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by Carina Dinis Santos

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„All men by nature desire to know“

Aristotle

Acknowledgments

In the first place I want to thank Alex, my supervisor, for all the patience and watch, and all the detailed explanations about the work. You were a God saver. Without you, of course I could never make it!

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Abbreviations

≥	greater than or equal
%	percent
ºC	degree Celcius
µg	microgram
µL	microliter
µm	micrometer
µM	micromolar
293T cells	human epithelial kidney cells
AB	Antibody
AcNPV	Autographa californica nuclear polyhedrosis virus
ATP	Adenosine-5'-triphosphate
BEVS	Baculovirus Expression Vector System
bp	base pairs
BSA	bovine serum albumin
C	molar concentration
C3	complement component 3
C4	complement component 4
cDNA	complementary DNA
CEACAM3	Carcinoembryonic antigen-related cell adhesion molecule 3
CFSE	Carboxyfluorescein succinimidyl ester
cm	centimeter
CPU	central processing unit
CR	complement receptor
CRs	complement receptors
CS	calf serum
C-terminal	carboxyl-terminus
CTLs	cytolytic T lymphocytes
Cy	cyanine
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
ddH_2O	double-destilled water

dGTP	deoxyguanosine triphosphate
DMEM	Dulbecco’s modified Eagle's medium
DNA	Deoxyribonucleic acid
dNTPs	deoxyribonucleosid triphosphates
dTTP	deoxythymidine triphosphate
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
EtBr	Ethidium bromide
EtOH	ethanol
FAK	focal adhesion kinase
FAT	focal adhesion targeting
FcR	Fc receptor
FcRs	Fc receptors
FCS	Fetal bovine serum
FITC	fluorescein isothiocyanate
fmol	femtomole
FPLC	fast protein liquid chromatography
FRNK	FAK related non-kinase
xg	acceleration
g	gram
GAP	glyceraldehyde 3-phosphate
GRB2	growth factor receptor-bound 2
GST	glutathione S-transferase
GTP	guanosine triphosphate
HBS	HEPES-buffered saline
HBSS	Hank’s Buffered Salt Solution
HEK	human epithelial kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
h.i.	heat inactivated
HMW	high molecular weight
HPLC	High-performance liquid chromatography
HRP	horseradish peroxidase

Ig	Immunoglobulin
IgG	Imumnoglobulin G
IgM	Immunoglobulin M
Igs	Immunoglobulins
Kb	kilo base pairs
KD	kinase domain
KDa	kilo Dalton
L	liter
LB	lysogeny broth
LMW	low molecular weight
LPS	lipopolysaccharides
M	molar
mA	milli Amperes
MAP	mitogen-activated protein
mg	milligram
MHC	major histocompability complex
min	minute
mL	milliliter
mM	millimolar
MOI	multiplicity of infection
Mpa	mega Pascal's
mRNA	messenger Ribonucleic acid
n	molar number
ng	nanogram
NK	natural killer
nm	nanometer
N-terminal	amino-terminus
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEEK	polyether ether ketone
PFA	Paraformaldehyde
Pfu	plaque-forming unit
PI3K	Phosphatidylinositol 3-kinase

PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethanesulfonylfluoride
PRRs	pattern recognition receptors
Psi	pound-force per square inch
PTKs	protein-tyrosine-kinases
PVDF	Polyvinylidene Fluoride
Pyk2	proline-rich tyrosine kinase 2
RIPA	Radioimmunoprecipitation assay
RNAse A	Ribonuclease A
Rpm	revolutions per minute
RT	room temperature
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sf	*Spodoptera frugiperda*
Sf9	*Spodoptera frugiperda* clonal isolate 9
SH	sulfhydryl
SH2	Src Homology 2
SH3	Src Homology 3
TAE	Tris-acetate-EDTA
TBS	Tris buffered saline
TBS-T	Tris buffered saline + 0.1% Tween20
TE	Tris EDTA
TEMED	Tetramethylethylenediamine
TRITC	Tetramethylrhodamine isothiocyanate
UV	ultraviolet
V	volume
V	Volt
Vis	visible
v/v	volume per volume
w/v	weight per volume
α- (in connection with antibodies)	anti-

Abstract

In all human beings, when a pathogen enters the body, specialized cells are recruited to the site of inflammation, where, by different pathways and with the help of different macromolecules, they will destroy the invading microorganism. Past and ongoing research in this field is directed toward understanding the molecular and cellular regulation of this process. One way to destroy pathogens is the phagocytosis and subsequent intracellular killing, where macrophages play an important role, both by recognizing these pathogens and ingest them. To recognize pathogens, the macrophages have different receptors on their surface, for example Fc receptors (FcR) and complement receptors (CR), which bind to immunoglobuline G (IgG) or complement factor 3b (C3b) opsonized particles, respectively. After the macrophages have recognized a pathogen, several signalling processes regulate the uptake of the bacteria by the phagocytes. For example, non-receptor tyrosine kinases may be recruited to the plasma membrane, where they transmit signaling by cell surface receptors, controlling processes as diverse as the immune response, cell adhesion and neuronal cell migration. One member of this group of proteins is Pyk2, which can be involved in cell growth, shape and tumor invasion. In this study it is demonstrated that Pyk2 is recruited to the site of uptake, when a C3b-opsonized particle is recognized by the CRs on mouse macrophages. Specificity of the Pyk2 antibody, used for this study, was assured by *in vitro* assays. When a mouse-specific CD11b antibody was used to block the complement receptor 3, the uptake and internalization of particles opsonized with complement, but not of particles opsonized with IgG, decreased significantly, proving that these particles were phagocytosed by the complement receptors. The functional role of Pyk2 in this issue has to be determined in further studies. Taken together, these results could improve the understanding of complement receptor mediated phagocytosis.

In a second approach, the Baculovirus expression was used to express functional human protein kinases in insect cells. Thus, different recombinant proteins were produced and purified, such as the Glutathione-S-Transferase (GST)-mFRNK-TAT and GST-Hck-kinase domain (KD). A Pyk2-KD and FAK-KD were also expressed by this system, purified, and successfully employed in in vitro kinase assays. Accordingly, this project covered all the steps necessary for the production of recombinant proteins and expression using the Baculovirus expression vector system. As the purified kinase domains showed activity, they will be useful tools for further biochemical *in vitro* studies.

Resumo

Em todos os seres humanos, quando um agente invasor entra no corpo, células especializadas são recrutadas para o sítio de inflamação onde, por diferentes vias e com a ajuda de diferentes macromoléculas, vão destrui-lo. Investigações nesta área são maioritariamente direccionadas para o entendimento da regulação molecular e celular deste processo. A fagocitose, processo pelo qual as partículas invasoras maiores que 0.5 μm de diâmetro são destruídas pelas células especializadas, tem um papel importante na imunidade inata, tanto por facilitar a remoção destas partículas como por iniciar a resposta imune adaptativa. É uma função fisiológica essencial, comum à maioria dos tipos de células eucarióticas e envolve a activação de complexas redes de sinalização. Como efetores críticos do sistema imunitário, os macrófagos usam processos de sinalização durante a adesão para concretizar muitas funções celulares essenciais, inclusivamente a fagocitose. Numerosos receptores fagocíticos estão presentes nos macrófagos, que se ligam directa ou indirectamente ao alvo. Destes receptores, os Fcγ e CRs são os mais estudados e exibem mecanismos diferentes de fagocitose e respostas celulares subsequentes que reflectem diferenças importantes nas suas vias de sinalização. Os Fc receptores (FcRs) servem de mediadores da fagocitose de partículas opsonizadas por IgG e os receptores de complemento (CRs) reconhecem as partículas opsonizadas com C3b/C3bi, componentes presentes no soro. Estes receptores juntam-se normalmente à volta da presa e induzem cascadas de sinalização intracelular que levam à activação e recrutamento de moléculas adaptadoras e de sinalização para sítios de ligação de partículas. A interacção das partículas opsonizadas com o complemento com CR3, desencadeia a imersão das partículas opsonizadas com o complemento e desempenha um papel importante na adesão celular, assim como na fagocitose. O papel da Pyk2, uma Proteína Tirosina Quinase não receptora, nas células da imunidade inata ainda não é claro. A Pyk2 não abriga os domínios SH2 ou SH3, mas contém vários sítios de ligação para diferentes proteínas de sinalização contendo SH2/SH3. Após a activação/autofosforilação dessa enzima em Tyr402, a Pyk2 pode ligar-se a proteínas do citoesqueleto (como a paxilina), de ligação (como a p130Cas), ou outras tirosina quinases (incluindo membros da família Src), que podem potencialmente impingir a Pyk2 numa variedade de vias de transdução de sinal. O sítio de autofosforilação da Pyk2 serve como um local de encaixe para o domínio SH2 da Src, permitindo-lhe alcançar uma conformação activa. Seguindo a activação, tanto a quinase Pyk2 como a Src funcionam em conjunto para activar a jusante moléculas de sinalização. Tem sido demonstrado que, juntamente com Src, a

Pyk2 funciona como um elo entre receptores acoplados à proteína G heterotrimérica e a via de sinalização da proteína quinase activada por mitógeno (MAP). Estudos anteriores mostraram que esta proteína é importante na activação dos macrófagos. Esta proteína foi mostrada ser uma molécula de sinalização intracelular crítica, integrando a estimulação de receptores de quemoquina e de factores de crescimento com várias vias a jusante. Anteriormente, a Pyk2 tem sido associada a processos de migração e adesão, através de sua activação em resposta a diferentes quemoquinas e integrinas. Foram produzidos ratos deficientes em Pyk2 e a sua análise mostra que se desenvolvem normalmente, excepto por exibirem migração de macrófagos com defeito. Além disso, a Pyk2, tal como a FAK, podem ser reguladas pela activação de receptores de integrina. No entanto, a Pyk2 não está localizada em contactos focais, mas sim concentrada na região perinuclear das células. Neste contexto, foi então estudado se esta proteína, presente em diferentes processos de sinalização, era recrutada durante a fagocitose mediada por receptores complemento. Para os receptores complemento presentes nos macrófagos reconhecerem as partículas acopladas com IgM, e activarem a fagocitose, estas partículas foram incubadas com soro de ratos, opsonizando deste modo as partículas com o factor C3b, que é reconhecido pelos receptores complemento. Foram usadas para este estudo partículas acopladas com Albumina e GST, que não devem ser reconhecidos por nenhum receptor, IgG que é reconhecida por receptores Fcγ mas não CRs, IgM que sem incubação com soro não é reconhecida por nenhum receptor e depois de incubação com soro é reconhecida pelos CRs e iC3b, um factor do complemento que é directamente reconhecido pelos CRs.

Depois de testado, o anticorpo usado para a detecção da Pyk2 nas células foi considerado específico para a proteína, provando que todo o sinal detectado pelo mesmo se devia à Pyk2. Foram também realizados testes para a optimização do processo de infecção e coloração das células, assim como para o processo de fagocitose. Também foi provado que as partículas eram de facto fagocitadas pelos macrófagos, já que foram detectados fortes sinais depois de coloração com actina, já demonstrada ser necessária ao processo de fagocitose. O estudo iniciou-se com ensaios intra e extracelulares, para quantificar a percentagem de internalização e adesão das partículas às células. Foi provada a internalização das partículas acopladas com IgM e incubadas posteriormente com soro pelos receptores complemento, usando um anticorpo de CD11b M1/7015. Este anticorpo foi estudado anteriormente e foi demonstrado por bloquear os receptores complemento. Ao usar este anticorpo antes da infecção, as partículas deixaram de ser internalizadas. Foi investigado então o recrutamento

da Pyk2 durante a fagocitose mediada por receptores complemento e foi detectado um claro sinal, provando a participação desta proteína neste processo. Foi também detectado aumento da autofosforilação desta proteína em células lisadas, depois de infecção com IgG e IgM mais soro.

Num outro projecto, a Expressão por Baculovírus foi usada para a expressão e produção de proteínas funcionais. O Sistema de Expressão por Baculovírus usando vectores (BEVS) é o sistema de expressão eucariótica mais poderoso e versátil disponível. Neste sistema, vários genes Baculovírus não essenciais ao ciclo de vida na cultura de tecidos podem ser substituídos por genes heterólogos. Já que o genoma Baculovírus é geralmente muito grande para facilmente inserir genes estranhos, genes heterólogos são clonados em vectores de transferência. A co-infecção do vector de transferência e do DNA AcNPV dentro de células *Spodoptera frugiperda* (*Sf*) proporciona a recombinação entre os sítios homólogos, transferindo o gene heterólogo do vector de transferência para o DNA AcNPV. O Vírus de Poliedrose Nuclear *Autographa californica* (AcNPV) é o tipo de Baculovírus mais extensivamente estudada. A infecção de células *Sf* com o AcNPV resulta na paragem da expressão dos genes do hospedeiro, o que permite uma elevada taxa de produção de mRNA recombinante e de proteína. O vírus recombinante induz a expressão da proteína alvo recombinante e também infecta as células de insecto adicionais, resultando em vírus recombinantes adicionais.

Para a expressão da GST-Hck-KD e GST-mFRNK-TAT, o cDNA da GST foi primeiro clonado num vector de transferência, pVL1392. Depois de obtido o novo vector de transferência, o cDNA da Hk-KD e mFRNK-TAT foi clonado nesse novo vector, pVL1392 N-term GST. Todas as construções foram sequenciadas e analisadas. As células *Sf9* foram então co-infectadas com os vectores finais e o DNA BaculoGoldTM, levando à produção de Baculovírus recombinantes e expressão das proteínas heterólogas. O vírus recombinante foi amplificado por vários ciclos até o título do vírus ser maior ou igual a 2x108 pfu/mL e o título foi determinado indirectamente por coloração e subsequente análise por microscopia florescente. O mesmo foi então expresso, durante um tempo determinado por western blot, as células lisadas e as proteínas purificadas por FPLC. Depois de obtida a proteína, foi feita uma diálise de aproximadamente 18 horas e a concentração determinada por um gel SDS. Para determinar se as proteínas quinase eram activas, foram realizados ensaios de quinase *in vitro*. Foram testadas duas proteínas expressas, que demonstraram ser activas.

Keywords

Pyk2

Phagocytosis

Complement receptors

Baculovirus expression

Recombinant proteins

Palavras-Chave

Pyk2

Fagocitose

Receptores complemento

Expressão por Baculovirus

Proteínas recombinantes

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1. Introduction

1.1 The immune system

Historically, immunity means protection from disease and, more specifically, infectious disease. The cells and molecules responsible for immunity constitute the immune system. Their collective and coordinated response to the introduction of foreign substances is called the immune response [1]. The immune system has evolved under tremendous selective pressure driven by pathogens. As a result, all multicellular organisms have developed the ability to recognize invading microbes and to eliminate them efficiently while avoiding massive damage to themselves. The problem with recognizing pathogens is their enormous variability and molecular heterogeneity, which is further aggravated by the high mutational rate characteristic of microorganisms and the use of a restricted number of receptors [2, 3]. This challenge has been partially met by the exploitation of conserved motifs on pathogens that are not found in higher eukaryotes. These motifs have essential roles in the biology of the invading agents, and are therefore not subjected to high mutation rates [3]. Vertebrates also have developed an additional way of dealing with the heterogeneity of pathogens by the way of the acquired immune system (see 1.1.1).

The physiologic function of the immune system is defense against infectious microbes. However, even non-infectious foreign substances as well as endogenous proteins can elicit immune responses. Furthermore, mechanisms that normally protect individuals from infection and eliminate foreign substances are also capable of causing tissue injury and disease in some situations. Therefore, a more inclusive definition of immunity is a reaction to foreign substances, including microbes, as well as to macromolecules such as proteins and polysaccharides, regardless of the physiologic or pathologic consequence of such a reaction [1].

1.1.1 Innate and adaptive immune response

Two general systems of immunity to infectious agents have been selected during evolution of vertebrates: the early reactions of innate immunity and the later responses of adaptive immunity (Figure 1) [1, 3, 4]. Both types of responses depend on the ability of the body to distinguish between own and foreign. Two categories of pathogens can be distinguished: those that occur primarily inside a host cell (all viruses, some bacteria, and certain protozoan parasites) and those that occur primarily in the extracellular

compartments of the host (most bacteria and other cellular pathogens) [5]. The essential difference between the two systems is the way by which they recognize microorganisms [4]. Innate immunity (also called natural or native immunity) consists of cellular and biochemical defence mechanisms to respond rapidly to infections. These mechanisms react only to microbes and not to non-infectious substances, and they respond in essentially the same way to repeated infections. The principal components of innate immunity are (1) physical and chemical barriers, such as epithelia and antimicrobial substances produced at epithelial surfaces; (2) phagocytic cells (neutrophils, macrophages) and NK (natural killer) cells; (3) blood proteins, including members of the complement system and other mediators of inflammation; and (4) proteins called cytokines that regulate and coordinate many of the activities of the cells of innate immunity. The mechanisms of innate immunity are specific for structures that are common to groups of related microbes and may not distinguish fine differences between foreign substances. Innate immunity provides the early lines of defence against microbes [1].

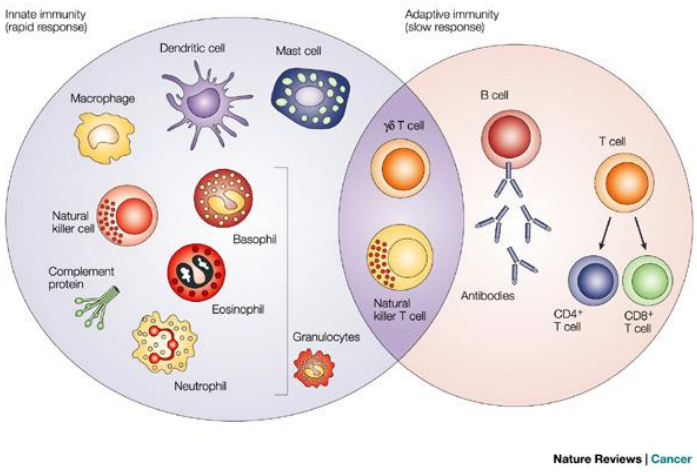


Figure 1 Innate and adaptive immunity. The mechanisms of innate immunity provide the initial defence against infections. Adaptive immune responses develop later and consist of activation of lymphocytes [6].

The adaptive immunity has been intensively studied with artificial antigens, such as haptens and foreign serum proteins, leading to a precise understanding of many of the cells, proteins, and genes involved [2]. The defining characteristics of adaptive immunity are exquisite specificity for distinct molecules and an ability to "remember" and respond more vigorously to repeated exposures to the same microbe. The adaptive immune system is able to recognize and react to a large number of microbial and non-microbial substances. In addition, it has an extraordinary capacity to distinguish among different, even closely related, microbes and molecules, and for this reason it is also

called specific immunity. Sometimes it is also called acquired immunity, to emphasize that potent protective responses are "acquired" by experience [1]. Adaptive immunity is mediated by T and B lymphocytes bearing clonally-distributed antigen receptors [2]. Foreign substances that induce specific immune responses and are the targets of such responses are called antigens [1]. The specificities of these receptors are generated by somatic mechanisms, and therefore are not products of natural selection directed by pathogens. Rather, several random processes contribute to the generation of the specificities of antigen receptors. Consequently, each lymphocyte can have a receptor with unpredicted specificity. In particular, a mature peripheral lymphocyte can have a receptor specific for a self-antigen. Therefore, a signal received through the antigen receptor is not sufficient on its own for the activation of naive lymphocytes. Indeed, it has been well documented that a second so-called co-stimulatory signal is required for lymphocyte stimulation [2].

Innate and adaptive immune responses are components of an integrated system of host defence in which numerous cells and molecules function cooperatively. The mechanisms of innate immunity provide effective defence against infections. However, many pathogenic microbes have evolved to resist innate immunity, and their elimination requires the powerful mechanisms of adaptive immunity. There are two important links between innate immunity and adaptive immunity. First, the innate immune response to microbes stimulates adaptive immune responses and influences the nature of the adaptive responses (Figure 2). Second, adaptive immune responses use many of the effector mechanisms of innate immunity to eliminate microbes, and they often function by enhancing the antimicrobial activities of the defence mechanisms of innate immunity [1]. The innate immune system is believed to have predated the adaptive immune response on several grounds [2].

Innate immunity is phylogenetically the oldest system of host defence, and the adaptive immune system evolved later. In invertebrates, host defence against foreign invaders is mediated largely by the mechanisms of innate immunity, including phagocytes and circulating molecules that resemble the plasma proteins of innate immunity in vertebrates. Adaptive immunity, consisting of lymphocytes and antibodies, first appeared in jawed vertebrates and became increasingly specialized with further evolution.

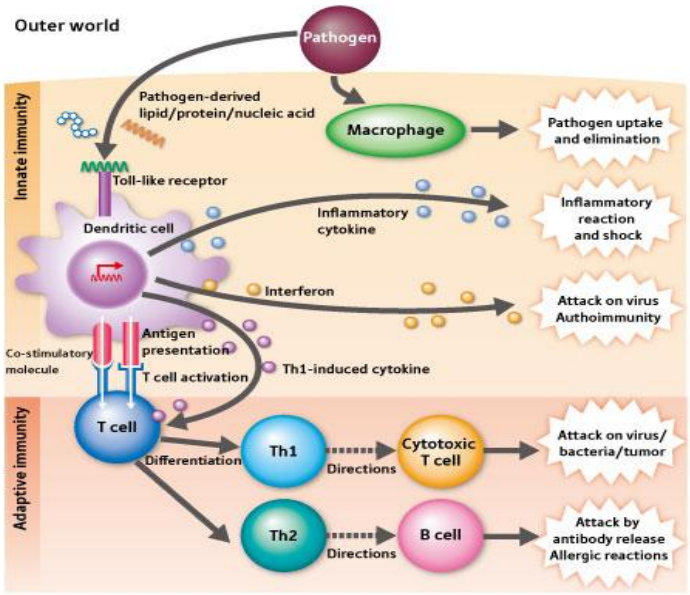


Figure 2 Innate and adaptive immunity. When a pathogen enters the body, mammals initially attempt to eliminate it by innate immunity. If the pathogen has previously infected (not correct, also in case of the first infection you will have an adaptive immune response) the organism, adaptive immunity then operates to specifically exterminate the returning invader [6].

There are two types of adaptive immune responses, called humoral immunity and cell-mediated immunity, which are mediated by different components of the immune system and function to eliminate different types of microbes (Figure 3).

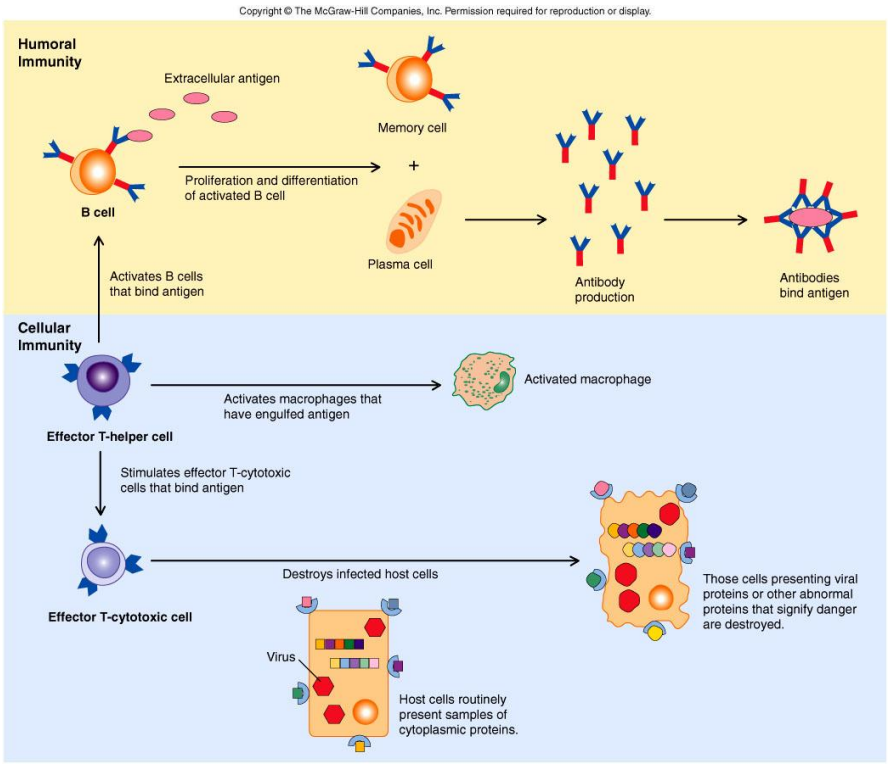


Figure 3 Types of adaptive immunity. In humoral immunity, B lymphocytes secrete antibodies that prevent infections and eliminate extracellular microbes. In cell-mediated immunity, T lymphocytes either activate macrophages to kill phagocytosed microbes or CTLs directly destroy infected cells [7].

Humoral immunity is mediated by the secretion of antibodies by B lymphocytes (also called B cells). Antibodies recognize microbial antigens, neutralize the infectivity of the microbes, and target microbes for elimination by various effector mechanisms. Humoral immunity is the principal defence mechanism against extracellular microbes and their toxins because secreted antibodies can bind to these microbes and toxins and assist in their elimination. Therefore, immunization schemes that trigger pathogen- or toxin-specific antibodies belong to the most successful prophylactic treatments in medical history. Antibodies themselves are specialized, and different types of antibodies may activate different effector mechanisms. For example, some types of antibodies promote phagocytosis, and others trigger the release of inflammatory mediators from leukocytes such as mast cells. Cell-mediated immunity**,** also called cellular immunity, is mediated by T lymphocytes (also called T cells). Intracellular microbes and viruses survive and proliferate inside phagocytes and other host cells, where they are inaccessible to circulating antibodies. Defence against such infections is a function of cell-mediated immunity, which promotes the destruction of microbes residing in phagocytes or the killing of infected cells to eliminate reservoirs of infection [1].

1.2 Phagocytosis

Phagocytosis, the process by which cells internalize particles larger than 0.5 μm in diameter, plays a critical role in innate immunity, both by facilitating the removal and killing of pathogens and by priming the adaptive immune response (Figure 4) [8, 9, 10, 11, 12, 13]. It is an essential physiological function, common to most eukaryotic cell types [14] and involves the activation of complex signalling networks within the host cell [15]. As critical effectors of the innate immune system, macrophages use signaling processes during adhesion to accomplish many essential cellular functions, including phagocytosis [16].

Despite the complexity associated with different phagocytic mechanisms, a number of shared features follow: Particle internalization is initiated by the interaction of specific receptors on the surface of the phagocyte (some capable of direct recognition, the so-called pattern recognition receptors (PRRs), and others that recognize opsonins on the pathogens) with ligands on the surface of the particle [8, 10]. This leads to the polymerization of actin at the site of ingestion, and the internalization

of the particle via an actin-based mechanism. After internalization actin is shed from the phagosome, and the phagosome matures by a series of fusion and fission events with components of the endocytic pathway, culminating in the formation of the mature phagolysosome. Since endosome-lysosome trafficking occurs primarily in association with microtubules, phagosome maturation requires the coordinated interaction of the actin and tubulin based cytoskeleton [10].

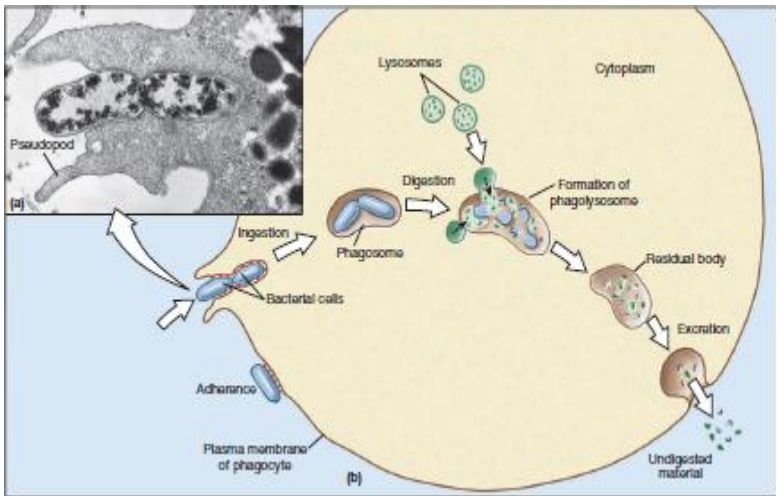


Figure 4 Phagocytosis of bacteria by a neutrophil. a) Extensions of cytoplasm, called pseudopodia, surround the bacteria. b) Steps of the process to destroy the invading bacteria [5].

