCCCCAGGATGTTGCCGTCCTCCTTGAAGTCGATGCCCTTCAGCTCGATGCGGTTCAC  493

Query 593 CAGGGTGTCGCCCTCGAACTTCACCTCGGCGCGGGTCTTGTAGTTGCCGTCGTCCTTGAA  652
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 492 CAGGGTGTCGCCCTCGAACTTCACCTCGGCGCGGGTCTTGTAGTTGCCGTCGTCCTTGAA  433

Query 653 GAAGATGGTGCGCTCCTGGACGTAGCCTTCGGGCATGGCGGACTTGAAGAAGTCGTGCTG  712
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 432 GAAGATGGTGCGCTCCTGGACGTAGCCTTCGGGCATGGCGGACTTGAAGAAGTCGTGCTG  373

Query 713 CTTCATGTGGTCGGGGTAGCGGGC-GAAGCACTGCACGCCGTAG-TGAAGGTGGTCACGA  770
          |||||||||||||||||||| | |||||||||||||||||| | | ||||||||||||
Sbjct 372 CTTCATGTGGTCGGGGTAGCGG-CTGAAGCACTGCACGCCGTAGGTCAGGGTGGTCACGA  314

Query 771 GGGTGGGCCAGGGCACGGGCAGCTTGCCGGTGGTGCAGATGAACTTCAGG-TCAGCTTGC  829
          ||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||
Sbjct 313 GGGTGGGCCAGGGCACGGGCAGCTTGCCGGTGGTGCAGATGAACTTCAGGGTCAGCTTGC  254

Query 830 CGTAG-TGGCATCGCCCTCGCCCTCGCCGGGACACGCTGAACTTGTGGCCGTTTACGTCG  888
          ||||| |||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct 253 CGTAGGTGGCATCGCCCTCGCCCTCGCCGG-ACACGCTGAACTTGTGGCCGTTTACGTCG  195

Query 889 CCGTCCAGCTCGACCAGGGATGGGCACCACCCCCGGTGAACAGCTCCTCGCC-TTGCTCA  947
          |||||||||||||||| |||||||||||| |||||||||||||||||| |||||||
Sbjct 194 CCGTCCAGCTCGACCAGG-ATGGGCACCACCCC-GGTGAACAGCTCCTCGCCCTTGCTCA  137

Query 948 C  948
          |
Sbjct 136 C  136
```

BLAST search results using sequence from CAPN1-GFP Lenti plasmid sequenced with Lenti SxW Reverse primer

```
Homo sapiens calpain 1, (mu/I) large subunit (CAPN1), mRNA
Length=3007

 Score =  789 bits (427),  Expect = 0.0
 Identities = 474/495 (96%), Gaps = 10/495 (2%)
 Strand=Plus/Plus

Query 18   AAATAGGAACACCACACTACTTGCGAAAAAAGAACC-GGCGGCGGGGGAGCACCGCGGGG  76
           |||| |||||||||||||| | | ||   || | ||| |||||||||||||||||||||||
Sbjct 1236 AAAT-GGAACACCACACT-C-TACG---AAGGCACCTGGCGGCGGGGGAGCACCGCGGGG1289

Query 77   GGCTGCCGAAACTACCCAGCCACCTTCTAGGGTGAACCCTCAGTTCAAGATCCGGCTGGA 136
           ||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct 1290 GGCTGCCGAAACTACCCAGCCACCTTCT-GGGTGAACCCTCAGTTCAAGATCCGGCTGGA1348

Query 137  TGAGACGGATGACCCGGACGACTACGGGGACCGCGAGTCAGGCTGCAGCTTCGTGCTCGC 196
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1349 TGAGACGGATGACCCGGACGACTACGGGGACCGCGAGTCAGGCTGCAGCTTCGTGCTCGC1408

Query 197  CCTTATGCAGAAGCACCGTCGCCGCGAGCGCCGCTTCGGCCGCGACATGGAGACTATTGG 256
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1409 CCTTATGCAGAAGCACCGTCGCCGCGAGCGCCGCTTCGGCCGCGACATGGAGACTATTGG1468

Query 257  CTTCGCGGTCTACGAGGTCCCTCCGGAGCTGGTGGGCCAGCCGGCCGTACACTTGAAGCG 316
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1469 CTTCGCGGTCTACGAGGTCCCTCCGGAGCTGGTGGGCCAGCCGGCCGTACACTTGAAGCG1528

Query 317  TGACTTCTTCCTGGCCAATGCGTCTCGGGCGCGCTCAGAGCAGTTTTTCTTTCTTTGCTA 376
           |||||||||||||||||||||||||||||||||||||||||||||||||  ||   ||  || |
Sbjct 1529 TGACTTCTTCCTGGCCAATGCGTCTCGGGCGCGCTCAGAGCAGTTCATCAACCT--GCGA1586