Numerous phagocytic receptors exist that can bind their target directly or indirectly through opsonins [13, 14]. Of these receptors, the opsonic Fcγ (FcγRs) and complements receptors (CRs) are the best described and exhibit different phagocytic mechanisms and subsequent cellular responses that reflect important differences in their signalling pathways [8, 17, 18, 19]. Ligand-bound receptors classically zipper around the phagocytic prey and induce intracellular signalling cascades that lead to the activation and recruitment of signalling and adaptor molecules at sites of particle binding. These locally assembled signalling complexes reorganize the actin cytoskeleton and regulate membrane dynamics underneath bound particles through the activation of Rho- and Arf-family GTP-binding proteins, respectively. According to the zipper model, phagocytosis of bound particles requires continuing ligation of phagocytic receptors around the whole phagocytic object, at least for spherical particles [10, 14]. The recognition mechanisms leading to phagocytosis occur either cellularly or humorally [10].

Fc receptors (FcRs) mediate phagocytosis of IgG opsonized particles and complement receptors (CRs) recognize the particles opsonized with C3b/C3bi,

components present in serum. Interaction of complement-opsonized particles with CR3 triggers the engulfment of complement opsonized particles and plays important roles in cell adhesion, as well as in phagocytosis [20, 21].

1.2.1 Fc Receptor mediated phagocytosis

Most of our understanding of the signaling pathways leading to phagocytosis in macrophages comes from studies of the FcRs [9]. In mammals, binding of immunoglobulins (Igs) to foreign particles leads to the prompt clearance of these particles from the organism. Igs act as opsonins, molecules that render the particle they coat more susceptible to the engulfment by phagocytic cells. The conserved Fc domain of the Igs is recognized by Fc receptors present on professional phagocytes, such as neutrophils and macrophages, and the opsonized particle is rapidly internalized. This internalization is characterized by a dramatic, actin-dependent extension of the plasma membrane around the particle and is followed by secondary activity, such as the production of reactive oxygen species and the release of inflammatory cytokines from the phagocyte [17]. Leukocyte Fc receptors promote the phagocytosis of opsonized particles and deliver signals that stimulate the microbicidal activities of the leukocytes. Fc receptors for different Ig heavy chain isotypes are expressed on many leukocyte populations and serve diverse functions in immunity. Of these Fc receptors, the ones that are most important for phagocytosis of opsonized particles are receptors for the heavy chains of IgG antibodies, called Fcγ receptors. There are three types of Fcγ receptors, which have different affinities for the heavy chains of different IgG subclasses and are expressed on different cell types [1, 10, 17, 22].

Phagocytosis of IgG-coated particles is mediated by binding of the Fc parts of opsonizing antibodies to Fcγ receptors on phagocytes. Therefore, the IgG subtypes that bind best to these receptors (IgG1 and IgG3) are the most efficient opsonins for promoting phagocytosis. Antibody molecules attached to the surface of a microbe or macromolecular antigen form multivalent arrays and are bound by phagocyte Fc receptors with much higher avidity than free circulating antibodies. Binding of multiple Fc receptors to antibody-coated particles leads to engulfment of the particles and their internalization in phagocytic vesicles. These phagosomes fuse with lysosomes, and the phagocytosed particles are destroyed in the phagolysosomes [1].

Binding of opsonized particles to Fc receptors, particularly FcγRI, also activates phagocytes by virtue of signals transduced by the FcR γ chain. These signals result in the activation of several tyrosine kinases in the phagocytes, which stimulate production of various microbicidal molecules [1, 23].

1.2.2 Complement receptors mediated phagocytosis

Complement-receptor mediated phagocytosis is morphologically distinct from that by FcRs, although both processes require actin polymerization. Complement-opsonised particles „sink" into the phagocyte. There is minimal membrane disturbance, and the internalization usually does not lead to an inflammatory response or oxidative burst [10]. Complement factors, present in serum, opsonize bacteria for phagocytosis by CRs on macrophages. Several receptors that participate in phagocytosis of complement-opsonized particles, including CR1, CR3 and CR4 are expressed on macrophages and neutrophils [10, 14]. CR3 represents a member of leukocyte β2-integrin family and contains multiple binding sites, which are able to interact with a range of ligands [22].

Microbes on which the complement system is activated by the alternative or classical pathway become coated with C3b, iC3b, or C4b and are phagocytosed by the binding of these proteins to specific receptors on macrophages and neutrophils. C3b and C4b (the latter generated by the classical pathway only) bind to CR1, and iC3b binds to CR3 (Mac-1) and CR4. By itself, CR1 is inefficient in inducing the phagocytosis of C3b-coated microbes, but its ability to do so is enhanced if the microbes are coated with IgG antibodies that simultaneously bind to Fcγ receptors. C3b-and iC3b-dependent phagocytosis of microorganisms is a major defense mechanism against infections in innate and adaptive immunity [1].

Integrins on the surface of macrophages are linked to actin filaments by actin-binding proteins such as vinculin and talin which are required for cytoskeletal rearrangement. While FcRs are constitutively active for phagocytosis, the CRs of residental peritoneal macrophages bind but do not internalize particles in the absence of additional stimuli [24, 25]. The activation of integrins is achieved via inside-out signaling, a process whereby the stimulation of cytokine and chemokine receptors activate intracellular signals (Figure 5). Those signals in turn regulate the avidity and/ or affinity of the integrins for the ligand, lateral mobility of integrins in the plasma membrane and subsequent binding of the cytoplasmic tails of integrins to cytoskeletal

proteins. Whilst the molecular details of inside-out signaling and the activation process of integrins are not precisely known, several potential elements in the mechanisms of activation have been identified. Cytokines (e.g. TNF-a and IFN-g) and phorbol esters (e.g. PMA) activate integrins via the activation of protein kinase C (PKC). PMA also induces an increase in phosphorylation of CD18. Examination of the cytoplasmic domains of CR3 and CR4 suggests several candidate sites for phosphorylation by PKC. PKC activation is associated with increased diffusion of the β2 chain of integrins. This suggests that phosphorylation of the β2 chain by PKC may serve as a regulatory mechanism for CR-mediated phagocytosis, and supports a role for β2 integrin phosphorylation by PKC that releases integrins from cytoskeletal constraints. Activation of integrins is known to be associated with a quantitative up-regulation of their functions. Upon activation, an integrin is thought to undergo a conformational change that enhances its ability to bind ligand. Following binding of the activated receptor to a Complement opsonized particle, CRs cluster and re-establish a firm link with the cytoskeleton. The few contact sites between the macrophage and Complement opsonized particle during the phagocytic process are rich in F-actin and cytoskeletal proteins such as vinculin, paxillin and a-actinin. CR-mediated phagocytosis results in the reorganization of polymerized actin at the site of ingestion and internalization of the opsonized particle. The signaling that induces actin polymerization and particle uptake by CR is not precisely known.

CR-mediated phagocytosis activates RhoA, a member of the Rho family of small GTPases (Figure 5). Rho and other GTPases are central in the regulation of the actin cytoskeleton, and in particular, RhoA stimulates contractile processes. Activation of RhoA results in the bundling of actin filaments and clustering of integrins. Rho-mediated signaling also promotes the assembly of stress fibres and focal adhesions, which regulate myosin filament formation and contractility. RhoA activation has been proposed to mediate integrin clustering at the site of ingestion, while regulating contractility of the actin cytoskeleton to allow the opsonized particle to sink inside the macrophage [25, 26].

The similarities in the roles of complement and Fc receptors in phagocytosis and inflammation raise the question of which is the more important effector mechanism of humoral immunity [1]. Also, IgM antibody-mediated protective immunity and pathologic reactions are dependent only on the complement system because IgM is an

efficient activator of the complement system but does not bind to leukocyte Fc receptors [1, 27]. To study complement mediated phagocytosis, there were used IgM coated particles which, therefore, were incubated with serum (which contains the complement components), leading to the recognition of these coated particles by complement receptors [28, 29, 30, 31].

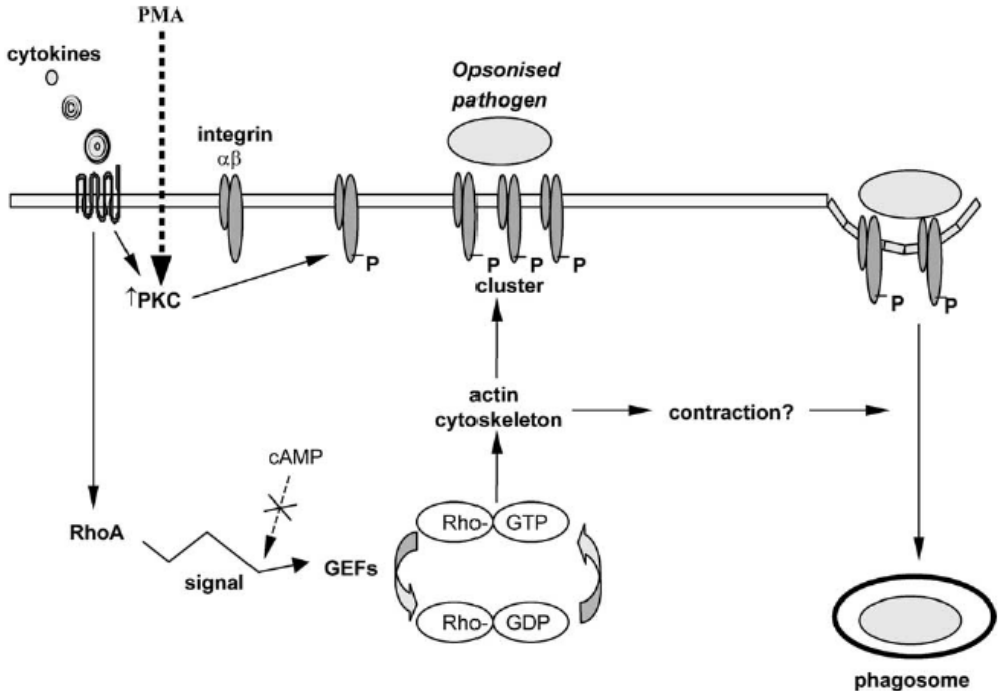


Figure 5 A model for complement-receptor mediated phagocytosis. CRs are inactive at the cell surface. Cytokines released at a site of infection activate macrophages through cytokine receptors. This activates PKC and RhoA. PKC phosphorylates the β chain and activates integrins. RhoA, under the regulation of GEFs, stimulates downstream effectors to promote reorganization of the actin based cytoskeleton. This results in the bundling of actin filaments and the clustering of integrins. It is also possible that RhoA activation stimulates contractility, allowing the opsonized particle to sink into the cytosol of the macrophage [25].

1.3 Tyrosine Kinases

Proteins are the most diverse of all macromolecules, and each cell contains several thousand different proteins, performing a wide variety of functions [32], where each protein has a unique and defined structure that enables it to carry out a particular function [13].

Protein kinases catalyze the transfer of the γ-phosphate group from ATP to the side chains of serine and threonine (protein-serine/threonine kinases) or tyrosine (protein-tyrosine kinases) residues. By contrast, protein phosphatases catalyze the

removal of phosphate groups from the same amino acids by hydrolysis. The combined action of protein kinases and protein phosphatases mediates the reversible phosphorylation of many cellular proteins. Frequently, protein kinases function as components of signal transduction pathways in which one kinase activates a second kinase, which may act on yet another kinase. The sequential action of a series of protein kinases can transmit a signal received at the cell surface to target proteins within the cell, resulting in changes in cell behaviour in response to environmental stimuli [32]. Protein-tyrosine phosphorylation is a mechanism for signal transduction that appeared with the evolution of multicellular organisms. Over 90 different protein-tyrosine kinases are encoded by the human genome. These kinases are involved in the regulation of growth, division, differentiation, survival, attachment to the extracellular matrix and migration of cells. Expression of mutant protein-tyrosine kinases that cannot be regulated and are constitutively active can lead to uncontrolled cell division and development of cancer.

Protein tyrosine kinases can be divided into receptor and non-receptor classes by virtue of whether they possess or lack extracellular ligand-binding and transmembrane domains. On the basis of sequence similarity in the catalytic kinase domain and the presence of common structural motifs, numerous families of non-receptor tyrosine kinases have been defined. Non-receptor tyrosine kinases may be recruited to the plasma membrane, where they mediate cellular signaling by cell surface receptors lacking intrinsic protein tyrosine kinase activities, controlling processes as diverse as the immune response, cells adhesion and neuronal cell migration. For instance, members of the Src family of protein tyrosine kinases are activated in response to stimulation of growth factor receptors, different G-protein-coupled receptors, and many other extracellular stimuli [13, 33].

One member of this group of kinases is the proline-rich tyrosine kinase (Pyk2, also designated RAFTK, CAK and CADTK). Pyk2 has an N-terminus with similarity to band 4.1 homology domain, a centrally located protein tyrosine kinase domain and two proline-rich regions at the C-terminus (Figure 6).

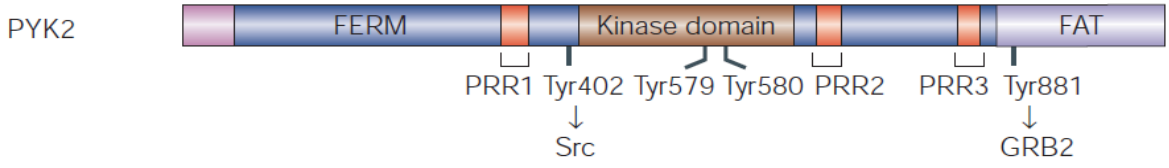


Figure 6 Non-receptor tyrosine kinase Pyk2. Proline-rich tyrosine kinase (Pyk2) has a central kinase domain, two proline-rich-regions (PRRs) and four tyrosine phosphorylation sites whereas the phosphorylation of Tyr402 and Tyr881 create binding sites for Src and growth factor receptor-bound 2 (GRB2), respectively. Pyk2 contains also a C-terminal focal adhesion targeting (FAT) domain that binds to paxillin. However, Pyk2 shows perinuclear distribution and is not strongly localized to focal contacts in many cells [34].

Proline-rich tyrosine kinase 2 is a non-receptor tyrosine kinase that is expressed in cells from neural, epithelial, and hematopoietic origin [35, 36, 37]. Pyk2 does not harbor SH2 or SH3 domains but contains several binding sites for different SH2/SH3-containing signaling proteins. Following activation/autophosphorylation of this enzyme at Tyr402, Pyk2 may bind cytoskeletal proteins (like paxillin), linkers (like $p130^{Cas}$), or other tyrosine kinases (including Src family members), which may potentially impinge Pyk2 in a variety of signal transduction pathways [35]. Also bind to Rho-GAP protein Graf and a LIM domain containing proteins, suggesting a role in regulation of the cytoskeleton and cellular morphology in response to extracellular stimuli [38, 39, 40]. The autophosphorylation site of Pyk2 serves as a docking site for the SH2 domain of Src [33], allowing it to achieve an active conformation. Following activation, both Pyk2 and the Src kinases function in tandem to activate downstream signaling molecules [41]. It has been shown that, together with Src, Pyk2 functions as a link between heterotrimeric G-protein-coupled receptors and the mitogen-activated protein (MAP) kinase signaling pathway [33].

The function of Pyk2 in innate immune cells is not clear. Previous work has shown that Pyk2 is important in macrophage activation. Pyk2-deficient macrophages exhibit impaired adhesion and migration owing to decreased Rho GTPase, and PI3K activation after integrin ligation. Previous studies using Pyk2 inhibitors suggested that this enzyme is critical for superoxide production in human PMNs stimulated with TNF-α. Furthermore, it was found that Pyk2 functions primarily in the integrin mediated signaling pathway and that Pyk2 deficiency results in reduced adhesion-mediated degranulation, which results in impaired host defense [41]. Also, studies with Yersinia (a genus of bacteria in the family Enterobacteriaceae) shown that Pyk2 function in Yersinia uptake by macrophages and, therefore, Pyk2 may be an integral part of the host response to Yersinia infections. Also in the same work, was found that Pyk2 autophosphorylation becomes elevated upon Yersinia infection in $FAK^{-/-}$ cells [42].

Pyk2 is also shown to be a critical intracellular signaling molecule, integrating chemokine and growth factor receptor stimulation with a variety of downstream pathways, including Ras, mitogen-activated protein (MAP) kinase, protein kinase C and inositol phosphate metabolism [36, 43]. Previously, Pyk2 has been linked to migration and adhesion processes as shown by its activation in response to different chemokines and integrins. Pyk2–deficient mice have been generated and analysis shows they develop normally except for exhibiting defective macrophage migration. Similarly, B lymphocytes derived from Pyk2–deficient mice displayed decreased motility (background migration in the absence of stimuli) that was accompanied with decreased responsiveness to a variety of chemokines [43].

Pyk2 can be activated by a variety of extracellular signals that elevate the intracellular calcium concentration. In addition, treatment with phorbol esters or agonists of G-protein-coupled receptors leads to Pyk2 tyrosine phosphorylation. Moreover, Pyk2, like FAK, can be regulated by the activation of integrin receptors. However, Pyk2 is not localized in focal contacts but rather concentrated in the perinuclear region of cells [33].

1.4 Recombinant protein expression in insect cells

The techniques developed in recombinant DNA technology have had an impact on every area of study in immunology. Genes can be cloned, DNA can be sequenced and recombinant protein products can be produced, providing components with which the structure and function of the immune system is studied [44]. These cloned genes can be transfected into cultured cells by several methods, expressing the recombinant proteins and purify them, afterwards [45]. It is known that insect cells, unlike bacteria, are capable of performing many of the processing events that are required for forming biologically active, foreign proteins [46]. Successful culture of insect cells requires a basic familiarity with insect cell physiology and general cell culture methods. The materials and methods for use with insect cell culture have evolved and contributed to the advancement of BEVS technology. The most common cell lines used for BEVS applications are Sf9, Sf-21, from *Spodoptera frugiperda* insect species, Tn-368 and BTI-TN-5B1-4, from *Trichoplusia ni* insect species. Of these, Sf9, a clonal isolate of the *Spodoptera frugiperd*a cell line IPLB-Sf21-AE, is probably the most widely used. Sf9 was originally established from ovarian tissue of the fall armyworm. Although there

is significant scientific data on the characteristics of this Lepidopteran cell line, it remains to be confirmed whether it is the best line for virus or recombinant protein production. Ongoing research suggests that different insect cell lines may support varying levels of expression and differential glycosylation with the same recombinant protein [47].

1.4.1 The Baculovirus Expression Vector System

The Baculovirus Expression Vector System (BEVS) is one of the most powerful and versatile eukaryotic expression systems available. In this system, several Baculovirus genes non-essential in tissue culture life cycle (polyhedrin, p10, basic) may be replaced by heterologous genes [46, 48, 49, 50].

Baculoviruses (family *Baculoviridae*) belong to a diverse group of large double stranded DNA viruses that infect many different species of insects as their natural hosts. Baculovirus strains are highly species-specific and are not known to propagate in any non-invertebrate host. The Baculovirus genome is replicated and transcribed in the nuclei of infected host cells where the large Baculovirus DNA (between 80 kb and 200 kb) is packaged into rod-shaped nucleocapsids. Since the size of these nucleocapsids is flexible, recombinant Baculovirus particles can accommodate large amounts of foreign DNA. *Autographa californica* nuclear polyhedrosis virus (AcNPV) is the most extensively studied Baculovirus strain. Its entire genome has been mapped and fully sequenced. Infectious AcNPV particles enter susceptible insect cells by facilitated endocytosis or fusion, and viral DNA is uncoated in the nucleus [46].

Since the Baculovirus genome is generally too large to easily insert foreign genes, heterologous genes are cloned into transfer vectors. Co-transfection of the transfer vector and AcNPV DNA into *Spodoptera frugiperda* (*Sf*) cells, after growing the cells from mid- to late-exponential growth phase, allows recombination between homologous sites, transferring the heterologous gene from the transfer vector to the AcNPV DNA [48, 51] (Figure 7). AcNPV infection of *Sf* cells results in the shut-off of host gene expression allowing a high rate of recombinant mRNA and protein production. Recombinant virus induces recombinant target-protein expression and also infects additional insect cells thereby resulting in additional recombinant virus [46, 51]. The recombinant Baculovirus system for expression of heterologous proteins in insect cells was developed in the laboratories of Max Summers and Lois Miller. Development

of this expression system was based on observations of the wild-type baculovirus life cycle [51].

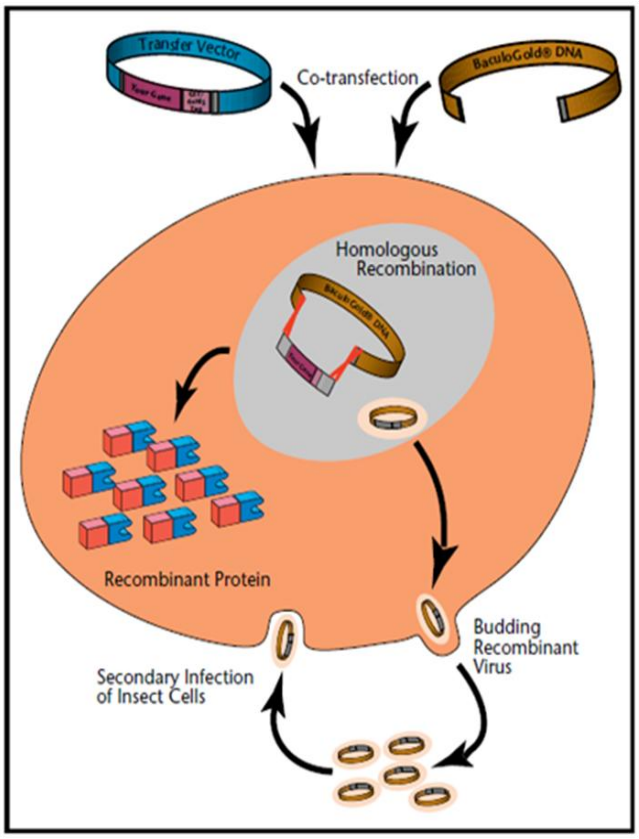


Figure 7 Generation of recombinant proteins with Baculovirus Expression Vector System. The cDNA to be expressed is cloned into a transfer vector (pVL1392) under the control of the polyhedrin promoter. This vector is co-transfected with linearized baculovirus DNA into the host insect cells. Through homologous recombination, the cDNA of interest replaces the polyhedrin cDNA [51].

Choosing the right system for foreign gene expression can be particularly important in obtaining biologically active recombinant protein [46, 48]. Several unique features of the BEVS have made it the system of choice for many applications. Some advantages of using this system are: the use of insect cell, functional activity of the recombinant protein, high expression levels, capacity of large inserts, ease of purification, between others [46, 52]. The MOI (multiplicity of infection) and the time of infection are two parameters that are easily manipulated and that may be important in optimizing heterologous protein yields [50].

1.5 Aims of the project

Several studies have shown that Pyk2 may bind cytoskeletal proteins (like paxillin), linker proteins (like $p130^{Cas}$), or other tyrosine kinases (including Src family members), which may potentially impinge Pyk2 in a variety of signal transduction pathways. Previous work has shown that Pyk2 is important in macrophage activation,

but the overall function of this protein in innate immune cells is still unclear. Pyk2 was also shown to be a critical intracellular signaling molecule, integrating chemokine and growth factor receptor stimulation with a variety of downstream pathways, including Ras, mitogen-activated protein (MAP) kinase, protein kinase C and inositol phosphate metabolism. As this protein is expressed in macrophages, and can be activated during phagocytosis, in the present project the aim was to know if this protein is recruited during complement receptor-mediated phagocytosis. First of all, the conditions should be improved, to further have a reliable Pyk2 staining and an efficient uptake of complement-opsonized particles. Pyk2 staining in macrophages, after activation of complement-mediated phagocytosis can show afterwards, if this protein is recruited or not.

In between, another project took place. The major aim was to express recombinant proteins with a Baculovirus expression vector system in insect cells Sf9. This way, active proteins could be produced, which can be further used in *in vitro* as well as *in vivo* studies. The TAT sequence was already shown to induce the uptake of proteins *in vivo* and FRNK (FAK-related non-kinase), can be used for studies of FAK, which is implicated in numerous cellular processes, including cell migration, differentiation, survival and proliferation. Thus, a recombinant protein bearing the TAT sequence, GST and FRNK wanted to be produced, to further investigate if the TAT sequence allows the uptake of FRNK and, if it this happens, this recombinant protein could be used for studies *in vivo*. Additionally, the kinase domain of Hck should be expressed as a GST-fusion protein in Sf9 and the kinase activity of the purified protein should be verified using an *in vitro* kinase assay.

2. Results

In the present work is separated in two different projects. Firstly, the analysis of the recruitment of Pyk2 in macrophages during complement receptor-mediated phagocytosis is presented. Secondly, the recombinant expression of different proteins in Sf9 insect using the baculovirus expression vector system is shown. The proteins were afterwards purified for further use in *in vitro* and *in vivo* assays.

2.1 Analysis of the recruitment of Pyk2 in macrophages after establishing IgG- versus complement-mediated opsono-phagocytosis assay

To analyze the recruitment of Pyk2 in during complement receptor-mediated phagocytosis, first of all the conditions had to be optimized to have a reliable Pyk2 staining and an efficient uptake of complement-opsonized particles. As macrophages are hard to transfect, HEK 293T cells were used. HEK 293T cells were transfected with Pyk2-HA, Pyk2-myc or the empty vector as a negative control. A western blot analysis of the transfected cells was made and it shows positive transfection for the two constructs (Figure 8). After staining, the cells were analyzed at the microscope. The results shows that the transfection worked, and as the cells had the same sites stained can be concluded that the Pyk2 antibody is specific for the protein (Figure 9).

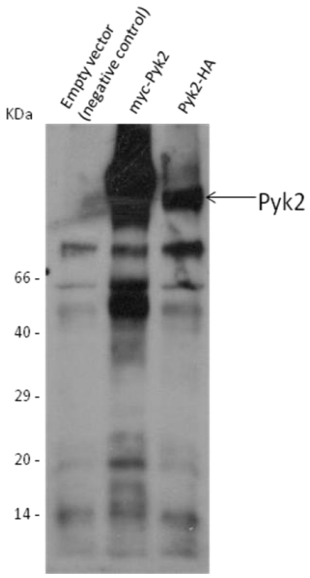


Figure 8 Transfection of 293T cells with Pyk2 constructs. 293T cells were transfected with the indicated constructs and were lysed 48 h later. Expression of the constructs was analyzed by western blotting using a Pyk2 AB 1:400 and an appropriate secondary antibody. Normally the size of the constructs should be nearly the same, as the single HA-tag compared to single myc-tag are similar in size but some difference is detected. This can be explained by the 6xmyc tag at the N-terminus of Pyk2 compared to the 2xHA tag of the HA-Pyk2 construct.

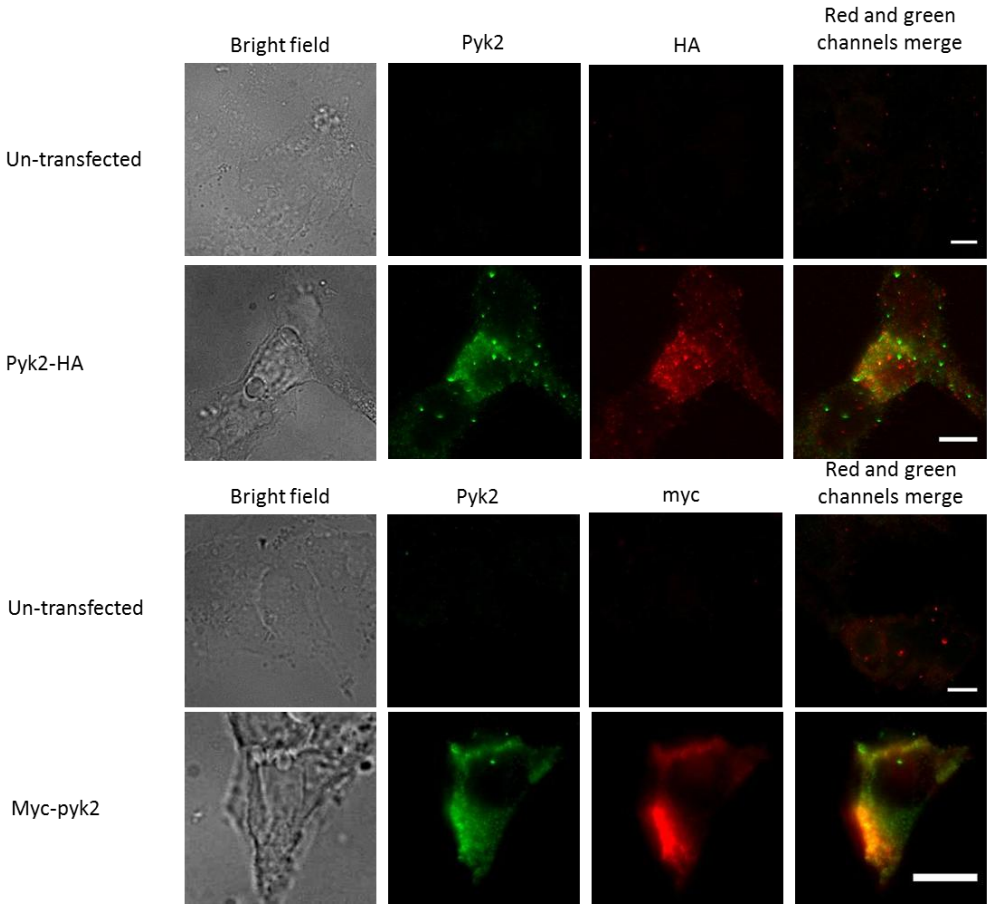


Figure 9 Analysis of Pyk2 expression by transfected cells using immunofluorescence. 293T cells were stained after fixation with 4% PFA and permeabilization with 10% FCS and 0.2% Saponin in PBS^{++}. The Pyk2, myc and HA AB were diluted 1:200 in blocking solution. The secondary antibodies used were a goat-anti-mouse Cy3 antibody for HA and myc and a goat-anti-mouse Cy2 antibody for Pyk2. Thus, the Pyk2 can be distinguished from the HA and myc as the Cy3 fluorescence is detected by a TRITC channel and the Cy2 fluorescence is green detected by a FITC channel. Scale bar 10 μm.

After this assay, the best procedure to fix and permeabilize the cells was determined. During this assay, the cells were stained for Pyk2 (1:50 in blocking solution) and four approaches were made:

1. 4% PFA 15 min at RT to fix the cells and ice-cold Acetone 10 min at -20ºC to permeabilize.
2. 4% PFA 15 min at RT to fix the cells and 0.4% Triton X-100 in PBS^{++} 3 min at RT to permeabilize.
3. 4% PFA 15 min at RT to fix the cells and 0.4% Saponin 10 min at RT to permeabilize.

4. MeOH ice-cold 5 min at -20ºC to fix the cells and 0.4% Triton X-100 in PBS $^{++}$ 3 min at RT to permeabilize.

After staining, the cells were analyzed using a fluorescence microscope (Figure 10). For all samples the same exposure times were used to allow comparison of the staining. It can be seen that the best way to fix and permeabilize the cells was the second approach, as with the same conditions for all approaches, this one has the highest signal in the green channel. The staining procedure is described in the methods section.

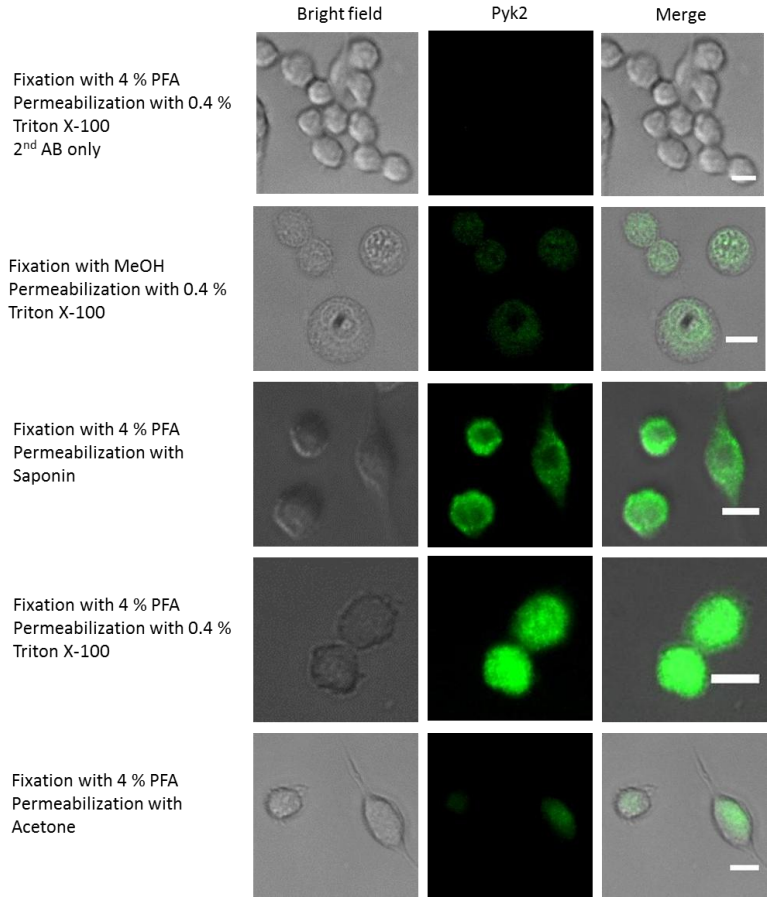


Figure 10 Analysis of fixation and permeabilization parameters for Pyk2 immunostaining. Raw macrophages were seeded on Poly-L Lysin/Fibronectin, get fixed and permeabilized as indicated and stained for Pyk2 using appropriate antibodies. Scale bar 10 µm.

It is described in the literature that macrophages need to be pre-activated before infection, and only this way bound particles will be phagocytosed by the complement receptors [14, 19]. To pre-activate the macrophages normally PMA or LPS are used [53]. Beside this, it was unclear which coating procedure assists maximal stimulation of the cells. In thus, Raw macrophages were seeded on fibronectin (1 µg/mL) or 0.1% of gelatine and maintained 2 hours at 37ºC. After this time, the cells were washed once with DMEM with 0.5% h.i. FCS and stored in this medium at 37ºC. The next day, the medium was supplemented with 150 ng/mL of PMA or 300 ng/mL of PMA in different wells and with 1 mM HEPES. The range of PMA concentration was as in the literature

[6, 11, 19, 53]. The cells were then incubated for 15 min at 37ºC and then analyzed at the microscope, taking pictures every 5 minutes for one hour. The movement of the cells and the spreading was analyzed by ImageJ (data not shown). It was seen that the cells spread better on Fibronectin, however, on Gelatine the cells seems to be more activated as they displayed increased migration. The cells not pre-activated showed only marginal movement. Therefore, to analyze the recruitment of Pyk2 in the cells during phagocytosis it was chosen to use Gelatine for coating and 200 ng/mL of PMA to pre-activate the cells, before infection.

It is well known that Actin plays an important role during phagocytosis [17, 54, 55] and there are made recent advances in this field, mostly related to FcR and CR3 [55]. Thus, the cells were infected with coupled beads and filamentous actin was stained to see if these beads were in fact phagocytosed by the cells (Figure 11). The beads were coupled with Albumin and GST, negative controls for phagocytosis as they don´t bind to receptors present in macrophages, IgG that, as described before, are internalized by the FcRs which allows have a positive control for phagocytosis, IgM that is showed to be internalized by the CRs after incubation with serum that contains the complement components, and iC3b that is a complement component and the macrophages has a iC3b receptor which recognize opsonized particles with this component. It was seen in all samples (except for Albumin as expected), that Actin was recruited to sites of infection. These results indicate that the beads are in fact being phagocytosed by the macrophages, which allows us to proceed to the analysis of Pyk2.

The Pyk2 staining did not showed any clear recruitment of this protein during phagocytosis (Figure 11). The analysis of Pyk2 continued with all conditions described before, and after three unsuccessful attempts was made an assay to see if the beads were in fact internalized by the cells. To this, the coupled beads were stained before infection with CFSE 1:1000 in PBS and after infection with antibodies against the coupled proteins to distinguish between intracellular beads from adhered, extracellular beads (Figure 12) (This procedure is described in the methods section).

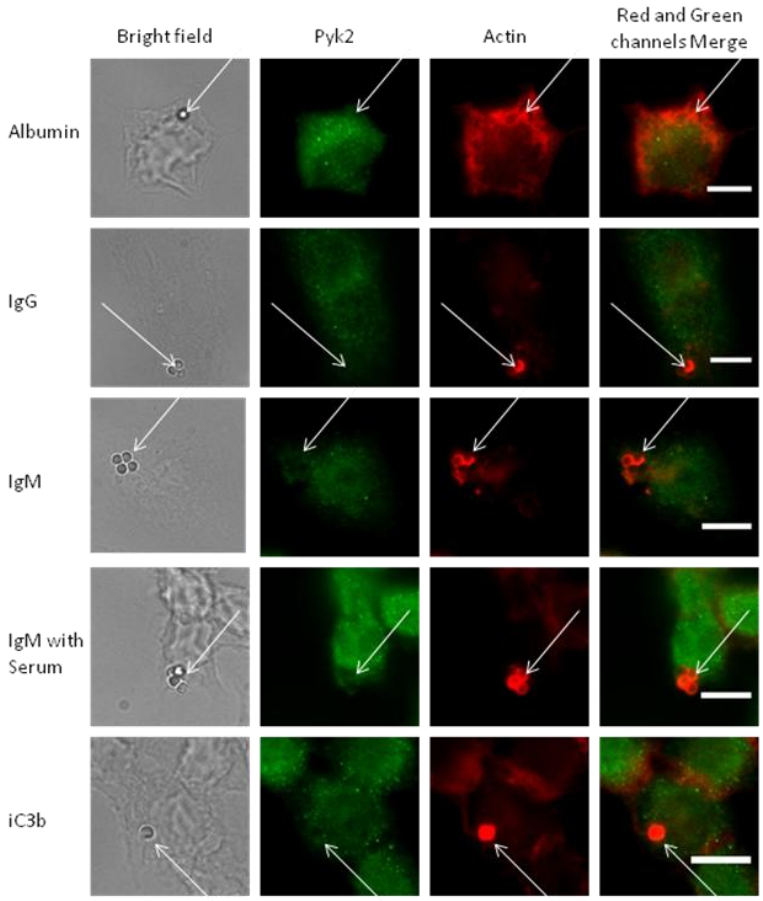


Figure 11 Actin recruitment during phagocytosis. Raw macrophages were incubated for 20 min with the indicated beads and fixed. Samples were stained for actin and Pyk2 staining. The intensity, exposure time and signal amplification in both channels were adjusted so that in the sample without staining there was no signal. Scale bars 10 μm.

The IgM beads were opsonized with complement components by incubating with mice serum at 37ºC for 1 h. The quantification of the first intra- and extracellular assay revealed the internalization of IgG and IgM with serum beads, as well as the IgM beads alone (Figure 13). The IgG beads are internalized by the Fc receptors [1, 10, 11, 31, 56] and the IgM with serum beads should be internalized by the CRs. But as we can see an IgM beads internalization it was not sure that the CRs are active for IgM with serum beads.

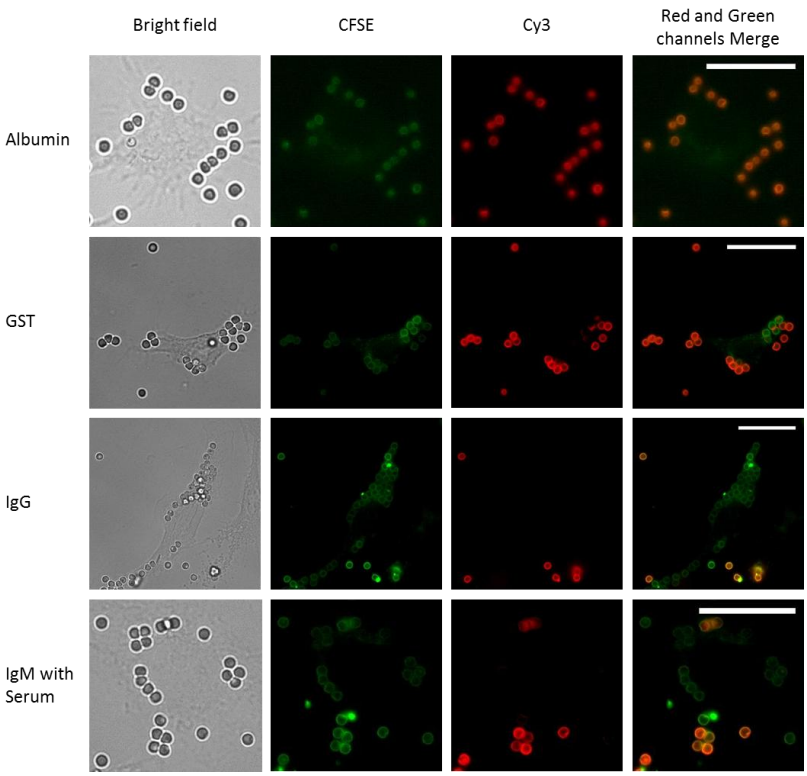
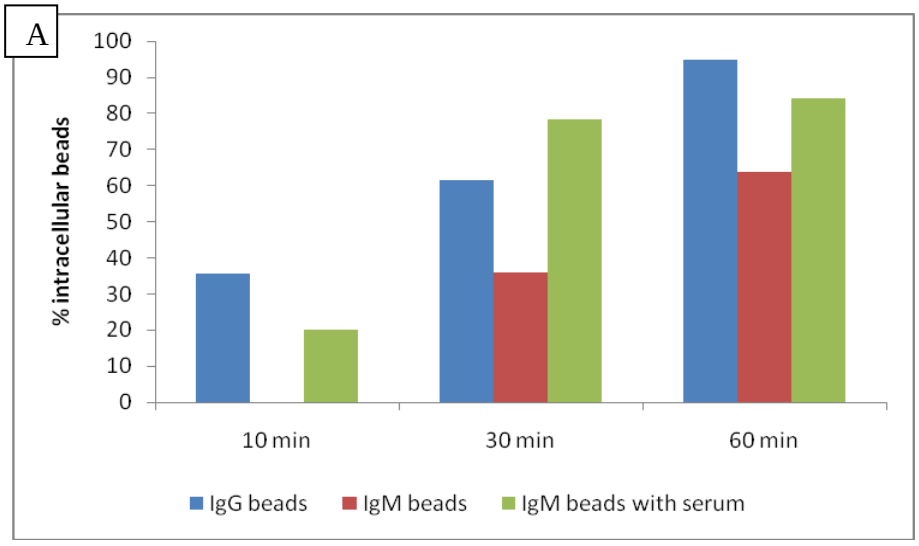


Figure 12 Internalization of coated latex beads by mouse macrophages. All the beads were stained with CFSE before infection and after infection and fixation the beads were stained again, and as there was no permeabilization only the extracellular beads should be stained. A streptavidin-Cy3 or Cy3 antibody was used, depending if the beads were biotinylated or not during CFSE staining (only the beads without antibody against the protein coupled were biotinylated, as the Albumin beads). The intra- and extracellular beads were distinguished afterwards. The beads in the cells were counted after analysis at the microscope, where the CFSE stained beads were detected with a FITC channel and the Cy3 stained beads with a TRITC channel. In this case the time of infection was 60 min. Scale bar 20 µm.



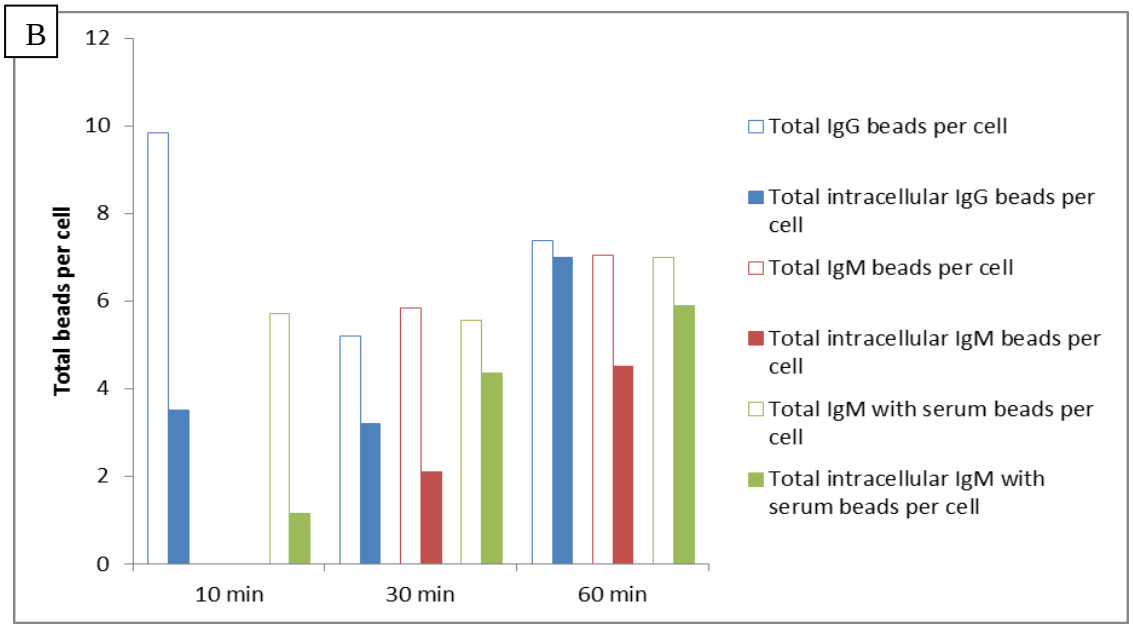
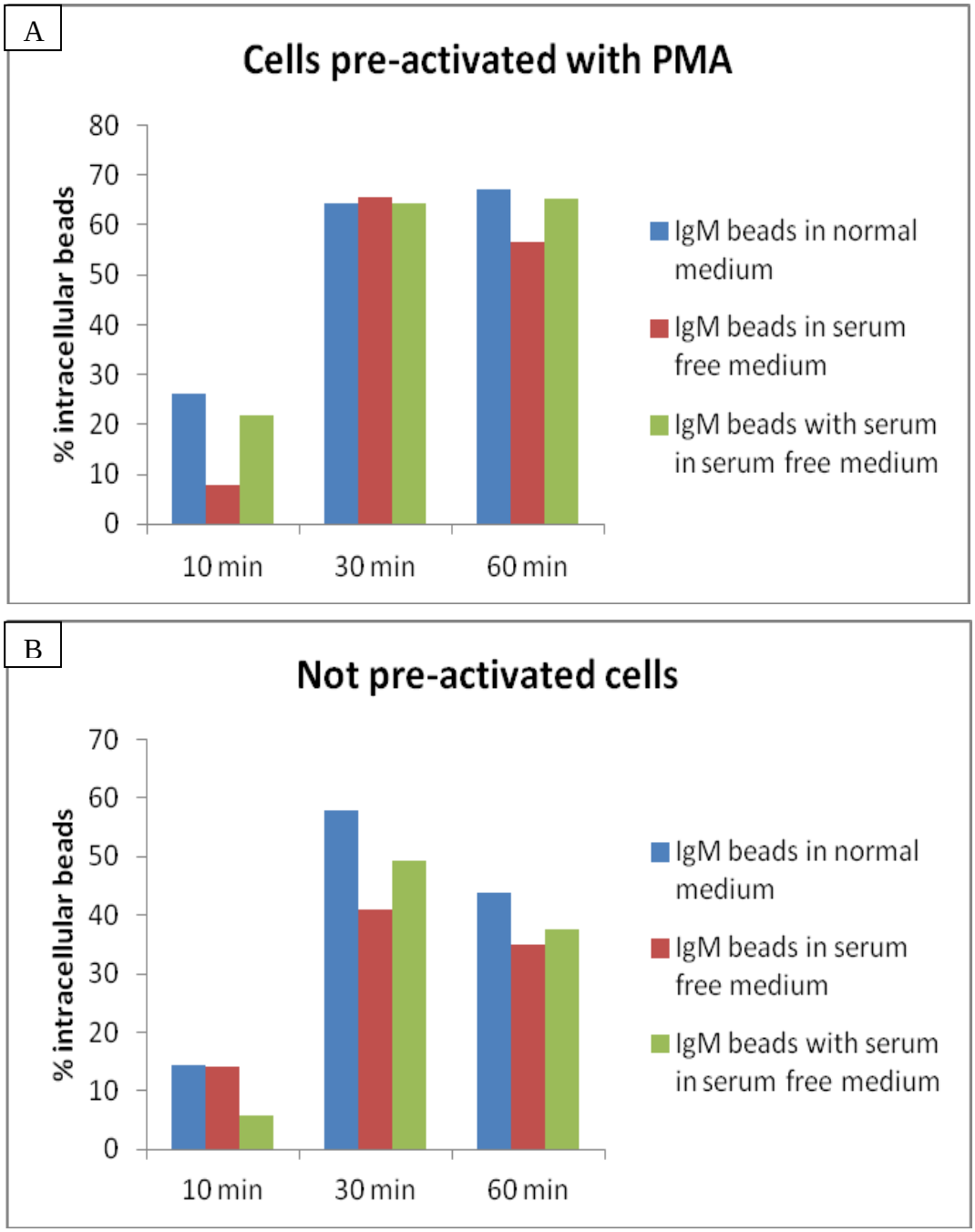


Figure 13 Internalization of coated latex beads by mouse macrophages. The cells were infected with MOI 40 at 37°C. After the indicated infection time the reaction was stopped with ice-cold PBS and washed with PBS. The cells were then fixed with PFA and the beads were stained (after blocking), as described before (Figure 12). Two different views of the results are presented where in A one can see the % of intracellular beads in each sample and in B the total and intracellular beads per cell in each sample.

The serum components present in IgM beads will activate the complement system and those beads should be recognized by the complement receptors and phagocytosed. The IgM beads only should not be internalized as they can not activate the CR, neither the FcR, but in Figure 13A a significant internalization of these beads can be seen. An hypothesis to this internalization was the FCS used in the medium. Thus, another assay was made, now with serum free medium (DMEM with 0.5% BSA). The cells were seeded the day before infection with normal medium and before infection the medium was changed. The serum incubation with IgM beads and the staining of the beads was done as before. To analyze whether the PMA really assisted the internalization of the particles, approaches with and without pre-activation with PMA were made. It was observed that PMA activation appears to induce the internalization of more beads in the IgM samples, so it was always used in the next experiments. Between the samples with IgM beads in normal and serum free medium, there was not a big difference. After 10 min there is more internalization with normal medium but after longer time points the internalization appears not to change

significantly (Figure 14). Thus, another hypothesis was that the IgM particles can be internalized by the Fc receptors or another pathway.



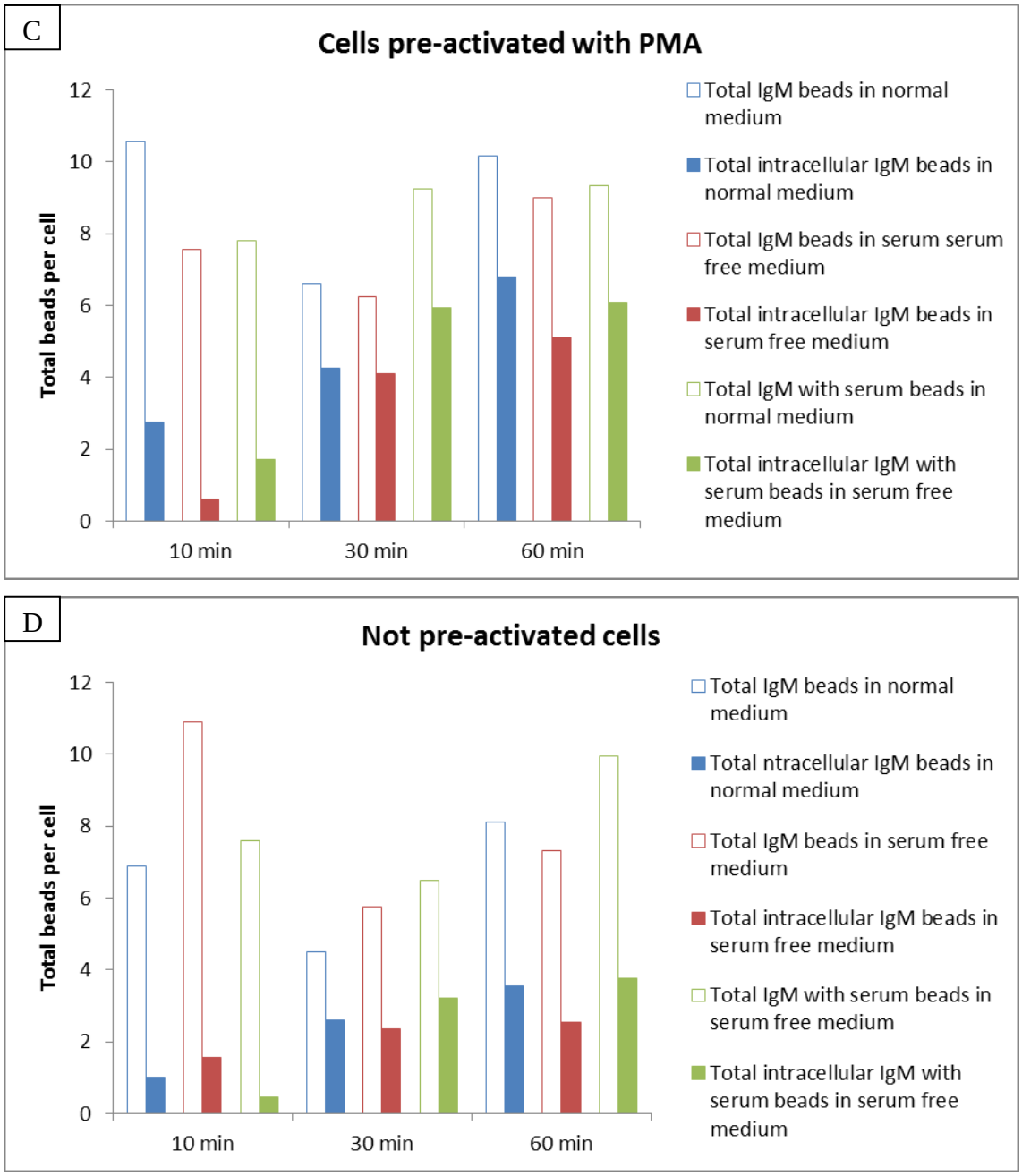


Figure 14 Intra- and extracellular assay with serum free medium. The cells were pre-activated or not with PMA and infected with MOI 40 at 37ºC. After the indicated infection time, the reaction was stopped with ice-cold PBS and washed with PBS. The staining procedure and the analysis by microscopy were as before (figure 12). As before, two different views of the results are presented. In A and B are the % intracellular beads with and without PMA, respectively, and in C and D the total and intracellular beads per cell with and without PMA, respectively.