Query 377  GAGGTCAGCACTCGCTTCCGCCTGCCTCCCGGGGAGTATGTGGTGGTGCCCTCCACCTTC 436
           |||||||||||| |||||||||||||| |||||||||||||||||||||||||||||||||
Sbjct 1587 GAGGTCAGCACCCGCTTCCGCCTGCCACCCGGGGAGTATGTGGTGGTGCCCTCCACCTTC1646

Query 437  GAGCCCAACAAGGAGGGCGACTTCGTGCTGCGCTTCTTCTCAGAGAAGAGTGCTGGGACT 496
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 1647 GAGCCCAACAAGGAGGGCGACTTCGTGCTGCGCTTCTTCTCAGAGAAGAGTGCTGGGACT1706

Query 497  GTGGAGCTGGATGAC  511
           |||||||||||||||
Sbjct 1707 GTGGAGCTGGATGAC  1721
```

BLAST search results using sequence from CAPND3-GFP Lenti plasmid sequenced with Lenti SxW Forward primer

```
Mus musculus transgenic enhanced green fluorescent protein (EGFP)
mRNA, complete cds
Length=1151

 Score = 1177 bits (637),  Expect = 0.0
 Identities = 702/729 (97%), Gaps = 21/729 (2%)
 Strand=Plus/Minus

Query 237 TTACTTGTACAGCTCGTCCATGCCGAGAGTGATCCCGGCGGCGGTCACGAACTCCAGCAG  296
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 852 TTACTTGTACAGCTCGTCCATGCCGAGAGTGATCCCGGCGGCGGTCACGAACTCCAGCAG  793

Query 297 GACCATGTGATCGCGCTTCTCGTTGGGGTCTTTGCTCAGGGCGGACTGGGTGCTCAGGTA  356
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 792 GACCATGTGATCGCGCTTCTCGTTGGGGTCTTTGCTCAGGGCGGACTGGGTGCTCAGGTA  733

Query 357 GTGGTTGTCGGGCAGCAGCACGGGGCCGTCGCCGATGGGGGTGTTCTGCTGGTAGTGGTC  416
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 732 GTGGTTGTCGGGCAGCAGCACGGGGCCGTCGCCGATGGGGGTGTTCTGCTGGTAGTGGTC  673

Query 417 GGCGAGCTGCACGCTGCCGTCCTCGATGTTGTGGCGGGTCTTGAAGTTCACCTTGATGCC  476
          |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct 672 GGCGAGCTGCACGCTGCCGTCCTCGATGTTGTGGCGGATCTTGAAGTTCACCTTGATGCC  613

Query 477 GTTCTTCTGCTTGTCGGCGGTGATATAGACCTTGTGGCTGTTGTAGTTGTACTCCAGCTT  536
          ||||||||||||||||  |||||||||| ||||||||||||||||||||||||||||||
Sbjct 612 GTTCTTCTGCTTGTCGGCCATGATATAGACGTTGTGGCTGTTGTAGTTGTACTCCAGCTT  553

Query 537 GTGCCCCAGGATGTTGCCGTCCTCCTTGAAGTCGATGCCCTTCAGCTCGATGCGGTTCAC  596
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 552 GTGCCCCAGGATGTTGCCGTCCTCCTTGAAGTCGATGCCCTTCAGCTCGATGCGGTTCAC  493

Query 597 CAGGGTGTCGCCCTCGAACTTCACCTCGGCGCGGGTCTTGTAGTTGCCGTCGTCCTTGAA  656
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 492 CAGGGTGTCGCCCTCGAACTTCACCTCGGCGCGGGTCTTGTAGTTGCCGTCGTCCTTGAA  433

Query 657 GAAGATGGTGCGCTCCTGGACGTAGCCTTCGGGCATGGCGGACTTGAAGAAGTCGTGCTG  716
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 432 GAAGATGGTGCGCTCCTGGACGTAGCCTTCGGGCATGGCGGACTTGAAGAAGTCGTGCTG  373

Query 717 CTTCATGTGGTCGGGGTAGCGGGC-GAAGCACTGCACGCCGTAGGTGAAGGTGGTCACGA  775
          |||||||||||||||||||| | |||||||||||||||||||| | ||||||||||
Sbjct 372 CTTCATGTGGTCGGGGTAGCGG-CTGAAGCACTGCACGCCGTAGGTCAGGGTGGTCACGA  314

Query 776 GGGTGGGCCAGGGCACGGGCAGCTTGCCGGTGGTGCAGATGAACTTCAGGGTCAGCTTGC  835
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 313 GGGTGGGCCAGGGCACGGGCAGCTTGCCGGTGGTGCAGATGAACTTCAGGGTCAGCTTGC  254