To investigate whether the uptake of IgM with serum beads is in deed based on CR mediated internalization a CD11b antibody against CRs was used to block them. It was shown previously that the CD11b antibody M1/70.15 can block the complement

receptors in macrophages using 10 µg/mL (10 min before infection), at room temperature [57, 58]. These conditions were used and the CD11b antibody was added during PMA activation, before infection.

In the first assay using the blocking procedure (data not shown) the IgG beads were blocked by the antibody. As the antibody was eluted in PBS with sodium azide, perhaps the inhibitory effect was due to the sodium azide, as this antimicrobial agent is toxic to most organisms as well it blocks the cytochrome electron transport system [59]. Therefore, the antibody was purified with a column, washing three times with PBS only, 10-15 min each time at 14000 rpm. After this purification, the concentration of the antibody was determined by a spectrophotometer (NanoDrop) and a new assay was made. To have a negative control for the blocking with CD11b antibody, an isotype-matched antibody was used (Rat IgG2b). These antibodies were also used to couple beads to have a better positive and negative control for the internalization, as the CD11b beads are only internalized by the CRs. To be sure that the Fc domains present in the antibodies were not exposed to be recognized by Fc receptors during infection, the beads were first coupled with Protein G that is shown to have great affinity to the Fc domains of IgG subclass 2b [60].

The IgG beads were not blocked as expected. Surprisingly, also the uptake of IgM only beads was not blocked. The IgM with serum beads are now blocked by the CD11b antibody but not by the Rat IgG2b (isotype from the CD11b). This shows that the IgM with serum beads uptake is really mediated by the complement receptors. The CD11b beads are internalized as the IgM with serum beads, which demonstrate that the complement receptors are active in the cells, and the RatIgG2b beads are a better negative control as they are less internalized than Albumin and GST beads (Figure 15).

After proving the complement receptor mediated internalization of the IgM with serum beads, the recruitment of Pyk2 to sites of infection was investigated again. Now, the tissue plates were centrifuged with the beads after infection for 3 min at 500xg, as in the intra- and extracellular assay, and the serum starvation was used. As expected no recruitment was found during the phagocytosis of Albumin, GST and Rat IgG2b beads. For IgG beads there was seen a recruitment in some cases. An approach was made blocking the cells with CD11b antibody before infection with IgM with serum beads and there was no recruitment of Pyk2, supporting that the recruitment of Pyk2 during the phagocytosis of these beads is due to the phagocytosis by the complement receptors.

The IgM with serum and CD11b beads show a strong recruitment of Pyk2 during phagocytosis (Figure 16).

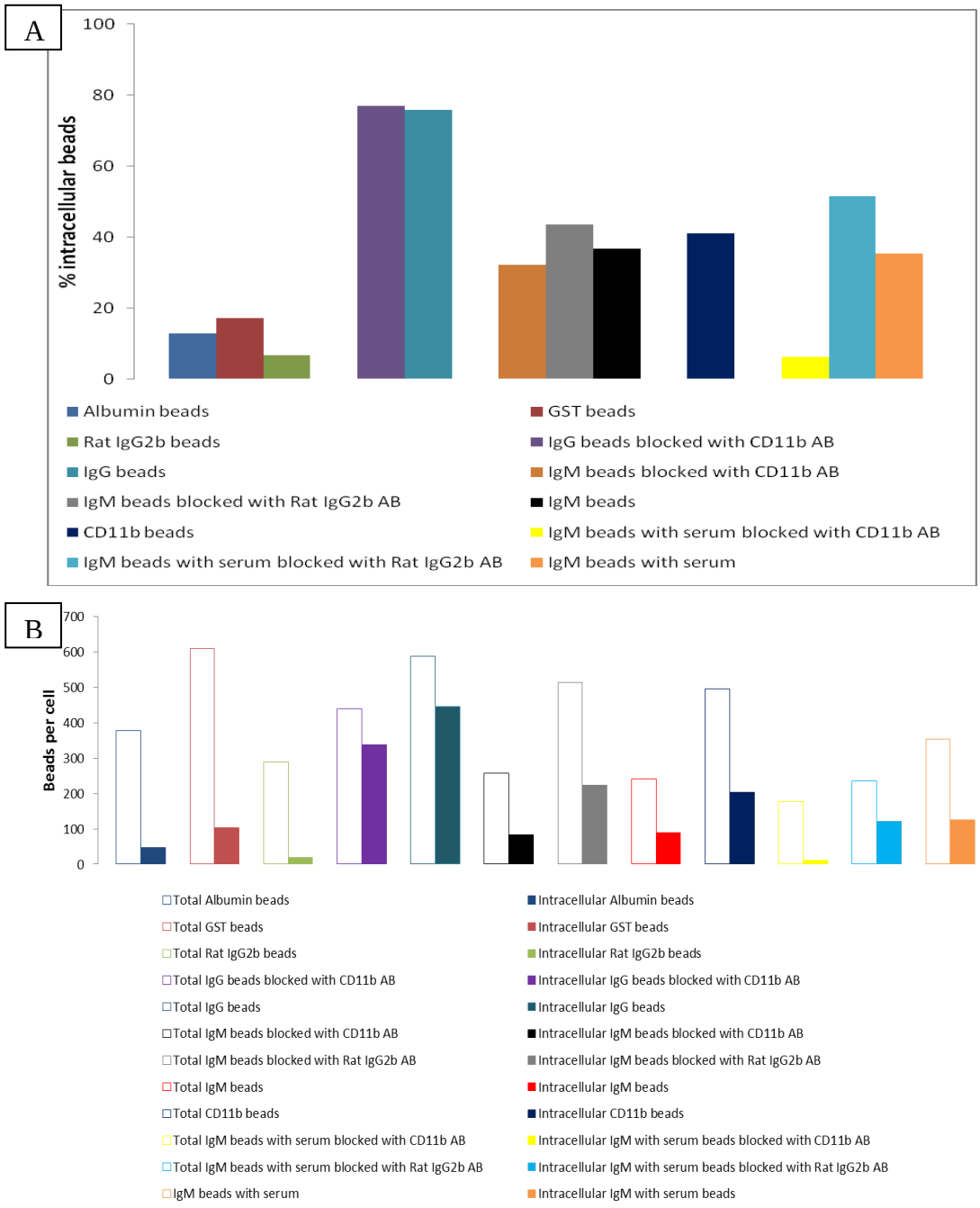


Figure 15 Intra and extracellular assay with CD11b blocking. The cells were seeded the previous day and the medium was changed to DMEM + 0.5% h.i. FCS. After the time of infection the cells were washed, fixed and the beads were stained as before. These results were obtained after 60 min of infection using MOI 40. In A is represented the % intracellular beads and in B the total and intracellular beads per cell.

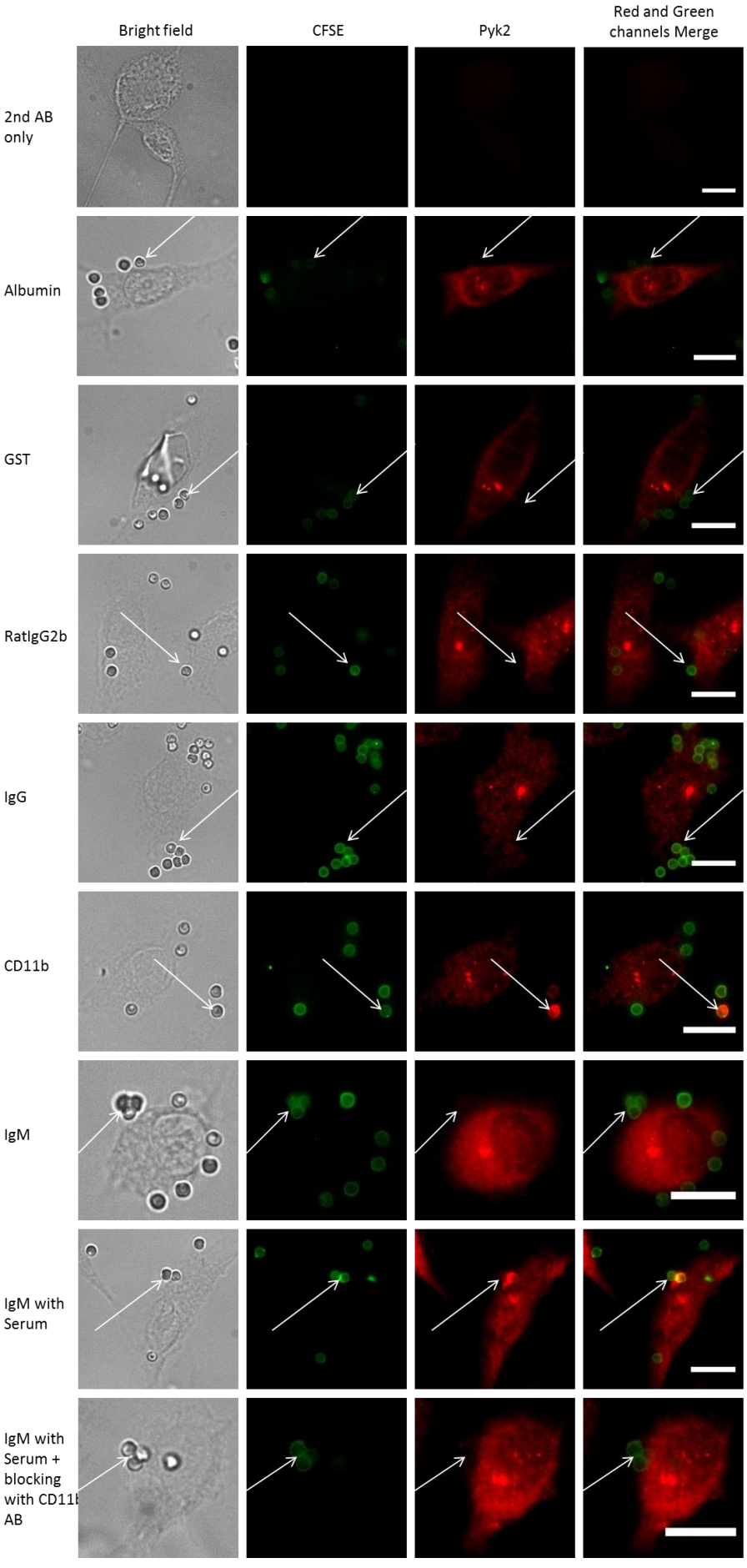

Bright field
CFSE
Pyk2
Red and Green channels Merge
2nd AB only
Albumin
GST
RatIgG2b
IgG
CD11b
IgM
IgM with Serum
IgM with Serum + blocking with CD11b AB

Figure 16 Recruitment of Pyk2 during CD11b or IgG-mediated phagocytosis. The cells were infected with MOI 15, centrifuged and after 30 min the reaction was stopped with ice-cold PBS. The cells were fixed, permeabilized, blocked, and stained for Pyk2 (1:50). It was used a Cy2 antibody to visualize the beads in the FITC channel and the pictures of Pyk2 recruitment were taken to have no signal in the non-stained cells. Scale bar 10 μm.

In the beginning of the work iC3b coated beads were used, but there was no clear recruitment, as for the other beads, and in the intra- and extracellular assay there was no internalization of the beads (data not shown). As now the conditions of the assays are stable, and we see clear recruitment, another approach with these beads was made. As before, no recruitment was found during the phagocytosis of Albumin and GST beads as expected. For IgG beads there was seen a clear recruitment. The IgM with serum beads have a clear and strong signal, as well as the iC3b beads (Figure 17).

To analyze the internalization of the iC3b beads, a new intra- and extracellular assay was made. Was seen that after 30 min of infection, the internalization of iC3b much intense than with the negative control beads (Figure 18). Also, after blocking the CRs with the CD11b antibody before cited, the internalization of these beads, as the IgM with serum beads, used as positive control, decreased significantly. Thus, it was clear that these beads also were internalized by the CRs as expected.

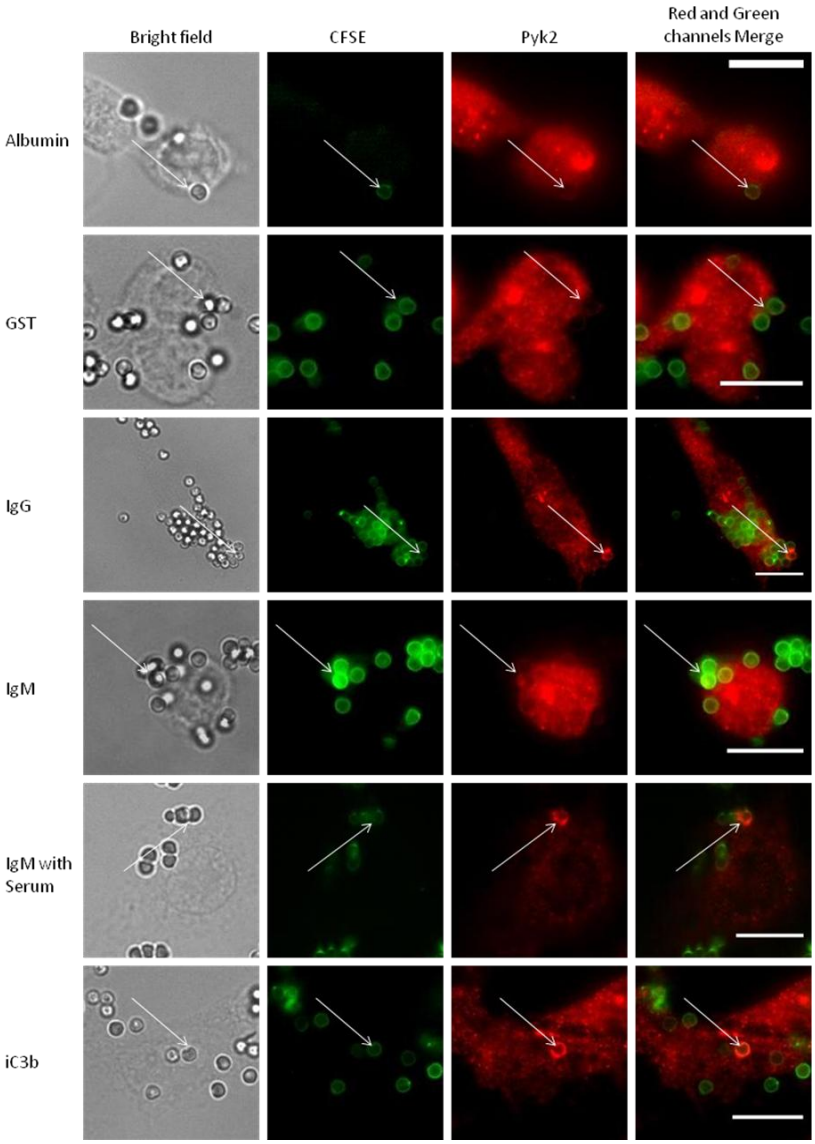


Figure 17 Recruitment of Pyk2 after infection with iC3b beads. The cells were infected with MOI 15, centrifuged and after 30 min the reaction was stopped with ice-cold PBS. The cells were fixed, permeabilized, blocked, and stained for Pyk2 (1:50). A Cy2 antibody was used to visualize the beads in the FITC channel and the pictures of Pyk2 recruitment were taken to have no signal in the non-stained cells. Scale bar 10 μm.

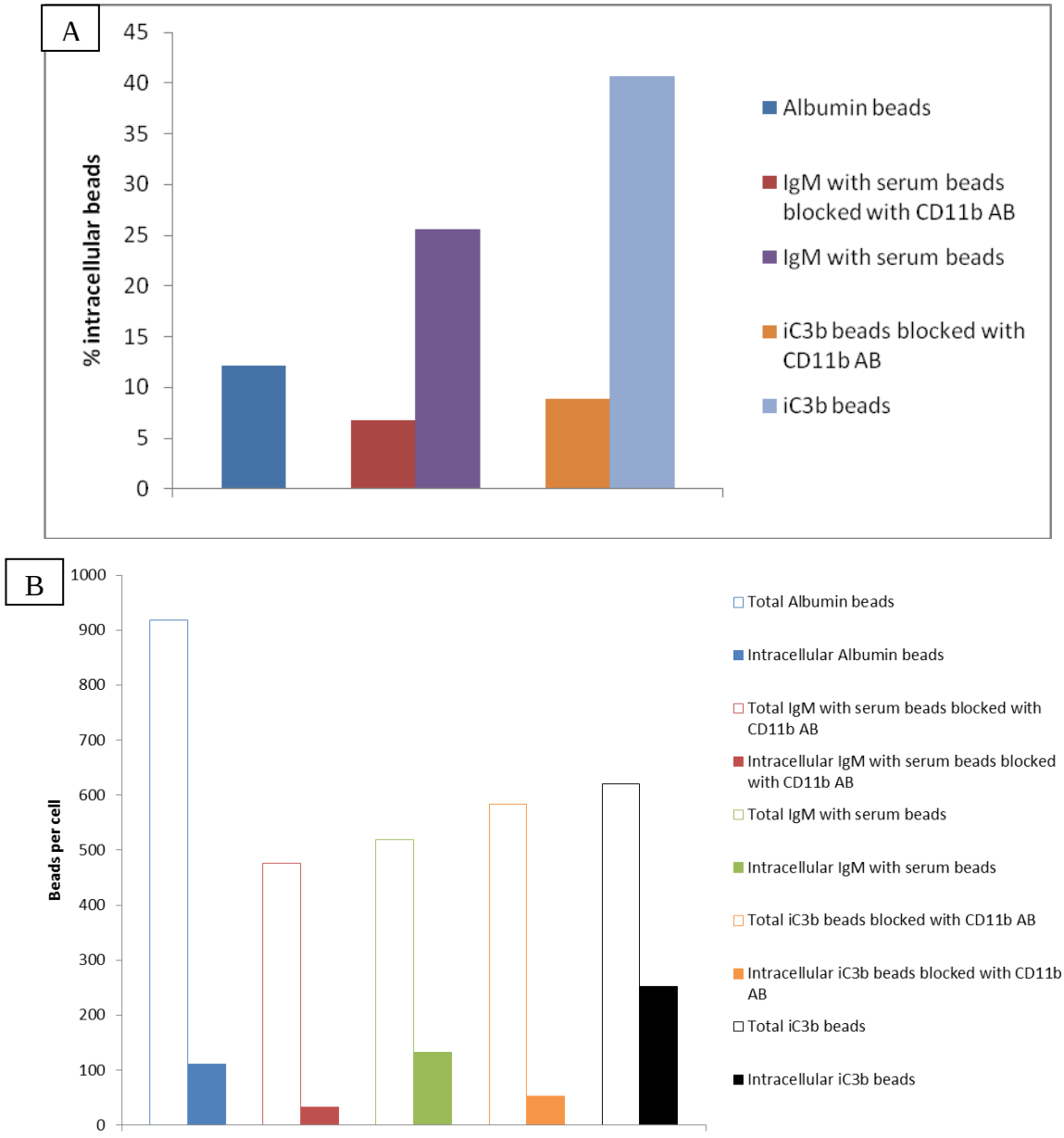


Figure 18 Intra- and extracellular assay with iC3b beads. The cells were seeded the previous day and the medium was changed to DMEM + 0.5% h.i. FCS. After the time of infection the cells were washed, fixed and the beads were stained as before. These results were obtained after 30 min of infection using MOI 40. In A is represented the % intracellular beads and in B the total and intracellular beads per cell.

Taken together the results reveal that Pyk2 is recruited during complement-mediated phagocytosis. Consequently, the next question is which functional role Pyk2 plays in this process. As Pyk2 needs to be phosphorylated to be active, the phosphorylation of Pyk2 in macrophages after infection with coupled beads was determined. It was seen, after infection with IgG and IgM with serum beads, the

increase of Pyk2 phosphorylation in the samples without PMA activation (Figure 19). The cells were seeded and infected as before, and after the time of infection they were lysed with Ripa buffer, and the lysates were analyzed by Western blot. Pyk2 phosphorylation was seen with the α-PTyr AB at around 120 KDa.

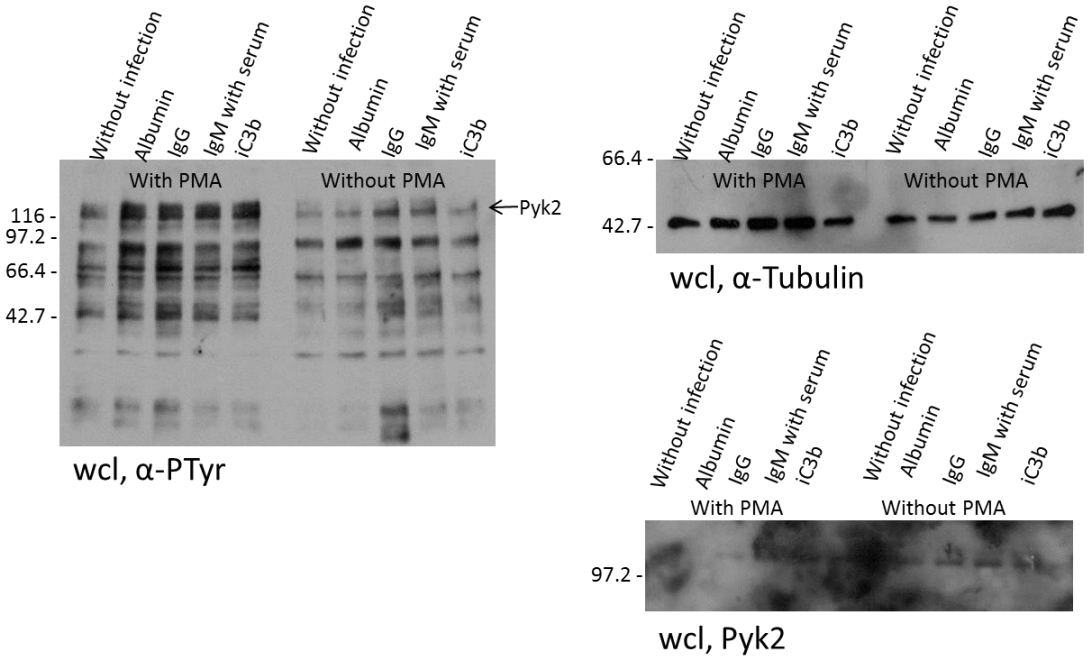


Figure 19 Test the phosphorylation of Pyk2. The cells were seeded as before, the medium was changed in the day before and the cells were infected for 30 min at 37ºC after PMA activation or not and centrifugation. Three antibodies were used to analyze the phosphorylation of the proteins in the samples (α-PTyr 1:1000), the amount of cell lysate added to the gel (α-Tubulin 1:1000) and the Pyk2 (1:400).

2.2 Recombinant expression in Sf9 insect cells

With the help of Baculovirus expression system, four distinct proteins were expressed in insect Sf9 cells and then purified. First, the GST-Hck-KD and GST-mFRNK-TAT constructs needed to be cloned by modifying the commercial vector with a GST-encoding sequence before combining that with the other sequences. In the case of Pyk2-KD and FAK-KD, the cDNAs had already been inserted in the transfer vector pVL1392.

2.2.1 Cloning of the cDNA into a Baculovirus transfer vector

For expression of GST-Hck-KD and GST-mFRNK-TAT, first the cDNA of GST was cloned into the transfer vector pVL1392. For amplification of the cDNA by PCR, two primers were used at which the 5'-primer had a BglII restriction site and the 3'- primer a NotI restriction site. A hot start PCR was carried out with these primers and the template plasmid which contains the GST cDNA. After the PCR, products were separated using a 1.4 % agarose gel (Figure 20). The samples containing the two most intensive bands were combined and purified using the QIAquick PCR Purification kit from Qiagen through the column.

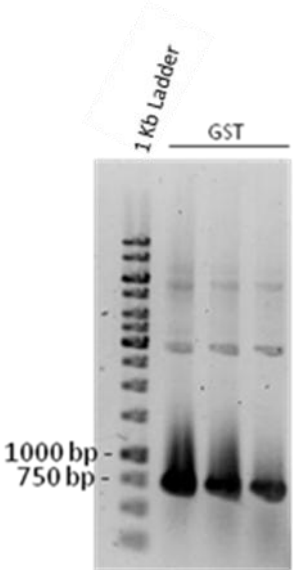


Figure 20 Amplification of GST cDNA by PCR. In all cases the expected band at about 700 bp is detected.

Subsequently, the insert and the transfer vector were digested with the same enzymes (BglII and NotI). The resulting fragments were separated in an agarose gels (Figure 21) and isolated using the QIAquick GelExtraction Kit from Quiagen.

Therefore, the two fragments (vector and insert) were ligated with T4 ligase, transformed into *E. coli* Nova blue and plated on LB plates with ampicillin and incubated over night. Colonies that grown were transferred to new LB plates and the next day plasmids were isolated. To ensure which clones had the insert, they were digested with ScaI. Figure 22 shows the six clones digested and non-digested and the 6th clone shows the two expected bands of 4690 bp and 5621 bp. The clone was sequenced to be sure that there were no mutations in the DNA and was frozen for long-term storage.

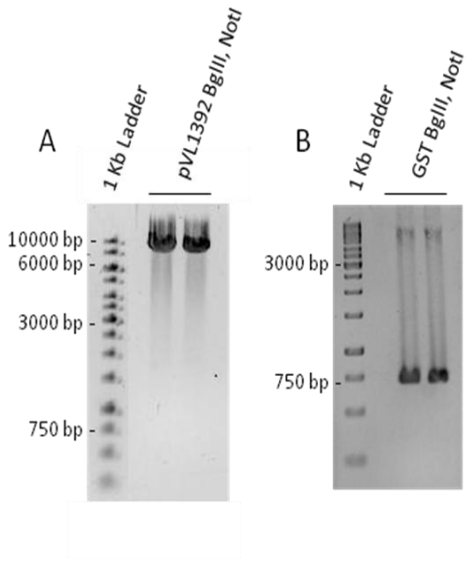


Figure 21 Digestion of:

A) **pVL1392 transfer vector with BglII and NotI.** In the two cases a major band of approximately 9000 bp is detected.

B) **GST cDNA with BglII and NotI.** In the two cases a major band of approximately 700 bp is detected.

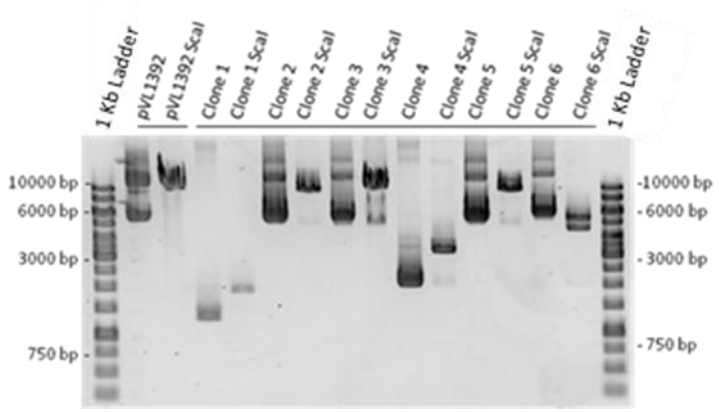


Figure 22 Verification of the pVL1392-GST clones by restriction digest. In the first rows there is the empty vector of pVL1392 as negative control of digestion where with ScaI the pVL1392 is linearized. Clone six shows the two expected bands of about 4690 bp and 5621 bp after ScaI digestion..

In the next step, the cDNA of Hck-KD and mFRNK-TAT had to be cloned in the new transfer vector pVL1392 N-term GST. As before, the cDNA was amplified with two primers each by PCR in which the 5'-primers had a NotI recognition site and the 3'-primers had an EcoRI recognition site. A hot start PCR was carried out with these primers and the template plasmid which contains the HcK-KD cDNA and the mFRNK-TAT cDNA. After PCR, products were separated on a 1.4 % agarose gel (Figure 23). The two most intensive bands were combined and purified using the QIAquick PCR Purification kit from Qiagen through the column.

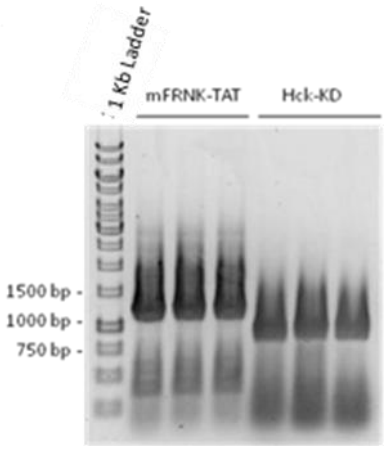


Figure 23 Amplification of mFRNK-TAT cDNA and Hck-KD cDNA by PCR. All approaches had the band size expected, for mFRNK-TAT about 1100 bp and for Hck-KD about 850 bp.

Subsequently, the insert and the transfer vector pVL1392 N-term GST were digested with the same enzymes. The resulting fragments were separated in agarose gels (Figure 24) and isolated using the QIAquick GelExtraction Kit from Quiagen.

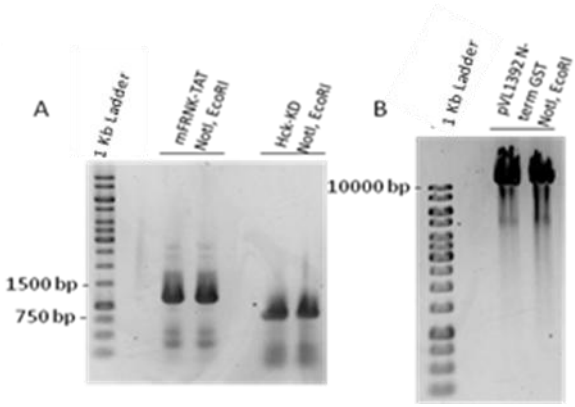


Figure 24 Digestion of:

A) **mFRNK-TAT and Hck-KD with NotI and EcoRI.** In the two cases for mFRNK-TAT a major band of approximately 1100 bp is detected and in the two cases for Hck-KD major band of approximately 850 bp is detected.

B) **pVL1392 N-term GST with NotI and EcoRI.** In the two cases a major band of approximately 10300 bp is detected.

Therefore, the two fragments (vector and insert) were ligated and transformed as before. For the Hck-KD construct, plasmids of 30 clones were isolated and analyzed by colony PCR. The figure 25 shows nine clones only and the clone 9 is supposed to be positive, as well as clones 12 and 27 (data not shown). To ensure that the clones had the insert, they were digested with BglII and EcoRI. The figure 26 shows the digestion of the three clones and the empty vector as a negative control. Only clone 27 shows the expected bands. Therefore, the clone was sequenced to be sure that there were no mutations in the DNA and was frozen for long-term storage. For the mFRNK-TAT

construct the clones were digested with XhoI, one positive was chosen, sequenced and frozen as for the other constructs. Four positive clones were obtained for this construct (Figure 27).

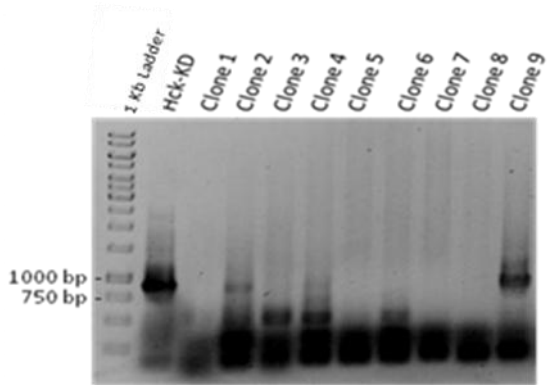


Figure 25 PCR products. As seen before, the Hck has a band size of about 850 bp. In the clone 9 this band can be seen as in the positive control.

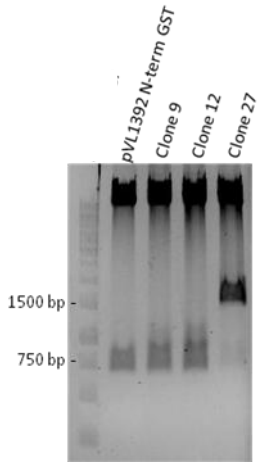


Figure 26 Verification of the clones by restriction digest. In the first column pVL1392 N-term GST was loaded as negative control. There is a band of about 700 bp as expected for the digestion with XhoI. In the other columns are the three clones positive for PCR but only one has the characteristic band for the digestion of the construct with Hck-KD at about 1500 bp.

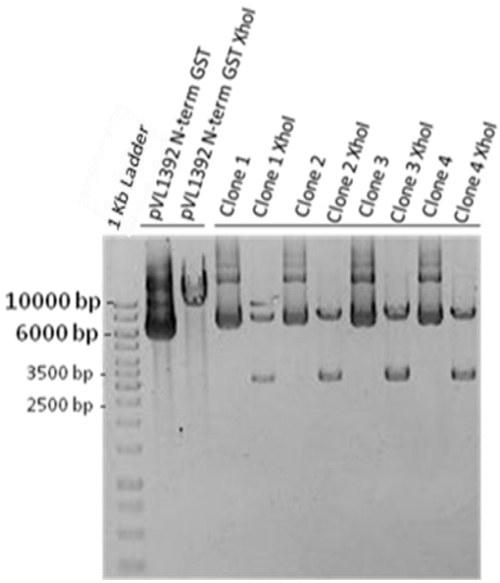


Figure 27 Verification of the clones by restriction digest. In the first column, pVL1392 N-term GST was loaded as a negative control. There is a band of about 10000 bp as expected for the digestion with XhoI. The other columns show four clones and all are positive, as they all have the two characteristic bands of about 8000 and 3300 bp with the digestion with XhoI.

2.2.2 Production of recombinant baculoviruses

Sf9 cells were co-transfected with the final transfer vectors and BaculoGold^{TM} DNA, leading to the production of recombinant baculoviruses and expression of the heterologous proteins.

2.2.3 Amplification and indirect titer determination of recombinant baculoviruses

A titer is a way of expressing concentration. Titer testing employs serial dilution to obtain approximate quantitative information. The titer corresponds to the highest dilution factor that still yields a positive reading, and, in this case, the viral titer is the lowest concentration of virus that still infects cells. The recombinant virus was amplified through several cycles until the titer was about $2x10^8$ pfu/mL. After each amplification round, the titer was determined indirectly by antibody staining and subsequent fluorescent microscopy analysis. Sf9 cells were seeded in 96 well-plates and after the cells attach to the plate, were infected with different dilution factors. The cells were afterwards stained, after an incubation time of 2 days at 27ºC, using an antibody which recognizes the C-terminal epitope tag added to each protein (see plasmid maps). Thus, only the cells expressing the protein were counted to the titer determination. Not-transduced cells were used to adjust background level in the Cy3 channel (Figure 28). Afterwards, samples with less than 10% of infected cells were chosen for quantification and six pictures were taken (three for each well). The cells were counted and from the ratio of transduced to non-transduced cells the titer was determined, as explained in the methods section. In each amplification, the viral titer typically increases an order of magnitude, for example, from $1x10^7$ to $1x10^8$.

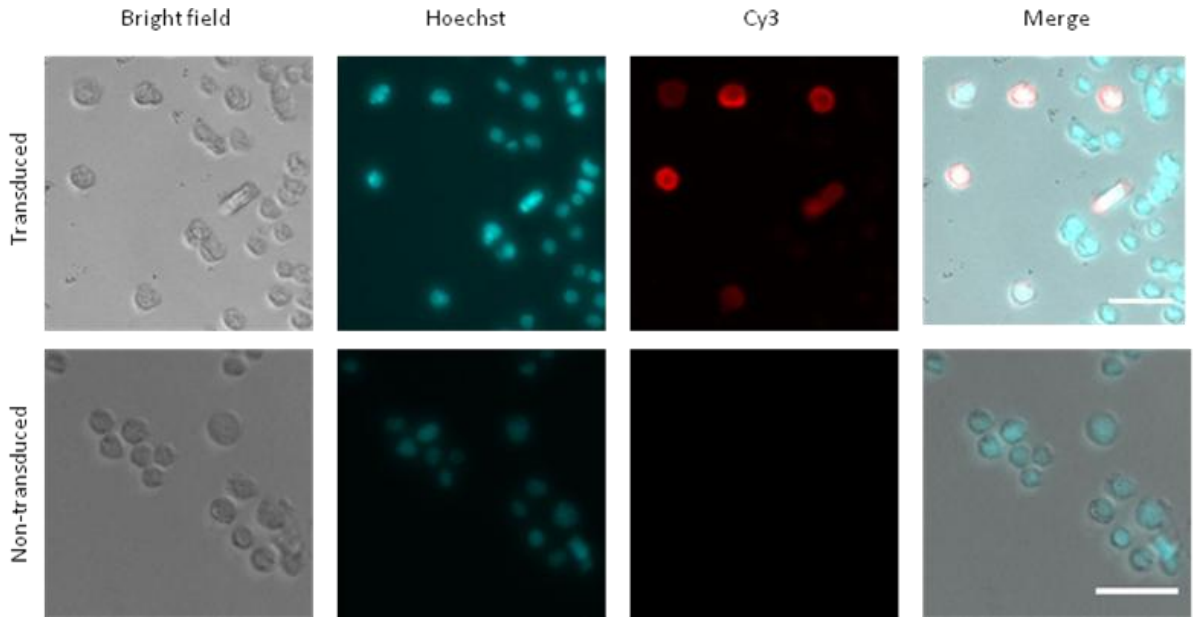


Figure 28 Indirect baculovirus titer determination by antibody staining. The bottom row shows pictures of non-transduced cells where no signal is observed in the Cy3 channel. In the top row infected cells appear red in the Cy3 channel due to the expression of the heterologous protein. This example is from baculovirus titer determination carrying the Pyk2-KD construct and the chosen dilution factor was 10^{-5}. Scale bar 50 μm.

2.2.4 Test of the best time to express the protein

Before starting the large scale expression of the proteins, it was necessary to know which time for expression for each protein was better to obtain the highest yield. The Sf9 cells were transduced and after 24, 48 and 72 hours they were harvested and lysed. An additional approach remained non-transduced cells and served as a negative control. The samples were analyzed for target protein expression by SDS-PAGE and subsequent Western blotting with the appropriate antibodies for each protein or a NEF antibody that recognizes the C-terminal domain present in all produced proteins. Using a HRP-coupled secondary antibody the primary antibody was visualized. For GST-Hck-KD was chosen 72 h to express the protein as the characteristic band for the protein at about 56 kDa is more intense, showing more amount of protein (Fig. 29A). For GST-mFRNK-TAT was chosen 48 h, as this sample showed a clear large amount of the protein, comparing with the 24 h (Figure 29B).

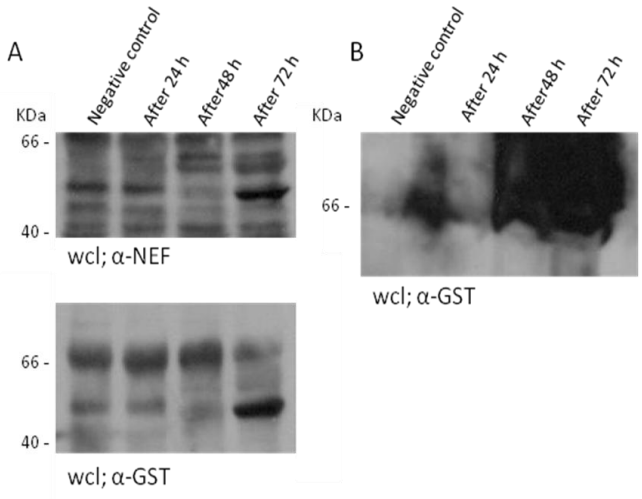


Figure 29 Analysis of target protein expression for:

A) **GST-Hck-KD with NEF antibody (up) and GST antibody (down).** Sf9 cells were transduced or left uninfected (negative control) and lysed after 24, 48 and 72 hours. After 72 hours we have a clearly band at about 56 KDa, time used, afterwards, during expression.

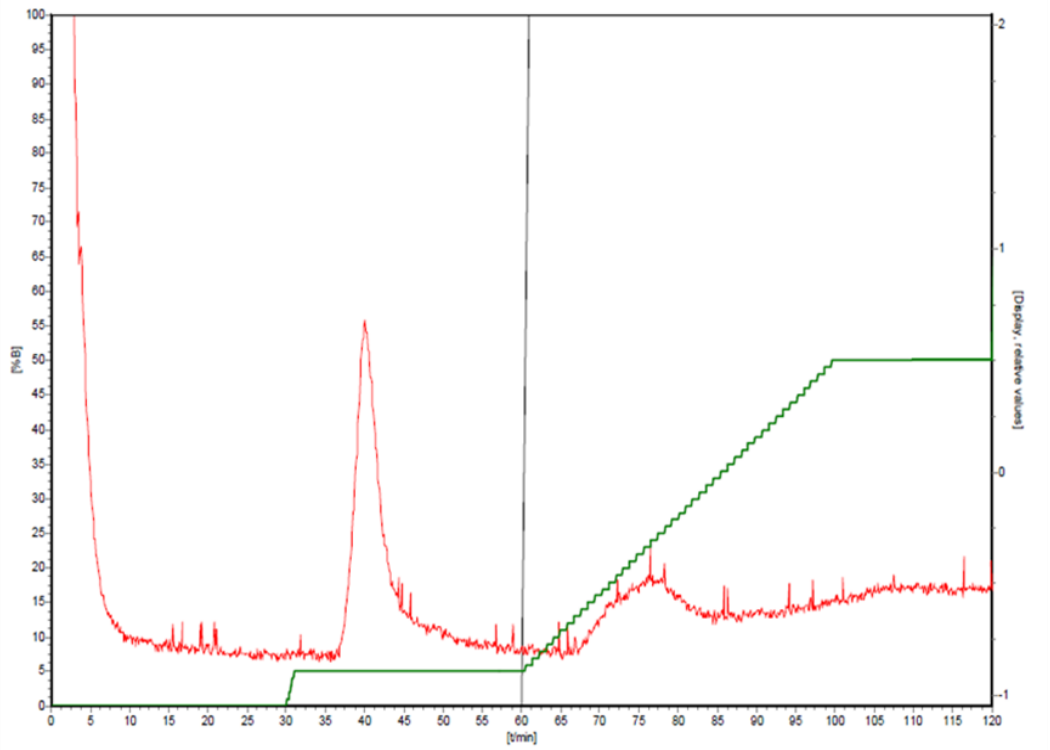
B) **GST-mFRNK-TAT with GST antibody.** Here we don't see clearly the bands but comparing the 48 hours with 24 hours we see a clear expression. In this case the 48 hours were chosen for expression. In this test expression was chosen to lyse the cells with 2xSDS. Perhaps the lysis with lysis buffer is better as we can see in the blot for GST-Hck-KD.

2.2.5 Expression and purification of the proteins

For expression of the protein in large scale, ten 10 cm cells culture dishes, each one with $8.8x10^6$ cells, were transduced with MOI 4, incubated at 27ºC for the best time of expression and lysed afterwards.

The lysates were then purified by Fast Protein Liquid Chromatography (FPLC). The columns were always equilibrated with the lysis buffer used. For Pyk2-KD and FAK-KD, the lysates were loaded onto a HisTrapFF crude column and washed with washing buffer. Subsequently, a washing step was followed by 5% elution buffer (500 mM imidazole) in washing buffer and finally the column matrix-bound protein was eluted. For GST m-FRNK-TAT and GST-Hck-KD another column was used (GSTrapFF). In this case the column was washed with 50 mM Tris pH 8 and 10 mM Glutathion. Figure 30 shows the different purification profiles for Pyk2-KD, GST-mFRNK-TAT and GST-Hck-KD, where the green lines represents the concentration of the elution buffer over time and the red line the absorption (A_{280}), indicating the protein concentration, over time.

A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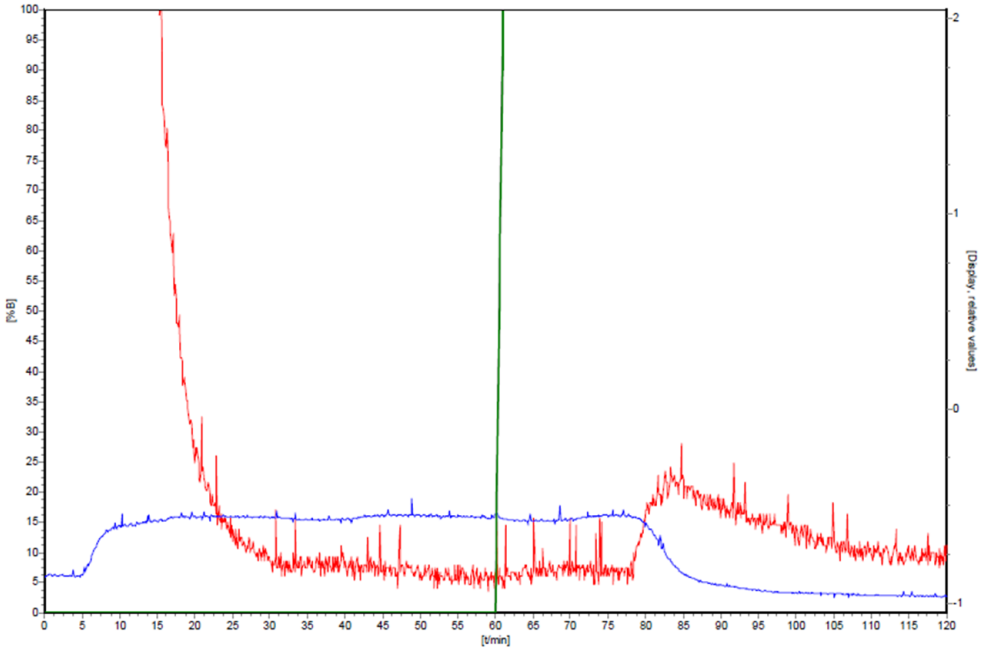
B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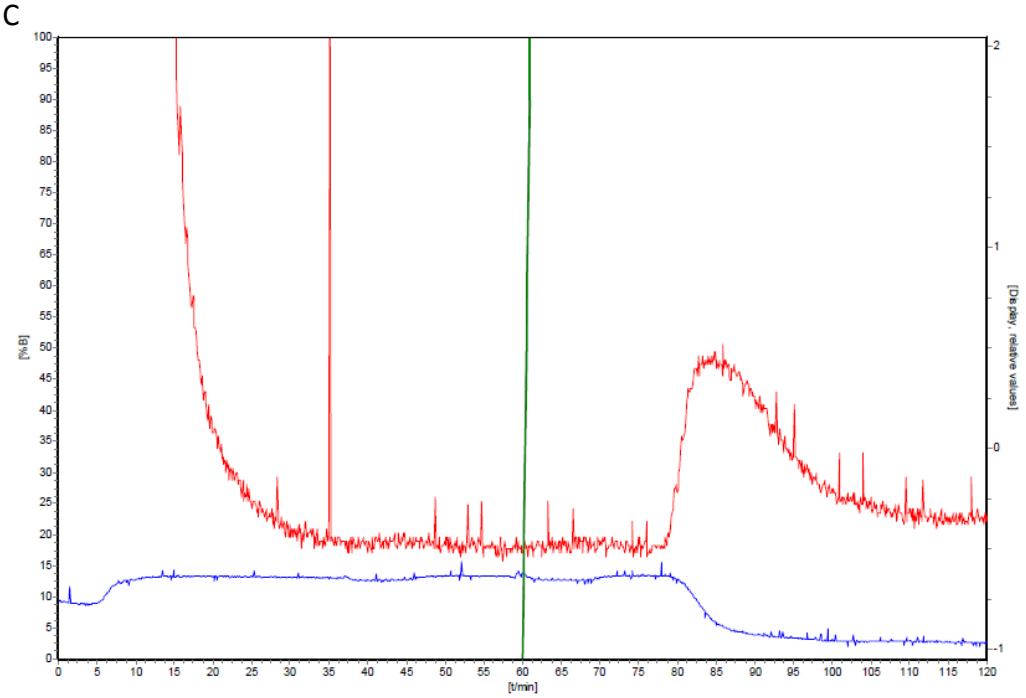


Figure 30 Profiles from the purification by FPLC of:

A) Pyk2-KD. Here we can see a peak at about 75 min correspondent to the time of elution of the protein. This tells us that the purification worked.

B) GST-mFRNK-TAT. Here we can see a peak at about 85 min correspondent to the time of elution of the protein which tells us that the purification worked.

C) GST-Hck-KD. Here we can see a peak at about 85 min correspondent to the time of elution of the protein which tells us that the purification worked.

After the elution all fractions were taken and analyzed for target protein by SDS-PAGE. As controls, the lysate was applied to the gel, followed by the flow through and the fractions from the washing steps. Figure 31 shows the different fractions analysis for FAK-KD, GST-m-FRNK-KD and GST-Hck-KD. The fractions containing the protein are clearly seen and those which have the highest staining were combined for dialysis.

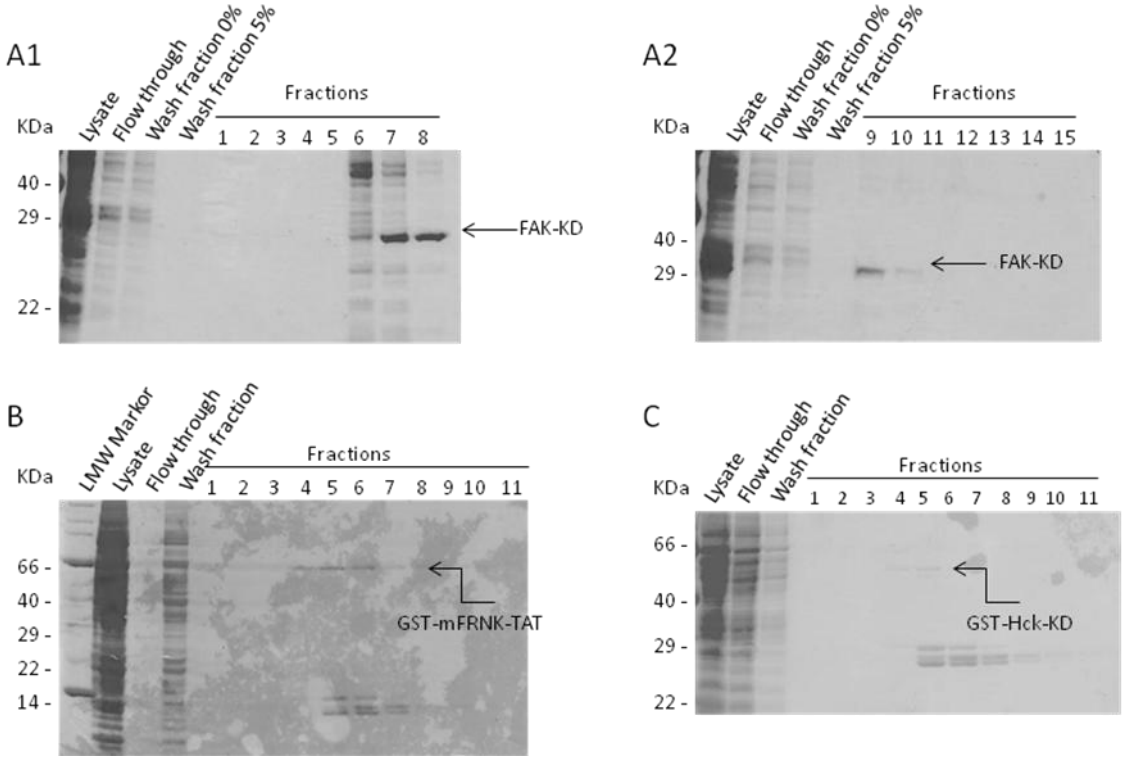


Figure 31 Fractions analysis for:

- **A) FAK-KD.** As shown in the figure there are three clear bands for FAK-KD at around 29 KDa. As we can see in the picture, this protein is not so pure, reason why the profile of the purification of this protein is not shown. As the purification did not work so well, these fractions were stored after dialysis but noted to be not pure.
- **B) GST-mFRNK-TAT.** In this case there are again three bands at around 66 KDa that belongs to this protein. The fractions 4 and 6 were combined for dialysis and the fraction 5 was combined with Rhodamine (1:1000) 30 minutes at room temperature and then dialysed. Thus, the protein gets already color and can be visualized by microscopy without being necessary to stain.
- **C) GST-Hck-KD.** Here the two bands at about 56 KDa in the four and five fractions are not clear has they are in the gel. As the Fraction 5 had some contaminants, the fractions were dialyzed separately.

After dialysis the concentration of the proteins was determined, before the aliquots were frozen in liquid nitrogen and stored at -80 ºC. The concentration of the purified protein was determined using a polyacrylamide gel, adding different concentration of BSA as control and the purified protein. After separation and coomassie staining, the band of the protein was compared with those of the BSA standard. As the concentration of BSA, in all approaches

was known, the same staining of bands indicates the concentration of the purified protein. Figure 32 shows the different protein concentration determination gels for Pyk2-KD, FAK-KD, GST-mFRNK-TAT and GST-Hck-KD.

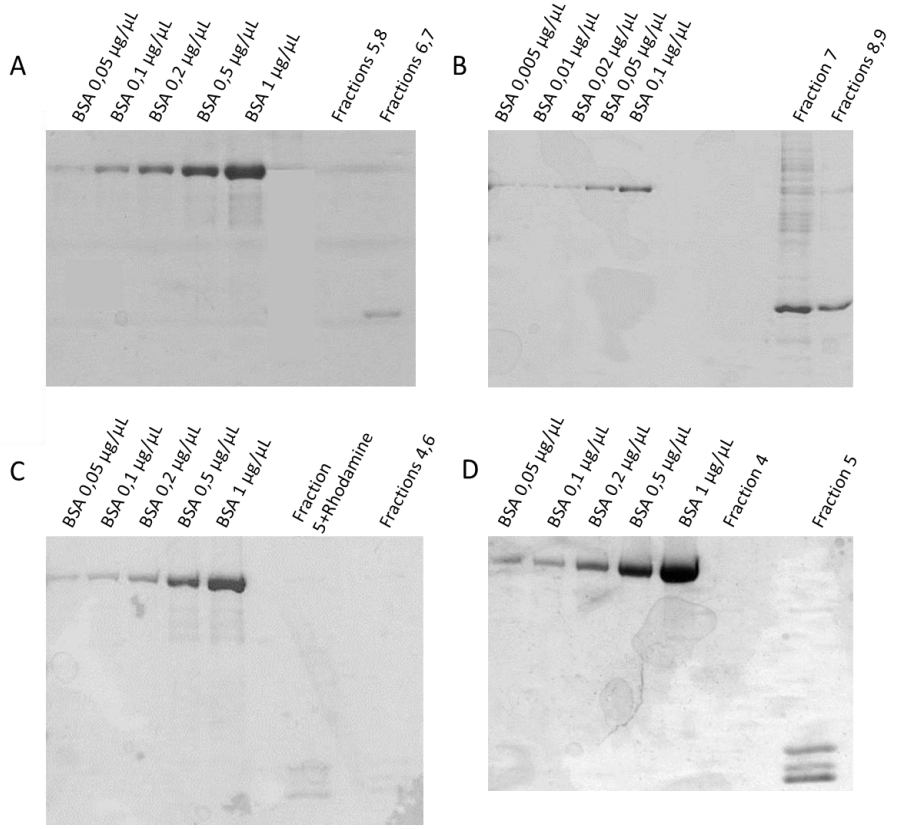


Figure 32 Quantification of:

A) Pyk2-KD. For this protein we see a band from the fraction 6 and 7 close to the band of BSA 0.1 µg/µL and more intense than for BSA 0.05 µg/µL. Therefore, it's assumed that the concentration is about 0.075 µg/µL. For the other fractions the band is not so clear in the picture but it's half of the band correspondent to BSA 0.05 µg/µL so the concentration is around 0.025 µg/µL.

B) FAK-KD. For this protein we see a band of the fraction 8 and 9 more intense than for BSA 0.1 µg/µL so the concentration should be 0.15 µg/µL. For fraction 7, the band appears to be the double of the band correspondent to BSA 0.1 µg/µL. Therefore the concentration is 0.2 µg/µL.