Query 836 CGTAAGGTGGCATCGCCCTCGCCCTCGCCCGGAACACGCCTGA-CTTGTGGCCCGTT-AC  893
          |||| |||||||||||||||||||||| ||| ||||| ||| ||||||||| ||| ||
Sbjct 253 CGTA-GGTGGCATCGCCCTCGCCCTCGCC-GGA-CACGC-TGAACTTGTGGCC-GTTTAC  199

Query 894 GTTCGCCGTC-AGCTCGACCAGGGAATGGGCACCCACCCCCCGGGTGAAACAGCTTCCCT  952
          || ||||||| |||||||||||| | |||||||| |||||  || |||| |||||| || |
Sbjct 198 GT-CGCCGTCCAGCTCGACCAGG-A-TGGGCACC-ACCCC--GG-TGAA-CAGCT-CC-T  149

Query 953 CGCCCCTTG  961
          ||||| |||
Sbjct 148 CGCCC-TTG  141
```

BLAST search results using sequence from CAPND3-GFP Lenti plasmid sequenced with Lenti SxW Reverse primer

```
calpain, small subunit 1 [Homo sapiens]

 Score = 1225 bits (663),  Expect = 0.0
 Identities = 669/672 (99%), Gaps = 0/672 (0%)
 Strand=Plus/Plus

Query 97  GGCGGCGGCGGTGGTGGAGGCGGCGGTGGCGGTGGAACGGCCATGCGCATCCTAGGCGGA  156
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 290 GGCGGCGGCGGTGGTGGAGGCGGCGGTGGCGGTGGAACGGCCATGCGCATCCTAGGCGGA  349

Query 157 GTCATCAGCGCCATCAGCGAGGCGGCTGCGCAGTACAACCCGGAGCCCCCGCCCCCACGC  216
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 350 GTCATCAGCGCCATCAGCGAGGCGGCTGCGCAGTACAACCCGGAGCCCCCGCCCCCACGC  409

Query 217 ACACATTACTCCAACATTGAGGCCAACGAGAGTGAGGAGGTCCGGCAGTTCCGGAGACTC  276
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 410 ACACATTACTCCAACATTGAGGCCAACGAGAGTGAGGAGGTCCGGCAGTTCCGGAGACTC  469

Query 277 TTTGCCCAGCTGGCTGGAGATGACATGGAGGTCAGCGCCACAGAACTCATGAACATTCTC  336
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 470 TTTGCCCAGCTGGCTGGAGATGACATGGAGGTCAGCGCCACAGAACTCATGAACATTCTC  529

Query 337 AATAAGGTTGTGACACGACACCCTGATCTGAAGACTGATGGTTTTGGCATTGACACATGT  396
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 530 AATAAGGTTGTGACACGACACCCTGATCTGAAGACTGATGGTTTTGGCATTGACACATGT  589

Query 397 CGCAGCATGGTGGCCGTGATGGATAGCGACACCACAGGCAAGCTGGGCTTTGAGGATTTC  456
          |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||
Sbjct 590 CGCAGCATGGTGGCCGTGATGGATAGCGACACCACAGGCAAGCTGGGCTTTGAGGAATTC  649

Query 457 TTGTACTTGTGGAACAACATCAAAAGGTGGCAGGCCATATACAAACAGTTCGACACTGAC  516
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 650 AAGTACTTGTGGAACAACATCAAAAGGTGGCAGGCCATATACAAACAGTTCGACACTGAC  709

Query 517 CGATCAGGGACCATTTGCAGTAGTGAACTCCCAGGTGCCTTTGAGGCAGCAGGGTTCCAC  576
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 710 CGATCAGGGACCATTTGCAGTAGTGAACTCCCAGGTGCCTTTGAGGCAGCAGGGTTCCAC  769

Query 577 CTGAATGAGCATCTCTATAACATGATCATCCGACGCTACTCAGATGAAAGTGGGAACATG  636
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 770 CTGAATGAGCATCTCTATAACATGATCATCCGACGCTACTCAGATGAAAGTGGGAACATG  829

Query 637 GATTTTGACAACTTCATCAGCTGCTTGGTCAGGCTGGACGCCATGTTCCGTGCCTTCAAA  696
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 830 GATTTTGACAACTTCATCAGCTGCTTGGTCAGGCTGGACGCCATGTTCCGTGCCTTCAAA  889

Query 697 TCTCTTGACAAAGATGGCACTGGACAAATCCAGGTGAACATCCAGGAGTGGCTGCAGCTG  756
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 890 TCTCTTGACAAAGATGGCACTGGACAAATCCAGGTGAACATCCAGGAGTGGCTGCAGCTG  949

Query 757 ACTATGTATTCC  768
          ||||||||||||
Sbjct 950 ACTATGTATTCC  961
```

BLAST search results using sequence from CAPNS1-GFP Lenti plasmid sequenced with Lenti SxW Forward primer