C) **GST-mFRNK-TAT.** For this protein we can't really see clear bands but it's clear that the band correspondent to the fraction 5 with Rhodamine is half of the band for BSA 0.05 µg/µL. Therefore the concentration is 0.025 µg/µL. For the other fractions the band is not clear enough for quantification.

D) GST-Hck-KD. Again the bands are not clear in this image, but was seen that for fraction 4 the concentration is 0.01 µg/µL and for fraction 5 0.02 µg/µL.

2.2.6 Verification of the functionality of the expressed proteins

To determine whether the kinase proteins were active, an *in vitro* kinase assay was performed. The procedure was conduced as described in the methods section. Two approaches were made with two different substrates (Table 1 and 2), and two kinase proteins were tested, FAK-KD and Pyk2-KD.

Table 1 Amounts of reagents for the *in vitro* kinase assay using GST-p130^{Cas} substrate.

	Substrat (GST-p130Cas)	*µM ATP*	*Kinase [µl]*	*Substrat [µL] from 0.1 µg/µL solution*	*Kinase-Buffer [µl]*
1	Substrat only (GST-p130Cas)	0	0	2	Up to 30 µL
2	Kinase only (FAK-KD)	0	4	0	Up to 30 µL
3	Kinase only (Pyk-KD)	0	4	0	Up to 30 µL
4	Substat +K (FAK-KD)	0	4	2	Up to 30 µL
5	Substat +K (PYk2-KD)	0	4	2	Up to 30 µL
6	FAK-KD +ATP	100	4	0	Up to 30 µL
7	Pyk2-KD + ATP	100	4	0	Up to 30 µL
8	Substat +ATP	100	0	2	Up to 30 µL
9	Substat +FAK-KD +ATP	100	4	2	Up to 30 µL
10	Substat +Pyk2-KD +ATP	100	4	2	Up to 30 µL

Table 2 Amounts of reagents for the *in vitro* kinase assay using GST-FAK-substrate.

	Substrat (GST-FAK)	*µM ATP*	*Kinase [µl]*	*Substrat [µL] from 0.1 µg/µL solution*	*Kinase-Buffer [µl]*
1	Substrat only (GST-FAK)	0	0	2	Up to 30 µL
2	Kinase only (FAK-KD)	0	4	0	Up to 30 µL
3	Kinase only (Pyk-KD)	0	4	0	Up to 30 µL
4	Substat +K (FAK-KD)	0	4	2	Up to 30 µL
5	Substat +K (PYk2-KD)	0	4	2	Up to 30 µL
6	FAK-KD +ATP	100	4	0	Up to 30 µL
7	Pyk2-KD + ATP	100	4	0	Up to 30 µL
8	Substat +ATP	100	0	2	Up to 30 µL
9	Substat +FAK-KD +ATP	100	4	2	Up to 30 µL
10	Substat +Pyk2-KD +ATP	100	4	2	Up to 30 µL

After analysis by western blot, it was seen that both kinase proteins tested were active *in vitro*, with an antibody which detects phosphorylated Tyrosins (α-PTyr 1:1000). To see the substrate in the samples a GST antibody (1:1000) was used and the kinases a NEF antibody (1:5000) (Figure 33).

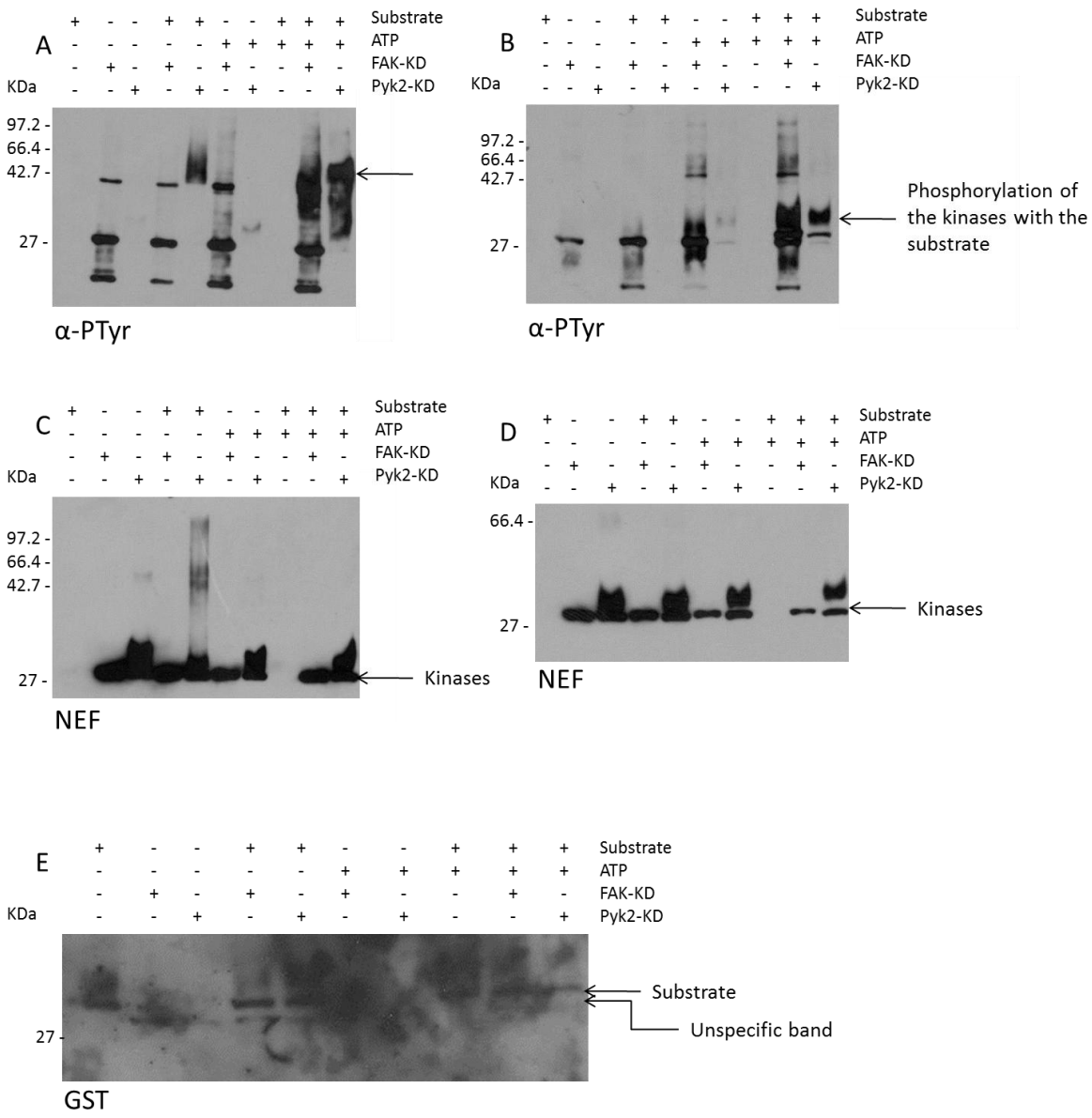


Figure 33 *In vitro* Kinase Assay. In A and C are presented the results for GST-p130^{Cas} substrate and in B, D and E for GST-FAK-substrate. Glutathione beads were used in this last substrate to fix to the substrate, and, after washing, remove the kinase from the substrate.

3. Discussion

3.1 Analysis of the recruitment of Pyk2 in macrophages during complement receptor-mediated phagocytosis

Pyk2, a non-receptor tyrosine kinase, is known to bind to cytoskeletal proteins, linkers and other tyrosine kinases, which may potentially impinge Pyk2 in a variety of signal transduction pathways [33].

During complement receptor-mediated phagocytosis, it has not been demonstrated yet that Pyk2 is recruited and functionally involved. Nevertheless, it was shown before that in response to Fc receptor-mediated phagocytosis, Pyk2 tyrosine phosphorylation is stimulated, and this kinase has been implicated in other processes involving reorganization of the cytoskeleton, such as migration and adhesion [20] as well as macrophage activation [39]. Also studies with Yersinia have shown an increase of Pyk2 autophosphorylation upon infection in $FAK^{-/-}$ cells. Thus, Pyk2 may be an integral part of the host response to Yersinia infections [40]. Pyk2 was also shown to be a critical intracellular signaling molecule and has been linked to migration and adhesion processes analyzed by its activation in response to different chemokines and integrins [34, 41].

Additionally, Pyk2 binds to the Rho-GAP Graf and LIM domain containing proteins, suggesting a role in regulation of the cytoskeleton and cellular morphology in response to extracellular stimuli [36, 37, 38]. CR-mediated phagocytosis activates RhoA, a member of the Rho family of small GTPases. Rho and other GTPases are central in the regulation of the actin cytoskeleton, and in particular, RhoA stimulates contractile processes. Activation of RhoA results in the bundling of actin filaments and clustering of integrins. Rho-mediated signaling also promotes the assembly of stress fibres and focal adhesions, which regulate myosin filament formation and contractility. RhoA activation has been proposed to mediate integrin clustering at the site of ingestion, while regulating contractility of the actin cytoskeleton to allow the opsonized particle to sink inside the macrophage [novo, RhoA]. As Pyk2 may play a role in regulation of the cytoskeleton, binding to Rho, which is known to be important in CR-mediated phagocytosis, Pyk2 may be also important in CR-mediated phagocytosis. As this protein is implicated in different signaling processes, the up regulation of the macrophages during this process can be done by the help of Pyk2.

Thus, it is important to study this kinase to have a better understanding of this small part of the immune system. In this aim, it was shown in the present work that Pyk2 is recruited during complement receptor-mediated phagocytosis (Figures 15 and 16).

To analyze Pyk2 localization during complement receptor-mediated phagocytosis mouse macrophages were used. These cells are known to phagocyte microbes when innate immune response is activated. PMA was used to activate the macrophages, as it was shown before that macrophages without stimulation or pre-activation are not able to phagocyte the microbes but only to bind them [14, 19]. To have a direct and certain ingestion of particles by complement receptors Polystyrene beads were coupled with IgM, which were incubated with serum to be opsonized with complement components that are, during infection, recognized by the complement receptors [26, 27, 28, 29]. Furthermore, a direct complement component, iC3b, was used to label the Polystyrene beads.

To have a reliable Pyk2 staining and an efficient uptake of complement-opsonized particles, the conditions were optimized. The Pyk2 antibody was shown to be specific for this protein, after transfect the cells with Pyk2 constructs and immunfluorescence staining (Figure 7). As showed before, actin plays an important role during many phagocytosis processes [17, 52, 53]. Thus, the actin was stained after infecting the macrophages with coupled beads, and it was seen a clear recruitment of Pyk2 at the site of ingestion. As the staining of Pyk2 in the beginning did not show any recruitment (data not shown), the internalization of the particles was studied. After staining the intra- and extracellular beads after different times of infection, it was shown that the beads were internalized efficiently (Figures 12 and 13). Also, to be sure that the opsonized IgM particles were internalized by the complement receptors, the complement receptor antibody CD11b M1/70.15 was used which is known to block complement receptor-mediated uptake [55, 56]. Only the particles opsonized with complement were blocked, clarifying the internalization of these particles by the complement receptors (Figure 14). During these assays, some unexpected results were obtained. The IgM beads which were not incubated with serum should not be internalized by any receptors in macrophages. Surprisingly, there was a significant internalization of these beads. It was proved that those beads were not internalized by the complement receptors but the pathway that IgM beads take after infection is not clear. After proving the phagocytosis of the beads by the complement receptors, except the negative controls and the IgG beads, the Pyk2 was stained to investigate whether this kinase was recruited during complement-mediated phagocytosis. In the first assays (data not shown), it was discovered that the best time to study the recruitment of Pyk2 was 30 min infection. After infecting the macrophages using all the optimized conditions and stain the Pyk2, a clear recruitment was observed (Figures 15, 16). It is also shown a clear recruitment of Pyk2 during Fc receptor-mediated phagocytosis, using the

IgG beads that were already shown to be internalized by this pathway in macrophages [1, 10, 11, 29, 54]. In previous studies in this group, it was shown that Pyk2 does not play a functional role during Fc receptor-mediated phagocytosis (A.-K. Fuchs, unpublished data). However, the recruitment of Pyk2 during this process was not analyzed.

To further investigate if Pyk2 is activated during complement-mediated phagocytosis, after proving the recruitment the phosphorylation of Pyk2 was investigated. The cells were infected, lysed and analyzed by western blotting. A clear phosphorylation of Pyk2 was seen in all samples and after infection with IgG particles and IgM opsonized with complement particles, an increase of this phosphorylation was observed (Figure 18).

3.2 Expression of kinase domains in Sf9 insect cells

Using the Baculovirus Expression Vector System (BEVS), the kinase domains of Pyk2 and FAK were expressed with a His-tag and the kinase domain of Hck and FRNK were expressed as GST-fusion proteins. The goals were the kinase domains for further testing *in vitro* and use these proteins for *in vitro* analysis. To express these proteins, a polyhedrin transfer vector was used, so that the gene is expressed under the polyhedrin promoter. It is known that the polyhedrin promoter is more active in the late stages of the infection cycle [44] and thus, 72 hours for expression were normally assumed, that the amount of expressed protein after infection time was highest. Nevertheless, a test expression was always made to be sure of the best time to express these proteins. All expressed proteins were purified by FPLC, which demonstrates success, except for FAK-KD where the purified protein had some contaminants (Figure 30). Accordingly, for this protein the use of additional purification could be useful, as there are more bands corresponding to other proteins. For the GST-tag purified proteins there are other bands to, which can be due to protein degradation during expression, as the bands exhibit the size of GST, Hck and FRNK alone. The flow through and wash fractions show bands corresponding to the protein. Perhaps a portion of the target protein in the purification under the conditions used got lost, but this could not be clarified by the Coomassie staining. This could be tested by a Western blot, using an antibody against the protein and seeing if these bands really belong to the target protein. After Dialysis, the concentration was determined and a test for the activity of the kinase domain was performed. The concentrations for Pyk2-KD, FAK-KD, GST-mFRNK-TAT and GST-Hck-KD were 0.1 mg/mL, 0.175 mg/mL, 0.025 mg/mL and 0.015 mg/mL, respectively (Figure 31), and in an *in vitro* kinase assay it could be shown that the isolated kinase domains of Pyk2 and FAK are

catalytically active (Figure 32). The two substrates used in this assay showed a good performance, however the GST-FAK-substrate has the same size as the kinases and it is necessary the use additional steps to get ride of the kinases.

3.3 Outlook

The aims of this study were accomplished, as the recruitment of Pyk2 during complement receptor-mediated phagocytosis was seen and this protein was phosphorylated during this process. The functional role of Pyk2 in this process is not clear yet, but further studies will be necessary to clarify this issue. The next steps with this protein can be the use of knock down macrophages to analyze if this protein is required for complement receptor-mediated phagocytosis. Also the use of Pyk2 inhibitors during complement mediated phagocytosis, displacing Pyk2 from their functions.

Since the expression and purification of the proteins were successful, following further investigations can be made, as the use of GST-mFRNK-TAT to see the uptake of FRNK (FAK non-related kinase), which can be used to study the well-known FAK, using the TAT sequence which is known to increase the uptake of proteins *in vitro*. Since its initial discovery as a substrate and binding partner for the Src oncogene, a role for FAK in cancer has been speculated. Thus, the regulation and role of this kinase in biological processes, important for the pathogenesis of cancer, may be important. Also, it is often desirable to attempt to measure and to modulate the activity of the protein kinases and phosphatases responsible for a given phenotypic change. Protein kinase can be used for more understanding in protein phosphorylation pathways and kinase signaling cascades.

4. Material

4.1 Cells

Table 3 Cells.

Raw 264.7	Mouse leukaemic monocyte macrophage cell line
Sf9	*Spodoptera frugiperda* clonal isolate 9
HEK 293T cells	human epithelial kidney cells

4.2 Medium

4.2.1 Medium for agar plates

Table 4 Medium for agar plates.

LB Medium	1% w/v Tryptone; 0.5% w/v yeast extract; 0.5% w/v NaCl; pH 7.0 with NaOH (autoclaved)
LB agar plates	1% w/v Tryptone; 0.5% w/v yeast extract; 0.5% w/v NaCl; 10 mM MgCl2; 1.2% w/v Agar-Agar; pH 7.0 with NaOH (autoclaved)
Refreezing medium	Refreezing medium 50% v/v LB medium; 50% v/v Glycerol (50%)

4.2.2 Medium for cell culture

Table 5 Medium for cell culture.

Raw 264.7 maintenance	DMEM 4.5 g/L Glucose + 10% v/v h.i. FCS (foetal bovine serum)
Raw 264.7 freezing	DMEM 4.5 g/L Glucose with 10% DMSO and 20% v/v h.i. FCS.
Sf9 maintenance	12.76 g IPL-41 Medium + 0.175 g $NaHCO_3$ (pH 6.0) + 10% FCS + 2% Yeastolate + 2% Lipid concentrate + 1% Penicillin/Streptomycin
Sf9 freezing	Sf9 maintenance medium with 10% DMSO and 20% FCS.
HEK 293T cells maintenance	DMEM 4.5 g/L Glucose + 7.5% v/v CS (calf serum)

4.3 Antibodies

4.3.1 Primary antibodies

Table 6 Primary antibodies.

Antigen	Name	Monoclonal/ Polyclonal, Imunogen	Species	Supplier

ATβ2	ATβ2	Monoclonal	Rat	AG Hauck
CD11b	M1/70.15	Monoclonal	Mouse	ImmunoTools
c-myc	9E10	Monoclonal	Mouse	Santa Cruz
c-Src	SRC-2	Polyclonal	Rabbit	Santa Cruz
GST	B-14	Monoclonal	Mouse	Santa Cruz
HA	F-7	Monoclonal	Mouse	Santa Cruz
His	H3	Monoclonal	Mouse	Santa Cruz
IgG2b	Rat IgG2b control	Monoclonal	Mouse	ImmunoTools
NEF	α-NEF	Hybrid clone	Mouse	AG Hauck
PTyr	α-PTyr	Monoclonal	Mouse	AG Hauck
Pyk2	H-102	Polyclonal	Rabbit	Santa Cruz
Tubulin	α-Tubulin	Monoclonal	Mouse	Development studies Hybidoma Bank/Joseph Frankel

4.3.2 Secondary antibodies

Table 7 Secondary antibodies.

Name	**Supplier**
Biotin-SP-conjugated AffiniPure Goat Anti-Mouse IgG	Jackson
Biotin-SP-conjugated AffiniPure Goat Anti-Mouse IgM	Jackson
Cy2-conjugated AffiniPure Goat Anti-Rabbit IgG	Jackson
Cy3-conjugated AffiniPure Goat Anti-Mouse IgG	Jackson
Cy3-conjugated AffiniPure Goat Anti-Rabbit IgG	Jackson
Cy5- conjugated AffiniPure Goat Anti-Mouse IgG	Jackson
HRP-conjugated AffiniPure Goat Anti-Mouse IgG	Jackson
HRP-conjugated AffiniPure Goat Anti-Rabbit IgG	Jackson
Rhodamine Red conjugated goat-α-Rat IgG	Jackson

4.3.3 Antibodies independent staining reagents

Table 8 Antibodies independent staining reagents.

Name	Supplier
Alexa-Fluor546-Phalloidin	Molecular Probes
Hoechst	Molecular Probes
CFSE	Molecular Probes
FITC-Streptavidin	ICN-MPBiomedicals
TAMRA Rhodamine (TRITC)-Streptavidin	Molecular Probes Jackson

4.4 Enzymes, proteins and complement components

4.4.1 Enzymes

Table 9 Enzymes.

Name	Supplier
Pfu-Polymerase	AG Hauck
Phosphatase	NEB
Restriction Enzymes	Fermentas or NEB
T4 Ligase	AG Hauck
Taq-Polymerase	AG Hauck
Trypsin-EDTA (1x)	PAA Laboratories

4.4.2 Proteins and complement components

Table 10 Proteins and complement components.

Name	Supplier
Albumin from chicken egg white	Sigma
Anti-Sheep Red Blood Cell Monoclonal Antibody-Ascites (IgM)	Cedarlane
Complement iC3b, Human	Calbiochem
FAK-KD	AG Hauck
Fibronectin	BD Bioscience
GST	AG Hauck
His-Pyk2	AG Hauck

Poly-L-Lysin	Sigma
Recombinant Protein G from *E. coli*	ZYMED Laboratories

4.5 Inhibitors

Table 11 Inhibitors.

Name	Target	Supplier
Leupeptin (Acetyl-L-leucyl-L-argininal hemisulfate)	Serine, cysteine and threonine proteases	Calbiochem
PMSF (phenylmethanesulfonylfluoride)	Serine proteases	Applichem

4.6 Primers

Table 12 Primers.

Name	Sequence	Template	Plasmid
GST pVL 1392 BglII_sense	5'-ACGAGATCTACCATGTCCC-CTATACTAGGTTATTG	pGEX-4T-1	pVL 1392
GST pVL 1392 NotI_anti	5'-ACTGCGGCCGCACGATCCA-CGCGGAACCAG		
Hck-KD pVL 1392 EcoRI_anti	5'-ACTGAATTCTTACCCATTCT-TGAAATATTCAGGCCCACGGT-TTTTCCAGCAGCG	human Hck cDNA	pVL 1392 pVL 1392 N-term GST
Hck-KD pVL 1392 NotI sense_neu	5'-ACTGCGGCCGCGGGAACG-ACGGGCTCTG		
mFRNK EcoRI anti	5'-ATCGAATTCTTAGCGGCGG-CGCTGGCGGCGTTTCTTGCG-GCCGTAGCCGCCGCGTGTCT-GCCCTAG	pAAVFRNK	pVL 1392 N-term GST
mFRNK NotI sense	5'-ACTGCGGCCGCGAATCCAG-AAGACAGGCTAC		

4.7 Plasmids

Table 13 Plasmids.

Vector	Inserts	Cloning Strategy or source
pOTB7	Hck	AG Hauck
pAAVFRNK	FRNK-HA	AG Hauck
pcDNA3.1(+) Hygro	Myc-Pyk2WT	AG Hauck

pcDNA31Hygro (empty vector)		AG Hauck
pEGFP C1 (positive for transfection)	GFP	AG Hauck
pGEX-4T-1		AG Hauck
pLP	hPyk2WT	AG Hauck
pLPS-3'EGFP	CEACAM3WT	AG Hauck
pVL 1392		AG Hauck
pVL 1392 N-term GST	mFRNK-TAT	Restriction digest with NotI/EcoRI and ligation with pVL 1392 His-Hck-KD-NEF NotI/EcoRI digested
pVL 1392	His-Hck-KD-Nef	Restriction digest with NotI/EcoRI and ligation with PCR product
pVL 1392	N-term GST	Restriction digest with NotI/BglII and ligation with PCR product
pVL 1392 N-term GST	Hck-KD-Nef	Restriction digest with NotI/EcoRI and ligation with pVL 1392 His-Hck-KD-NEF NotI/EcoRI digested

4.8 Media and Buffer

Table 14 Media and Buffer.

Name	Preparation
Anode buffer (5x)	125 mM Tris-Base 200 mM 6-aminohexanacid pH 9.4
Birnboim-Doly P1	50 mM Tris/HCl 10 mM EDTA 100 mg/mL RNAse A
Birnboim-Doly P2	0.2 mM NaOH 20% w/v SDS
Birnboim-Doly P3	3 M potassic acetate pH 5.5 with acetic acid
Blocking buffer	10% v/v h.i. CS in PBS^{++}
Blotto	2% w/v BSA

	0.05 %w/v Sodium azide in 1x TBS-T
Borate buffer	0.2 M borate buffer prepare by adding 1 M NaOH to boric acid until pH 8.5
Carbodiimide	2% N-(3-Dimethylaminopropyl)-N'-ethyl-carbodiimide hydrochloride in 0.1 M MES buffer
Carbonate buffer	0.1 M Na_2CO_3 in 0.1 M $NaHCO_3$ until pH 9.6
Cathode buffer (5x)	125 mM Tris-Base pH 10.4
Coomassie Stain	25% v/v Isopropanol 10% v/v Pure acetic acid 3% w/v coomassie
Destain	45.5% v/v Methanol 9.1% v/v Pure acetic acid
DNA-Ladder (1 kb Marker)	1 kb DNA-Ladder in 6x loading dye solution
dNTPs [20 mM]	20 mM dGTP 20 mM dATP 20 mM dCTP 20 mM dTTP in ddH_2O
Dialysis Buffer	20 mM Tris HCl, pH 7.5 0.5 M NaCl 0.1 mM β-Mercaptoethanol 5 L total
Ethidium bromide	10 mg/mL EtBr
FACS buffer	5% heat inactivated FCS 0.1% Sodiumazide in 1x PBS
CFSE	4 mg/mL CFSE in DMSO
GEBS	20% w/v Glycerol 0.5% w/v Sarkosyl 50 mM EDTA 0.05% bromphenol blue
H_2O_2 solution	30% v/v H_2O_2
HBSS (Hanks' Balanced Salt Solution)	PAA Laboratories
Kinase Buffer	20 mM HEPES; pH 7,3 10 mM $MgCl_2$ 10 mM $MnCl_2$

	150 mM NaCl 10% Glycerol
Lysis buffer for Sf9 cells (1)	25 mM Tris pH 7.5 1 mM EDTA 0.33% Tween 20 500 mM NaCl 100 µM PMSF 1 mM β-Mercaptoethanol 5 µg/mL Leupeptin 1 mM $NaVO_3$
Lysis Buffer for Sf9 cells (2)	10 mM Tris pH 7.5 130 mM NaCl 0.5% Triton X-100 10 mM NaF 10 mM NaPi 10 mM NaPPi 100 µM PMSF 5 µg/mL Leupeptin
MES buffer	0.1 M MES in water pH 5.2-6
PBS (10x)	1.37 M NaCl 26.8 mM KCl 14.7 mM KH_2PO_4 78.1 mM Na_2PO_4
PBS^{++}	0.9 mM Ca^{++} ($CaCl_2$) 0.5 mM Mg^{++} ($MgCl_2$) in 1xPBS
PFA 4%	4% w/v PFA in bidest H_2O pH 7.4
Pfu-buffer	10 mM Tris/HCl pH 7.5 50 mM KCl 1.5 mM $MgCl_2$
PMA (Phorbol 12-myristate 13-acetate)	200 mM in DMSO
Poly-L-Lysin	10 mg/mL in PBS
RIPA-buffer	1% Triton X-100 50 mM Hepes 150 mM NaCl 10% Glycerol 1.5 mM $MgCl_2$ 1 mM EGTA 10 mM Sodium pyrophosphate 100 mM NaF 1 mM Natrium Orthovanadate

	5 µg/mL Leupeptin 10 µg/mL Aprotinin 10 µg/mL Pefablock 5 µg/mL Pepstatin 10 µM Benzamidin 0,1% w/v SDS 1% v/v Deoxycholin acid
Running-buffer (10x)	3% w/v Tris Base 14.4% w/v Glycine 1% w/v SDS
Sample buffer 2x/4x	4% w/v SDS 20% w/v Glycerol 125 mM Tris HCL 10/20% v/v β-Mercaptoethanol 1% w/v Bromphenol blue pH 6.8
SDS-PAGE-Marker (HMW)	0.5 mg/mL Horseradish-peroxidase (40 kDa) 0.5 mg/mL Bovine Serum Albumin (66 kDa) 0.5 mg/mL Lipoxidase (96 kDa) 0.5 mg/mL -Galactosidase (116 kDa) 0.5 mg/mL Myosin rabbit muscle (205 kDa) in Triton-buffer
SDS-PAGE-Marker (LMW)	0.5 mg/mL Lysozym (14.4 kDa) 0.5 mg/mL Soybean Trypsin Inhibitor (22 kDa) 0.5 mg/mL Horseradish-peroxidase (40 kDa) 0.5 mg/mL Bovine Serum Albumin (66 kDa) 0.5 mg/mL Lipoxidase (96 kDa) in Triton-buffer
Sodium dodecyl sulfat (SDS)	20% w/v SDS
Stacking gel buffer	0.5 M Tris Base pH 6.8
Storage buffer	0.01 M NaH_2PO_4 (pH 7.4) 1% BSA 0.1% sodium acid 5% glycerol
TAE-buffer (50x)	500 mM TrisBase 50 mM EDTA 5.7% w/v acetic acid
TBS (10x)	500 mM Tris Base 1.5 M NaCl pH 7.5
TBS-T	0.05% Tween in 1x TBS
TE-buffer	10 mM TrisBase 1 mM EDTA

	pH 8.5 (autoclaved)
T4 Ligase buffer	50 mM Tris/HCl pH 7.5 10 mM $MgCl_2$ 10 mM Dithiothreitol 1 mM ATP
Triton-buffer	1% v/v Triton X-100 50 mM Hepes 150 mM NaCl 10% Glycerol 1.5 mM $MgCl_2$ 1 mM EGTA 10 mM Sodium pyrophosphate 100 mM NaF 1 mM Sodium Orthovanadat 5 µg/mL Leupeptin 10 µg/mL Aprotinin 10 µg/mL Pefablock 5 µg/mL Pepstatin 10 µM Benzamidin
Western transfer buffer	0.3% w/v Tris Base 1.44% w/v Glycine 21.5% v/v Methanol 10% w/v SDS 30 min devolatilization

4.9 Chemicals

Table 15 Chemicals.

Name	**Manufacter**
Acetic acid	Roth
Aceton	Merck
Acrylamid	Roth
Agarose	Roth
Albumin from chicken egg white	Sigma
Biotin	Thermo Scientific
Boric Acid	Merck
BSA	AppliChem
Calcium chloride	Sigma
Copper(II) sulfate	Merck

Ethanol	Sigma
Ethanolamine	Merck
Gelatine	Merck
Glycerin	Roth
Hydrogen peroxide	Merck
Leupeptin	Calbiochem
Magnesium chloride	Roth
β-Mercaptoethanol	Merck
MES	AppliChem
Methanol	Riedel-de Haën
mounting Medium	Dako
N-(3-Dimethylaminopropyl)-N'-ethyl-carbodiimide hydrochloride	Fluka
Sodium Azide	Riedel-de Haën
Sodium carbonate	Riedel-de Haën
Sodium chloride	Roth
Sodium dihydrogen phosphate	Merck
Sodium fluoride	AppliChem
Sodium hydrogen carbonate	AppliChem
Sodium hydroxide	Riedel-de Haën
Sodium orthovanadate	AppliChem
Sodium pyrophosphate	AppliChem
TEMED	Roth
Tris-Base	AppliChem
Triton-X-100	Roth
Trypton	Roth
Tween-20	Roth

4.10 Kits

Table 16 Kits.

Name	Application	Manufacter
BD BaculoGold™ Transfection Kit	Transfection of Sf9 cells	BD Biosciences
QIAquick Gel Extraction Kit	Gel Extraction	Qiagen
QIAprep Spin Miniprep Kit	Plasmid isolation	Qiagen

4.11 Equipment

Table 17 Equipment.

Name	Notation	Manufacter
Autoclave	Autoclave	Sauter, Sulgen Switzerland A.Hartenstein
Balance	Kern 440-47N / Kern 440-35N	PeqLab
Blotter	Perfect Blue Semi-Dry Elektroblotter	Eppendorf
Centrifuges	5415R / 5417R / 5702 Multifuge 1s-R	Hereaus Instruments
Digestor	Dry-block DB-3	Techne
Electrophoresis System (agarose power supply)	EV343 / E132 / EV202	Consort
Electrophoresis System (SDS)	Mini Protean 3Cell	BioRad
Gel Documentation	GelDoc XR System	BioRad
Incubator (bacteria)	Celsius 2005	Memmert
Incubator (cells)	Innova Co 170 CO2	New Brunswick Scientific
Microscopes	ECLIPSE TS100	Nikon
	AF 6000LX	Leica
Microwave	646223M	Privileg
Mini UV Table	60 ECX 20M	PeqLab
Neubauer Chamber	Neubauer Chamber	Roth
PCR Cycler	Primus 25 advanced	PeqLab
pH Meter	Seven Easy	Mettler Toledo

Rotator	SB 3	Stuart
Spectrophotometer	NanoDrop ND-1000	PeqLab
Spectrofluorometer	Varioskan Flash	Thermo Scientific
X-ray Cassette		Rego

4.12 Material

Table 18 Material.

Product	**Supplier**
Plastic Ware	Greiner Bio-one
Cover Slips	WTC Binder
Eppendorf tubes	Eppendorf
Glass Ware	Schott
Object Slides	Menzel Gläser
Pipettes	Gilson
Pipettes Tips	Sarstedt
PVDF-Membranes	Millipore
Sterile Filter (Poresize: 0.2 µm & 0.45 µm)	Schleicher & Schuell
X-Ray Films	Typon Imaging AG

4.13 Software

Adobe Photoshop 6.0, Bio Edit, Clone Manager Professional 9, ImageJ, LAS software, MS Office and Quantity One.

5. Methods

5.1 Methods with cells

5.1.1 Culturing

Sf9 cells were cultured in 10 cm dishes with pre-warmed Sf9 medium and kept in water saturated atmosphere at 27ºC. Cells were splitted every two to three days, normally 1:3, scratching the cells, resuspend them in the dish and transferred them to a sterile 15 mL tube. Cells were centrifuged 3 min at 800 rpm, the old medium was aspired and new medium was added. The cells were then seeded with the appropriate dilution.

Raw 264.7 cells were cultured in 10 cm dishes with pre-warmed medium and kept in water saturated 95% air / 5% CO_2 atmosphere at 37 ºC. Cells were splitted every two to three days, normally 1:5, scratching the cells, resuspend them in the dish and transferred them to a sterile 15 mL tube. Cells were centrifuged 3 min at 800 rpm, the old medium was aspired and new medium was added. The cells were then seeded with the appropriate dilution. To analyze the cells by staining in slides, the plates needed to be coated. Coverslips and 500 µL of 0.1 % Gelatine per well were added to the plate and incubated for 2 hours at 37 ºC. After this time the Gelatine was aspired and the cells were seeded.

HEK 293T cells were cultured in 10 cm dishes with DMEM + 7.5% v/v CS and kept in water saturated 95% air / 5% CO_2 atmosphere at 37ºC. Cells were splitted every two to three days, normally 1:5, adding 5 mL of PBS to wash them, after aspiring the medium, and 1 mL of Trypsin EDTA, after aspiring the PBS, until the cells were detached. The reaction of Trypsin is stopped by adding 4 mL of fresh medium. The cells were resuspended and then centrifuged, in a clean sterile 15 mL tube, 3 min at 800 rpm. The medium was aspired and new medium was added. Cells were seeded using the desired dilution.

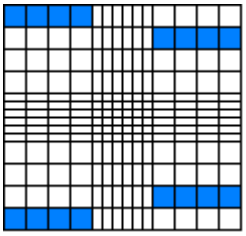
5.1.2 Unfreezing and refreezing of cells

To unfreeze one vial of cells was removed from liquid nitrogen. The vial was thawed in a 37 ºC water bath. After thawing, the entire vial was sprayed with 70% EtOH. The cells in the vial were gently suspended and transferred to a sterile 15 mL tube. 5 mL of pre-warmed medium were added slowly and the cells were centrifuged for 3 min at 600 rpm. The supernatant was aspired and the pellet was resuspended in 10 mL of fresh medium. The suspension was transferred to a 10 cm dish and the cells were incubated in the appropriate atmosphere.

Only well growing cells with good viability and low passaging number were used for freezing. The cells were harvested and centrifuged. The pellet was resuspended in 1 mL of specific medium and transferred to a sterile cryovial. The cryovial was transferred to an Isopropanol box and stored at -80 ºC to allow slow freezing. After 24-72 hours, cells were transferred to liquid nitrogen storage.

5.1.3 Counting of cells

If necessary for the experiment, cells were counted before seeding into well-plates/dishes. After transferring the cells to a sterile 15 mL tube, 10 µL of the suspension were added to a Neubauer chamber (Figure 34).



The cells in the blue squares (Figure 34) were counted and the total number was multiplied by 1 x 10^4 to receive the concentration of cells per mL.

5.1.4 Transfection of Sf9 cells

A clean plasmid preparation was needed for each transfection sample. 7x10^5 cells/mL were seeded in each dish (3.5 cm dishes). The cells were incubated at 27ºC until they stick to the dish (≥15 min). One negative control was used to compare transfected cells. In the meantime, 2 µL of BaculoGold DNA was combined with 1 µg of the plasmid DNA in a clean sterile tube and mixed. After 5 min, 0.4 mL of the Transfection Buffer B were added. The medium in the dish with the attached cells was aspired and 0.4 mL of the Transfection Buffer A were added. The sample in tube was added to the dish dropwise and mixed. The dishes were incubated at 27ºC for 4 h. After that time the supernatant was aspired and the cells were washed in 1.5 mL of fresh medium and were kept with 1.5 mL of fresh medium in the appropriate atmosphere for 5 days.

After 5 days, the cells were collected and centrifuged (2000xg, 5 min). The supernatant was then transferred to another clean sterile tube and stored at 4 ºC.

Transfection supernatants should be amplified to produce high titer virus stocks that are used for recombinant protein production.

5.1.5 Transfection of HEK 293T cells

One day before transfection, the cells were seeded with low density in 10 cm dishes. First 500 µL of sterile H_2O were added to a 14 mL tube and 5 µg of appropriate plasmid DNA were added. 500 µL of 2x HBS-Buffer were added to the mixture and mixed by vortexing. 50 µL of sterile 2.5 M $CaCl_2$ were added dropwise while vortexing the tube. The samples were incubated 15 min at room temperature to allow DNA precipitation. Meanwhile, 10 µL of chloroquine (25 mM) were added to the cells. After the incubation time, 1 mL of DNA from the tubes was added to the cells, drop by drop and the dishes were incubated 6-8 hours at 37ºC. After this time, the medium was aspired and new medium was added to the cells. The cells were then incubated 2 days at 37 ºC.

5.2 Recombinant protein expression with BEVS

5.2.1 Amplification of Baculovirus

For each amplification MOI 1 was used. The volume of the needed virus supernatant to infect the cells was calculated as follows:

$C = \frac{n}{V}$, where C is the virus titer and n is the number of the cells in the dish.

If the virus titer was unknown (transfection supernatants), the cells were infected with 200 µL. In this case the number of the cells in each 10 cm dish was $8{,}8x10^6$.

$5x10^6$ cells were seeded in one 10 cm dish and the cells were kept in the appropriate environment until they were attached to the dish. After this time, the cells were infected with the virus and incubated at 27ºC during 5 days.

After 5 days, the supernatant was collected by centrifuge the culture medium in the dish 5 min at 2000 x g. The supernatant was stored at 4ºC.

To determine the titer of the virus, cells were seeded in a 96 well-plate ($2.5x10^5$ cells/mL in each well) and, after they were attached, the cells were infected with different dilutions of the virus in duplicate. Normally, the dilutions used were 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}. The well-plate was incubated at 27ºC during 2 days and then the cells were analyzed by fluorescence microscopy after antibody staining.

5.2.2 Expression of the protein

Initially the time of expression for each protein had to be determined. $2.5x10^6$ cells were seeded in 6 cm dishes and transduced with the virus concentrate carrying the appropriate construct at MOI 4. One negative control was used (without infection) and for each construct

3 dishes were infected (24, 48 and 72 hours). The cells were then incubated at 27ºC. After the 24 h the negative control dishes and the 24 hours dishes were removed from the incubator and the cells were counted. The lysis was done with 2xSDS buffer or Sf9 lysis buffer. The cells were centrifuged 3 min at 800 rpm and the supernatant was aspired. The sample with lower cell number was resuspended in 100 µL of 2x SDS buffer. In the other sample the addition of 2x SDS buffer needed to be in the same proportion of the number of cells. The samples were boiled 5-10 min and stored at -20 ºC until analysis. In the next days the procedure was the same but the addition of 2x SDS was related to the 100 µL added in the first day. The samples were then analyzed by western blot for target protein expression.

After determination of the best expression time, the cells were seeded in 10 cm dishes (10 dishes for each expression). The number of cells seeded was $8.8x10^6$ per dish. Each dish was infected with the virus concentrate at MOI 4 and then the cells were incubated at 27ºC.

5.2.3 Sf9 Lysis

The transduced cells were harvested, counted and the total cell number was calculated. The cell suspension was centrifuged (5 min at 800 rpm), the supernatant was aspired and the pellet was resuspended in lysis buffer (1 mL lysis buffer / $2x10^7$ cells). The lysate was incubated for 20 min at 4ºC under constant rotation and then centrifuged 30 min at 13000 rpm at 4ºC (Thermo centrifuge, Rotor SS34). The supernatant was transferred to clean Eppendorf tubes and stored at -80ºC (after shock freezing with liquid nitrogen) for long-term storage or at -20ºC for overnight storage.

5.2.4 Purification of the protein

For all proteins was used FPLC (Fast protein liquid chromatography) technique. Two different columns were used. For the His column, the column was washed with 50 mM NaPi (pH 8) and 1 M NaCl after addition of the protein. This connects the protein to the niquel that stay stacked to the membrane in the column. After this, starts the second washing step with 0.5 M Imidazol, 0.5 M NaCl and 50 mM $NaH_2PO_4.H_2O$. This separate the protein of the niquel and the protein was collected in glass tubes. The same was made for GST column, with 50 mM Tris pH 8 and 10 mM GSH to wash the column.

4 kDa dialysis membranes were swelled in ddH_2O and the fractions with the protein were added to the membrane. Dialyis was performed 4x each one with a new basin during 1.5

-3 hours (the last one was always made over night). In the final dialysis step the buffer was supplemented with 10% Glycerin. All steps were performed at 4ºC under mixing.

The protein was collected from the dialysis membrane and aliquoted in clean 1.5 mL Eppendorf tubes. The protein was shock-freezed in liquid nitrogen and stored at -80ºC.

5.3 Microscopy

5.3.1 Staining of Sf9 cells

The cells were washed 3x with PBS^{++} (100 µL/well) and then fixed for 15 min with 4% PFA. Then the cells were washed 1x with PBS^{++} and permeabilized for 5 min with 0.5% Triton X-100 in PBS. Again the cells were washed 1x with PBS^{++} and blocked for 10 min. The first antibody was added (α-NEF 1:250 in blocking solution) and the well-plate was incubated for 45 min at room temperature in the dark. After this time the cells were washed 2x with PBS^{++} and blocked again for 10 min. The second antibody was added (α-Mouse IgG-Cy3 1:300 in blocking solution) and the well-plate was incubated for 45 min at room temperature in the dark. The cells were washed 2x with PBS^{++} and incubated for 15 min with Hoechst (1:10000 in PBS^{++}) at room temperature in the dark. The cells were washed 2x with PBS^{++} and then stored at 4ºC in PBS^{++} until analysis.

5.3.2 Baculovirus Titer determination

The baculovirus titer was determined by staining of infected Sf9 cells for target protein expression and subsequent analysis by fluorescence microscopy. The properties of the channels were adjusted to the controls and then the dilution was chosen for quantification with less than 10% of infected cells. Three images were taken for each well and the total cell number in each image was determined as well as the infected cells only. The titer was calculated as shown below.

$$MOI = -\ln\left[1 - \frac{M_2}{M_1 + M_2}\right]$$

, where M_1 is the number of uninfected cells and M_2 is the number of infected cells.

The MOI was calculated for each well and then the mean MOI was used to calculate the titer of the virus as described below.

$$Titer\,(pfu) = \frac{MOI \times 2 \times 10^5}{dilution}$$

, and the result come in mL^{-1}.

To amplification was repeated until a titer of $2x10^8$ pfu/mL was reached.

5.3.3 Staining of Raw 264.7 and HEK 293T cells

The cells were washed 3x with PBS^{++} and fixed for 15 min at room temperature with 4% PFA. After this time, the cells were washed again 3x with PBS^{++} and permeabilized for 3 min with 0.4% Triton X-100 in PBS (if the procedure was only for staining of the extracellular particles the cells were not permeabilized). The cells were washed again 3x with PBS^{++} and incubated for 10 min with blocking solution. After this time, the blocking solution was aspired and 25 µL of the first antibody was added to each well (1:100 – 1:400 in blocking solution). The well-plate was incubated for 1 h at room temperature in camera humidified box. After this incubation time, the cells were washed 3x with PBS^{++} and blocked again for 10 min. The second antibody was added (1:100 – 1:200 in blocking solution) and the well-plate was again incubated in the humidified box for 45 min at room temperature. Finally, the cells were washed 3x with PBS^{++}.

5.3.4 Staining of the beads

For direct labeling of the protein-coated polystyrene beads, the beads were incubated for 30 min at 37ºC under constant shaking with CFSE 1:1000 in PBS. Afterwards, the beads were washed three times with PBS to remove residual CFSE. After each washing step the beads were centrifuged at 1000xg for 1 min and the supernatant was discarded.

To stain the extracellular beads in the cells, after fix them, some protein don't have an antibody, as for Albumin as example. Thus, for the intra and extracellular assay, the beads were biotinilated with Sulfo-NHS-LC-Biotin (Thermo Scientific), 0.5 mg/mL in PBS, during staining with CFSE.

5.4 Molecular-biological methods

5.4.1 Polymerase chain reaction (PCR)

All PCRs were performed under "hot-start" conditions. A typical sample recipe is described in Table 19.

Table 19 Typical recipe for PCR.

Reaction mix		Enzyme mix	
H_2O	32 µL	H_2O	8 µL
10xBuffer (+ $MgCl_2$)	4 µL	10xBuffer (+ $MgCl_2$)	1 µL
Primer 1 (5')	1 µL (10 pM)	Pfu-Polymerase	0.5 µL

Primer 2 (3')	1 µL (10 pM)	Taq-Polymerase	0.5 µL
Template	1 µL (~100 ng)	Total	10 µL
dNTPs	1 µL (20 mM)		
Total	40 µL		

Reaction and Enzyme mix were always prepared separately. The program of the PCR was conducted as described in Table 20.

Table 20 PCR program.

1. Heat Lid to 110ºC.
2. Initial denaturation of the template DNA: 94ºC for 1 min.
3. Pause at 94ºC (addition of the enzyme mix to the reaction mix).
4. Start Loop, 34x.
5. Denaturation of the DNA: 94ºC for 15 seconds.
6. Annealing: 50 ºC – 65 ºC for 25 seconds (depending on the melting temperature of the used primers. Generally, an annealing temperature of about 6°C below the melting temperature was used)
7. Elongation: 72ºC for 1 min – 2 min per 1kb.
8. Close Loop.
9. Final elongation: 72ºC for 8 min.
10. Store forever at 8ºC (until further processing).

5.4.2 Agarose Gel-Electrophoresis

Depending on the size of the analyzed DNA gels with a agarose concentration between 0.7% and 2% were used. A 0.7% gel will show good separation (resolution) of large DNA fragments (5–10 kb) and a 2% gel will show good resolution for small fragments (0.2–1kb). Typically, a band is easily visible if it contains about 20 ng of DNA, depending on the number and size of the bands expected. The suitable amount of agarose was solved in 1x TAE buffer by heating and poured into the gel chamber after cooling down to about 50°C. Slots were formed by a comb. The DNA samples were mixed with 6x GEBS, applied to the slots and then separated in 1x TAE at 90 V (for analytical gels) or 60 V (for preparative gels). A DNA

standard (Gene Ruler DNA Ladder) that was also applied in one slot facilitated a direct comparison of the fragment sizes. DNA bands were visualized by staining the gel in an ethidium-bromide (EtBr) bath (10 min), as after intercalation into the DNA, EtBr fluoresces under UV light.

5.4.3 Recovery of DNA from agarose gels (Gel extraction)

The band of interest was visualized by ethidium bromide staining on a UV light-box (a trans-illuminator). As the UV mutagenizes the DNA at a measurable rate, the time that the gel was exposed at UV light was kept at a minimum. A scalpel blade was used to cut out the band of interest and the fragment was placed in a clean 1.5 mL Eppendorf tube. The DNA was purified with the help of a kit (QIAquick gel extraction/PCR purification) according to the manufactures instructions.

5.4.4 PCR-Products purification

The PCR fragments were purified with the help of a kit (QIAquick gel extraction/PCR purification) according to the manufactures instructions.

5.4.5 Restriction digest of DNA

Depending on the restriction sites appropriate restriction enzymes and buffers were used. A typical recipe used in this procedure is described in Table 21.

Table 21 Typical recipe for DNA digestion.

DNA	3 µg
Buffer (10x)	1 µL
Restriction enzyme 1	1 µL
Restriction enzyme 2	1 µL
H_2O	dependent
Total	10 µL

If necessary, the samples were supplemented with 100 µg/mL BSA. Depending on the total volume of the recipe the volume of buffer was adapted. The volume of the enzyme used depended on the time of digestion, the amount of DNA and the efficiency of the enzyme in

the used buffer. Typically, the time of digestion was 3 h at 37ºC. If the time of digestion was over night the amount of used enzyme was normally 0.3 µL. The digestion fragments were analyzed by agarose gel electrophoresis after purification with the help of a kit (QIAquick gel extraction/PCR purification).

5.4.6 Quantification of nucleic acids

Quantification of DNA was conducted by a spectrophotometer (NanoDrop) using 1.2 µL of the sample. Sample buffer only was used for blank measurement.

5.4.7 Dephosphorylation of DNA fragments with alkaline phosphatase

To dephosphorylate the 5´-positions of the backbone DNA, the samples were treated by alkaline phosphatase. A typical recipe used is described in Table 22.

Table 22 Typical recipe for alkaline phosphatase treatment.

Phosphatase Buffer (10x)	depending
Alkaline Phosphatase	1 µL
Volume of digestion	All

The samples were mixed and kept for 15 min at 37 ºC and then heat inactivated at 65ºC for 10 minutes.

5.4.8 Ligation of prepared vectors and DNA fragments

The digested and dephosphorylated backbone DNA (vector) as well as the digested cDNA insert were ligated using T4 Ligase. In all the experiments 10 and 40 fmol of vector and insert, respectively, were used. A typical recipe used is presented in Table 23.

Table 23 Typical recipe for ligation.

Vector	10 fmol
Insert	40 fmol
T_4 Ligase	1 µL
T_4 Ligase Buffer (10x)	1 µL

H_2O	dependent
Total	10 µL

As a negative control always a ligation sample was made with the vector only. After adding all the volumes to clean 1.5 mL Eppendorf tube, the samples were kept for 30 min at room temperature. If the ligation was made over night, the samples were kept at 4ºC. Afterwards, the ligation samples were transformed in *E. coli* Nova Blue.

5.4.9 Transformation

100 µL of chemically competent cells (Nova Blue) were added to 14 mL tubes (one tube for each sample) and the plasmid DNA to be transformed was added to the competent cells. The tubes were incubated on ice for 30 min and then heat shocked for 75 seconds at 42ºC. 1 mL of LB medium was added to each tube and they were incubated for 1 hour at 37 ºC under constant rotation (225 rpm). After this time, the whole volume in each tube was transferred to a clean Eppendorf tube and centrifuged at 3000 rpm for 3 min. The supernatant was discarded with exception of about 100 µL and the pellet was resuspended in the left supernatant. This volume was plated on an agar plate with the appropriate selection antibiotic (depending on the resistance of the transformed plasmids). The plates were then incubated at 37ºC over night.

5.4.10 Preparation of Plasmid DNA (Mini-prep)

Plasmid isolation from *E. coli* was conducted as described in Table 24.

Table 24 Isolation of Plasmid DNA.

1. The bacteria were scratched from the plate and suspended in 300 µL of buffer P1.
2. 300 µL of buffer P2 were added and the tubes were inverted 10 times.
3. 300 µL of buffer P3 were added and the tubes were inverted 10 times.
4. The samples were centrifuged for 10 min at 4 ºC (14000 rpm).
5. The supernatant was transferred to clean eppendord tubes and the pellet was discarded. To each sample 560 µL of isopropanol were added and the tubes were inverted three times.
6. Centrifuge for 15 min.
7. The supernatant from this centrifugation was aspired. The pellets were washed with 500 µL of EtOH each and centrifuged again for 5 min.
8. The supernatant was aspired again and a new centrifugation took place during 2 min.

9. The pellet was then incubated 20 min at 37 ºC (to dry the left EtOH).
10. After this time, the pellet was resuspended in 40 µL of TE Buffer.

5.4.11 DNA Sequencing

For sequencing, 2 µg of Plasmid DNA were added to a clean Eppendorf tube and sterile H_2O was added to a total volume of 30 µL. Samples were sent to GATC, Konstanz and the obtained sequence was compared afterwards with the aid of the clone manager.

5.5 Protein-biochemical analysis

5.5.1 SDS-Polyacrylamide gel-electrophoresis (PAGE)

The concentration of acrylamide determines the resolution of the gel - the greater the acrylamide concentration so much the better the resolution of lower molecular weight proteins. The lower the acrylamide concentration so much the better is the resolution of higher molecular weight proteins. Depending on the size of the protein of interest different concentrations of acrylamide were used.

The gel used for protein separation was prepared in two steps: the separating gel was prepared first and added into the space occupied by the cassettes, overlayed with ddH_2O and left by 20 min until it get polymerized. The second gel, the stacking gel, was added into the left space between the cassettes and the teeth were added into this gel. 20 more minutes were needed until the gel get polymerized. The samples were mixed with 2x SDS-buffer and were boiled together with a protein standard for 5-10 min at 95°C. The samples and the protein standard were loaded onto the gel and the separation was conducted at 120 V for 2 h. A typical recipe of the gel is presented in Table 25.

Table 25 Typical recipe for preparation of a SDS-PAGE gel.

Separation gel (12.5 %)	4.3 mL dH_2O; 2.5 mL Tris (1,5 M; pH 8,8); 3.1 mL Acrylamide (40%); 50 µL Sodium dodecyl sulfate (SDS; 20%); 30 µL Ammonium Persulfate (APS; 10%); 15 µL TEMED
Stacking gel (5 %)	6.15 mL dH_2O; 2,5 mL Tris (0,5 M; pH 6,8); 1.25 mL Acrylamide (40%); 50 µL Sodium dodecyl sulfate (SDS; 20%); 30 µL Ammonium Persulfate (APS; 10%); 15 µL TEMED
Running buffer	25 mM Tris-HCl; 192 mM Glycine; 0.1% SDS; 2 L dH_2O

5.5.2 Western Blot (Semy-Dry)

For Western Blots, PVDF membranes were used which were activated in Methanol first and than rinsed in Anode buffer. Three filter papers were wetted in Anode buffer and put in the Semy-Dry Blotting chamber. After adding the pre-activated membrane, the gel which was rinsed in Cathode buffer was put on top. Finally, three filter papers were added which were wetted in Cathode buffer before. The transfer was conducted at 75 mA per membrane for 2 hours. After this time, the membrane was stained with Coomassie blue and de-stained afterwards. The bands of the protein standard were marked on the membrane with an usual pen. After this, of the membrane was washed once in TBS-T and than it was incubated in blocking solution for 1 h at room temperature to prevent non-specific antibody binding in the following steps. After blocking, the membrane was incubated with the primary antibody (generally between 0.5 and 5 µg/mL in blocking solution) at 4°C over night.

After washing the membrane three times with TBS-T to remove unbound and non-specific bound primary antibody, the membrane was exposed to another antibody, directed at a species-specific portion of the primary antibody. The secondary antibody was diluted 1:3000 in TBS-T. It is usually linked to biotin or to a reporter enzyme such as alkaline phosphatase or horseradish peroxidase. This means that several secondary antibodies will bind to one primary antibody and enhance the signal. Usually the secondary antibody was incubated with the membrane for 1 hour at room temperature. After this time the membrane was washed three times with TBS-T and incubated for 2 min with 10 mL of ECL-medium and 3 µL of 35% H_2O_2. The membrane was then closed into an X-ray cassette and developed in a darkroom using a AGFA Curix60.