```
Mus musculus transgenic enhanced green fluorescent protein (EGFP)
mRNA, complete cds
Length=1151

 Score = 1218 bits (659),  Expect = 0.0
 Identities = 711/733 (97%), Gaps = 15/733 (2%)
 Strand=Plus/Minus

Query 237 TTACTTGTACAGCTCGTCCATGCCGAGAGTGATCCCGGCGGCGGTCACGAACTCCAGCAG  296
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 852 TTACTTGTACAGCTCGTCCATGCCGAGAGTGATCCCGGCGGCGGTCACGAACTCCAGCAG  793

Query 297 GACCATGTGATCGCGCTTCTCGTTGGGGTCTTTGCTCAGGGCGGACTGGGTGCTCAGGTA  356
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 792 GACCATGTGATCGCGCTTCTCGTTGGGGTCTTTGCTCAGGGCGGACTGGGTGCTCAGGTA  733

Query 357 GTGGTTGTCGGGCAGCAGCACGGGGCCGTCGCCGATGGGGGTGTTCTGCTGGTAGTGGTC  416
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 732 GTGGTTGTCGGGCAGCAGCACGGGGCCGTCGCCGATGGGGGTGTTCTGCTGGTAGTGGTC  673

Query 417 GGCGAGCTGCACGCTGCCGTCCTCGATGTTGTGGCGGGTCTTGAAGTTCACCTTGATGCC  476
          |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct 672 GGCGAGCTGCACGCTGCCGTCCTCGATGTTGTGGCGGATCTTGAAGTTCACCTTGATGCC  613

Query 477 GTTCTTCTGCTTGTCGGCGGTGATATAGACCTTGTGGCTGTTGTAGTTGTACTCCAGCTT  536
          ||||||||||||||||  |||||||||| ||||||||||||||||||||||||||||||
Sbjct 612 GTTCTTCTGCTTGTCGGCCATGATATAGACGTTGTGGCTGTTGTAGTTGTACTCCAGCTT  553

Query 537 GTGCCCCAGGATGTTGCCGTCCTCCTTGAAGTCGATGCCCTTCAGCTCGATGCGGTTCAC  596
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 552 GTGCCCCAGGATGTTGCCGTCCTCCTTGAAGTCGATGCCCTTCAGCTCGATGCGGTTCAC  493

Query 597 CAGGGTGTCGCCCTCGAACTTCACCTCGGCGCGGGTCTTGTAGTTGCCGTCGTCCTTGAA  656
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 492 CAGGGTGTCGCCCTCGAACTTCACCTCGGCGCGGGTCTTGTAGTTGCCGTCGTCCTTGAA  433

Query 657 GAAGATGGTGCGCTCCTGGACGTAGCCTTCGGGCATGGCGGACTTGAAGAAGTCGTGCTG  716
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 432 GAAGATGGTGCGCTCCTGGACGTAGCCTTCGGGCATGGCGGACTTGAAGAAGTCGTGCTG  373

Query 717 CTTCATGTGGTCGGGGTAGCGGGC-GAAGCACTGCACGCCGTAG-TGAAGGTGGTCACGA  774
          |||||||||||||||||||| | |||||||||||||||||| | | ||||||||||
Sbjct 372 CTTCATGTGGTCGGGGTAGCGG-CTGAAGCACTGCACGCCGTAGGTCAGGGTGGTCACGA  314

Query 775 GGGTGGGCCAGGGCACGGGCAGCTTGCCCGGTGGTGCAGATGAACTTCAGGGTCAGCTTG  834
          |||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct 313 GGGTGGGCCAGGGCACGGGCAGCTTGCC-GGTGGTGCAGATGAACTTCAGGGTCAGCTTG  255

Query 835 CCGTAGGTGGCATCGCCCTCGCCCTCGCCCGGACACGCTGAAACTTGGTGGACCGTTTAC  894
          |||||||||||||||||||||||||||| |||||||||||| |||| ||| ||||||||
Sbjct 254 CCGTAGGTGGCATCGCCCTCGCCCTCGCC-GGACACGCTGAA-CTTG-TGG-CCGTTTAC  199

Query 895 GTTCGCCCGTCCAGCTCGACCAGGATGGGGCACCAACCCCCGGTGACCAGCTCCTCGCC-  953
          || |||| ||||||||||||||||||||| ||||| |||| ||||| ||||||||||| 
Sbjct 198 GT-CGCC-GTCCAGCTCGACCAGGATGGG-CACCA-CCCC-GGTGAACAGCTCCTCGCCC  144

Query 954 TTGCCTCACCATG  966
          |||| ||||||||
Sbjct 143 TTGC-TCACCATG  132
```

BLAST search results using sequence from CAPNS1-GFP Lenti plasmid sequenced with Lenti SxW Reverse primer