5.5.3 Cell Lysis

All steps were performed at 4°C. The culture medium was discarded and PBS was added to wash the cells. The PBS was then aspired, taking care to not aspire the cells, and 1 mL of RIPA buffer was added. The cells were maintained 2-5 min in this buffer and were scratched from the dish. The lysates were then transferred to clean Eppendorf tubes. To break the DNA, the lysates were passed 6-10 times through a fine needle using a syringe. After this, 50 µL of sepharose beads were added to the tubes and get mixed. The tubes were left 5 min in the shaker and centrifuged for 10 min at 13000 rpm and 4ºC. The supernatant from the centrifugation step was collected to a new tube and the lysate was stored at -80ºC.

5.5.4 Protein concentration determination

To determine the concentration of purified proteins a SDS-PAGE procedure was used. The samples with unknown protein concentration were added to the gel with samples with known concentration (usually BSA in different concentrations). After coomassie staining and de-staining of the gel the band of the protein was compared to the bands of BSA and the closest match was used to calculate the concentration of the purified protein. Alternatively, the concentration was determined with a spectrophotometer (NanoDrop) against an appropriate blank.

5.6 *In Vitro* Kinase Assay

In this assay, two substrates were used, GST-p130^{Cas} and GST-FAK. The adaptor protein p130^{Cas} (Cas) has been shown to be involved in different cellular processes including cell adhesion, migration and transformation. This protein has a substrate domain with up to 15 tyrosines that are potential kinase substrates, able to serve as docking sites for proteins with SH2 or PTB domains. Cas function is dependent on tyrosine phosphorylation of its substrate domain and can be used to study the activity of kinases *in* vitro. This substrate was already shown to be useful to test the kinase activity of FAK [Cas as substrate]. The C-terminal part of substrate domain of this substrate is from rat p130^{Cas} (a.a. S^{325} to R^{516}). FAK can also be phosphorylated by other kinases, being useful to study the kinase activity *in vitro*. This substrate is a peptide around autophosphorylation site of human FAK (a.a. N^{378} to T^{406}).

In clean Eppendorfs were added the kinase, the substrate and the ATP, dependent from the control to the kinase buffer. The samples were then incubated 30 min at 30ºC. In the meantime, Glutathione beads were washed three times at 2500xg, 1 min each wash, at 4ºC in PBS and resuspended afterwards in kinase buffer. After the incubation time, the samples with GST-FAK substrate were mixed with 2xSDS buffer 1:2 and stored at -20ºC. The samples with GST-p130^{cas} substrate were mixed with Glutathione beads 1:2 after stopping the kinase activity with 1 mM EDTA, and incubated 1 h at 4ºC under constant rotation. After this time, the samples were centrifuged and the supernatant was transferred to new Eppendorf tubes and mixed with 2xSDS buffer 1:2. The beads were then washed three times with PBS, resuspended in kinase buffer and mixed with 2xSDS buffer 1:2. All samples were then boiled at 95ºC and loaded onto a 12.5% SDS gel, and run at 120 V. The gel was then transferred to a membrane by Semi-Dry Western blotting. The antibodies used in this assay were the

Phospho-Tyrosine to detect the phosphorylation of the kinase and the substrate, the GST to detect the substrates and NEF to detect the kinases.

5.7 Covalent coupling of proteins to latex beads

For each protein to be coupled, 200 µl of a 2.5% solution of carboxylated polybead 2 µm microspheres (in short: latex beads) were added to a 2 mL Eppendorf tube with 1 mL of carbonate buffer. The beads were mixed with this buffer and then centrifuged for 6 minutes at 10000 rpm. The supernatant was discarded and the latex beads were resuspended in 1 mL of carbonate buffer and washed again. After this step, the latex beads were washed four times with 1 mL of MES buffer. After the fourth washing step the beads were resuspended in 0.625 mL MES buffer and 0.625 mL of a freshly prepared 2% carbodiimide solution were added, drop by drop. The latex beads were then kept, at a maximum of 4 h, on a rotor at room temperature, under constant inverting. After this time, the latex beads were centrifuged. The supernatant was discarded and the beads were washed three times with 1 mL MES buffer. Thereafter, the latex beads were resuspended in 600 µL of borate buffer and 200-400 µg of protein to be coupled were added. The beads were then incubated on the rotor at room temperature over night.

In the next day, 25 µL of ethanolamine solution were added and the bead solution remained inverting for 30 minutes on the rotor. This way, the latex beads which have not reacted with the protein to be coupled will be blocked. Thereafter, the latex beads were centrifuged for 10 minutes and resuspended in 1 mL of borate buffer with 1 mg/mL BSA and incubated for 30 minutes at room temperature on the rotor. The latex beads were washed once with 1 mg/mL BSA in borate buffer and then resuspended in 200 µL of storage buffer.

To control if the coupling worked, 10 µL of the beads were added to a clean Eppendorf tube and washed 3x with PBS-T. After these washing steps, the beads were stained for the proteins that were coupled and the fluorescence intensity was measured with a spectrofluorometer.

6. Literature

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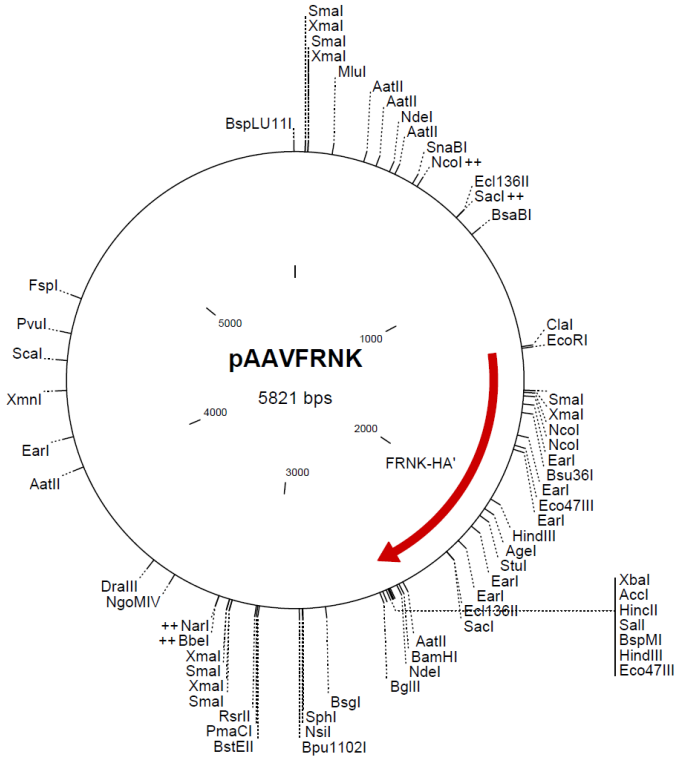
Plasmid maps

1. pAAVFRNK



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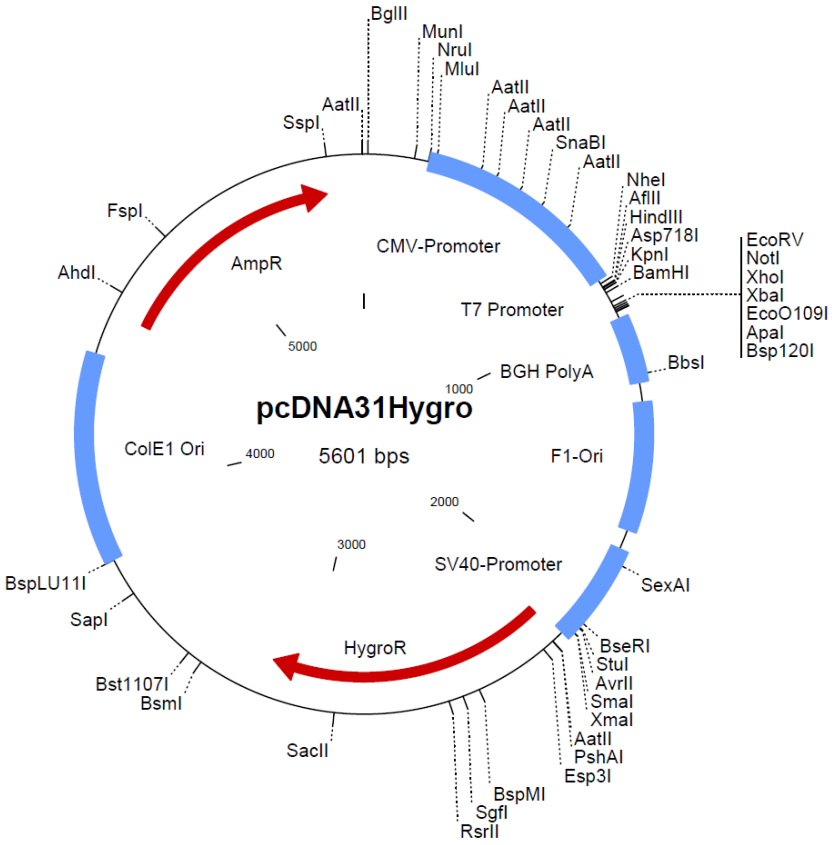
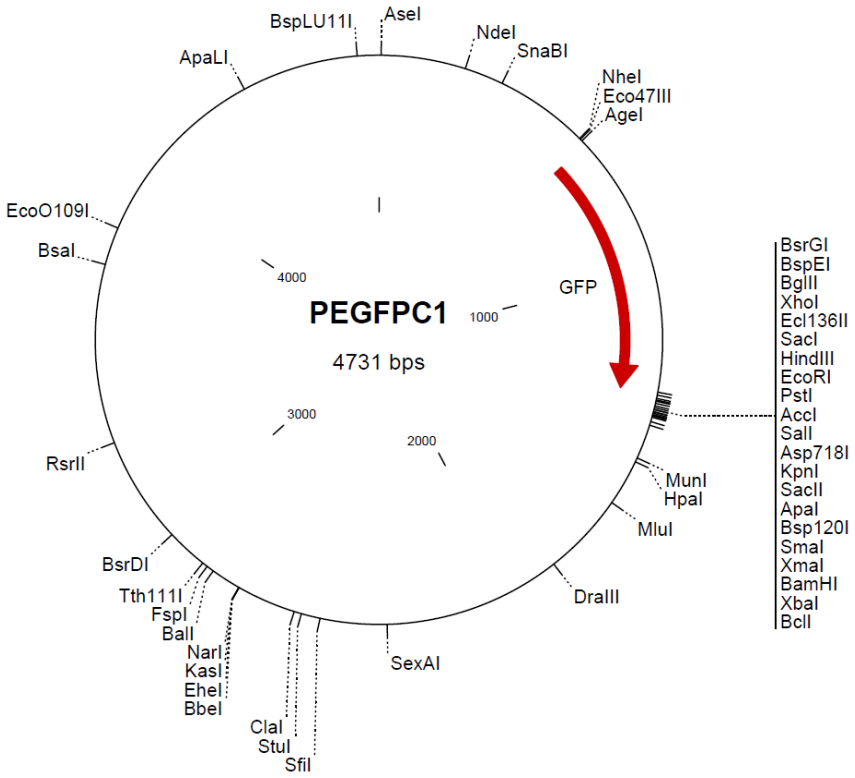


3. pEGFP-C1



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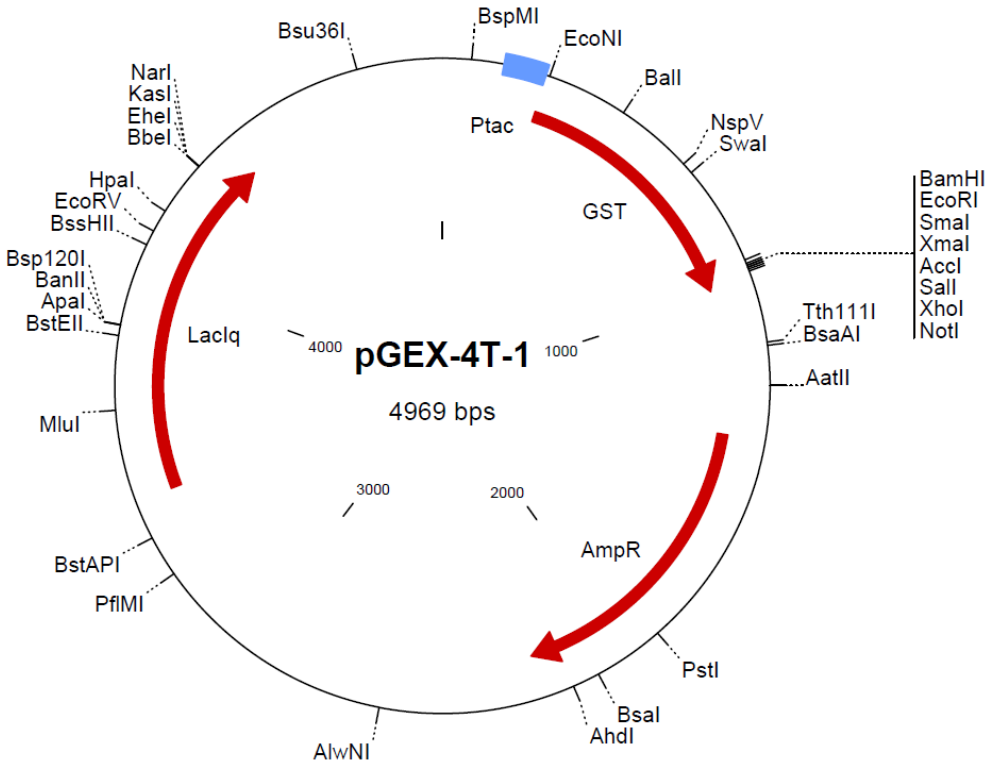

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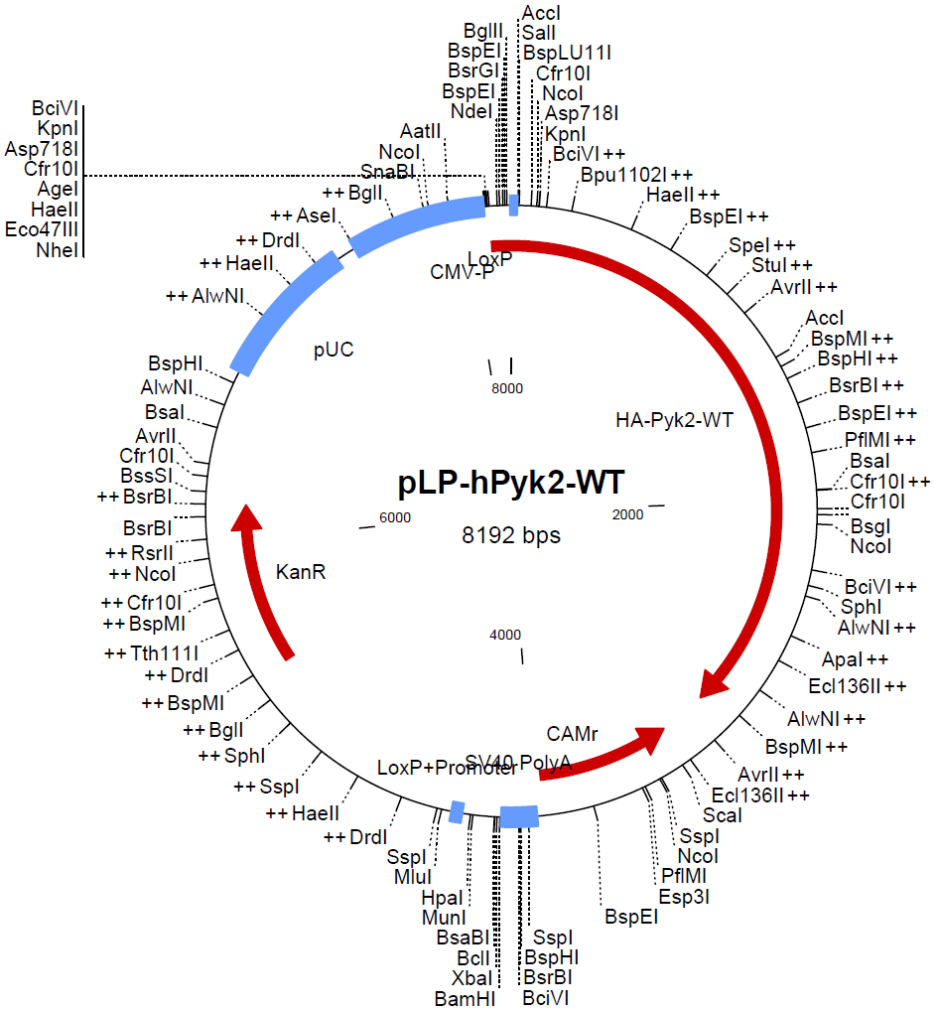

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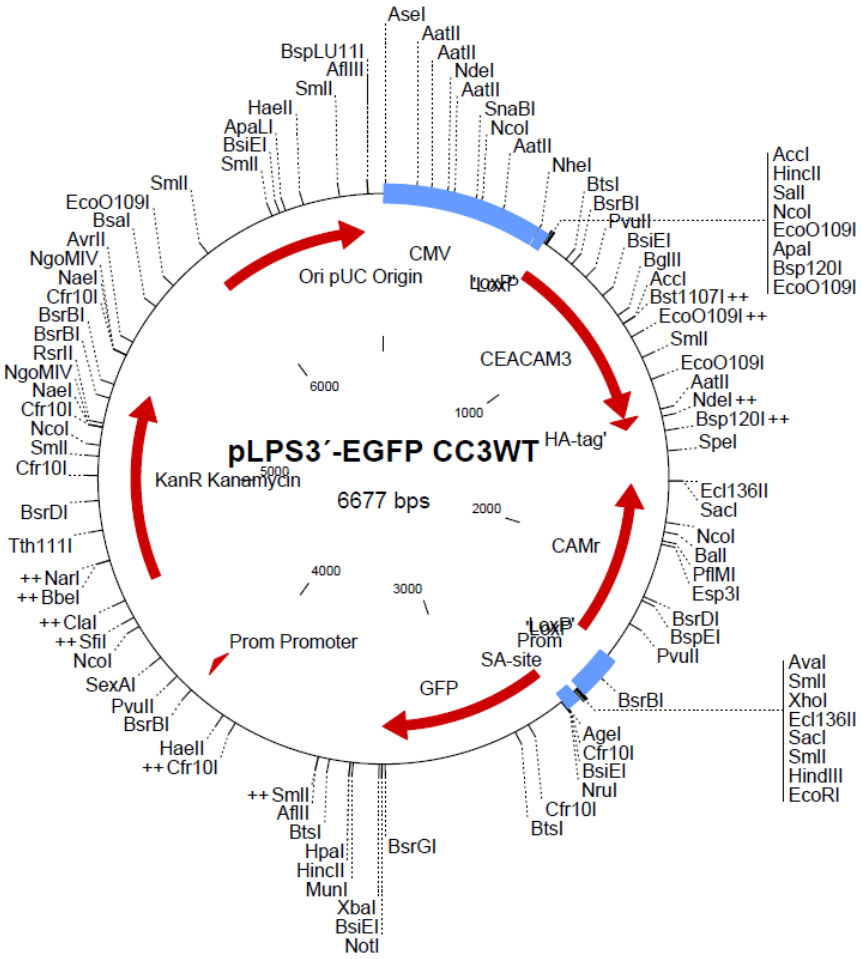
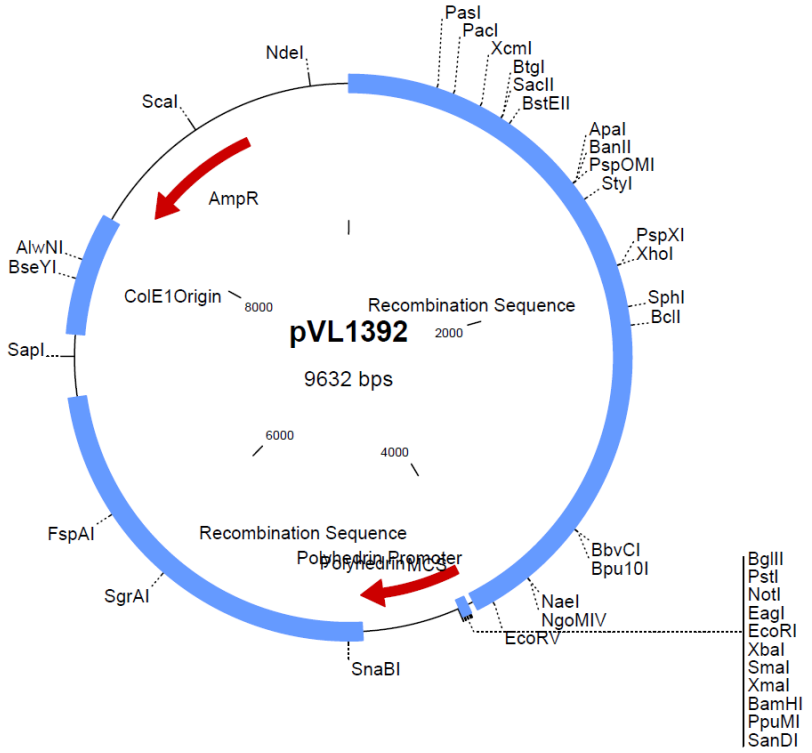


7. pVL1392



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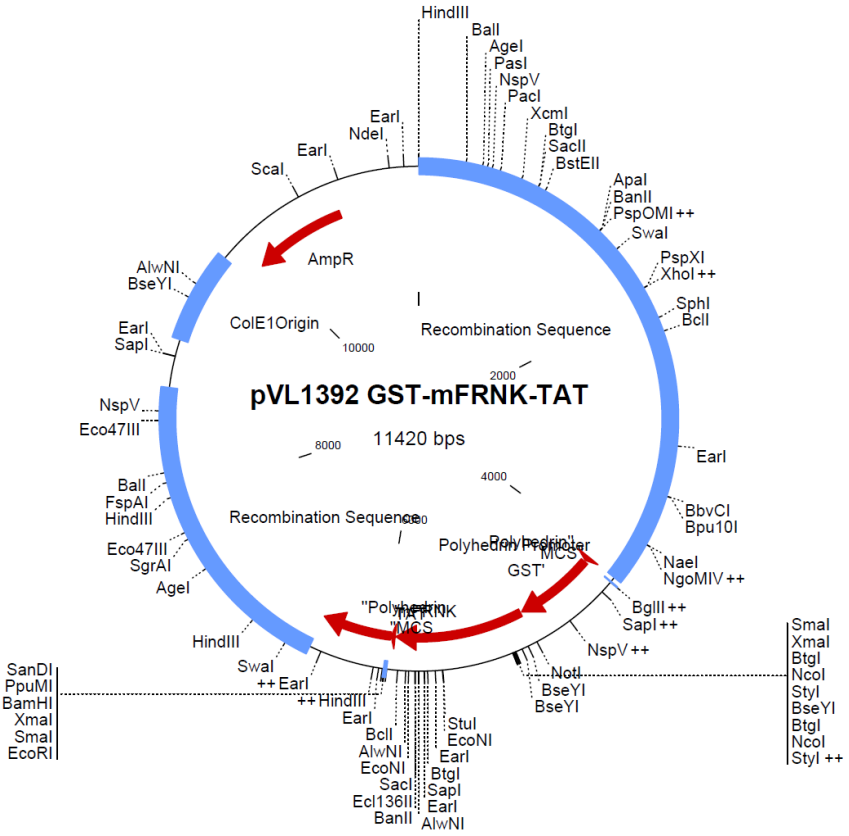

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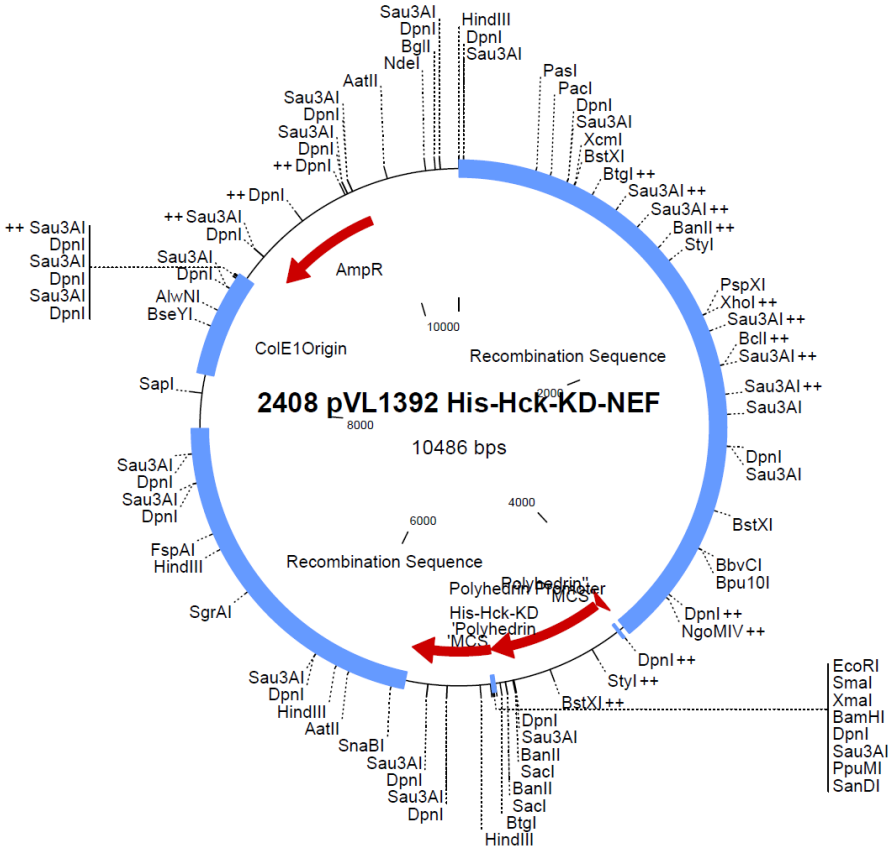

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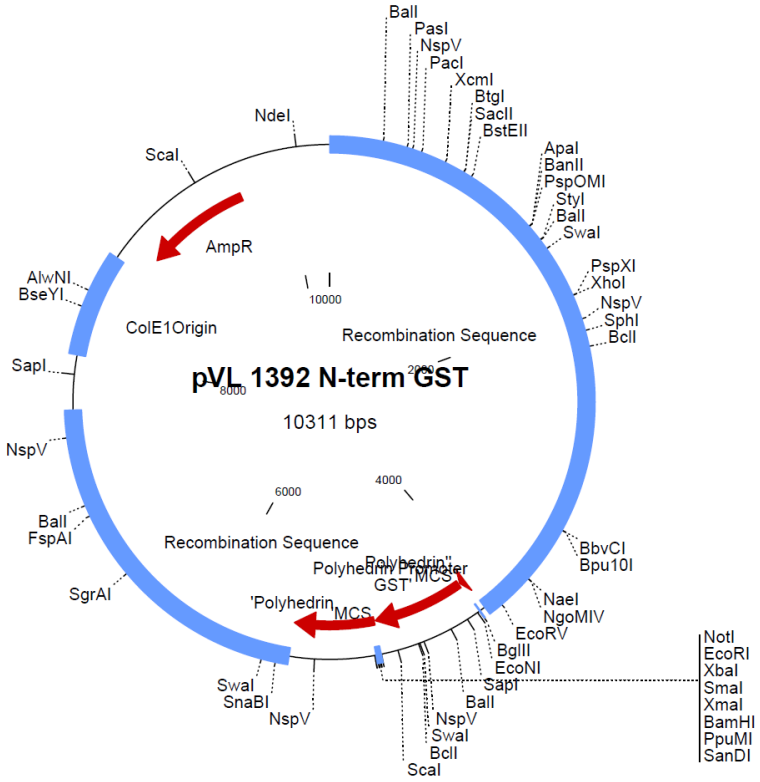

11. pVL1392 N-term GST Hck-KD