Assessing the Role of Amino Acid Residues in The Transmembrane Domains Of The α- and γ-Chains Of The High-Affinity Receptor Complex For Immunoglobulin E In Signal Transduction

By

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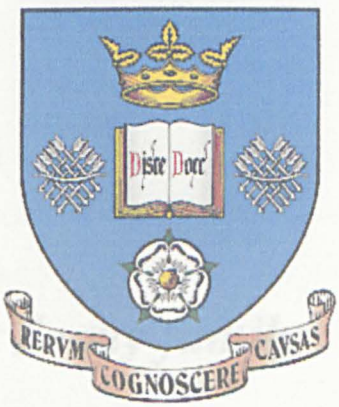

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To my family

Abstract

The dramatic rise in the incidence of allergic/asthmatic disorders in the past three decades has placed a major socio-economic burden on global health care. Exocytosis of mediators causing allergic responses follows the binding, and subsequent receptor cross-linking by cognate allergen of immunoglobulin (Ig) E antibodies to Fc-receptors (FcεRI), expressed predominantly on mast cells and basophils.

Fc-receptors have an invariant predominantly hydrophobic amino acid motif (LFAVDTGL) in their transmembrane (TM) domain but contain a hydrophilic aspartic acid residue (D194). The function of this potentially energetically unstable residue in the TM of the human (hu) FcεRIα subunit was targeted by transfecting the Rat Basophilic Cell line (RBL-2H3.1) with cDNA constructs encoding the gene for native and mutant huFcεRIα subunits and assessing receptor expression and signalling events. RBL-2H3 transfected with cDNA constructs encoding a medium-sized polar residue huFcεRIα (D194T) demonstrated the formation of a functional rat/human chimeric receptor complex which, when activated via huIgE and antigen, supports mediator release, intracellular calcium mobilisation and tyrosine phosphorylation of γ-chain and Syk kinase. Transfection with mutant huFcεRIα subunit cDNA constructs encoding non-polar Ile (D194I) and V (D194V), larger sized polar Arg (D194R), smaller sized polar Ser (D194S) and non-polar Ala (D194A) abrogated surface expression of huFcεRIα and degranulation. An established RBL γ-chain deficient cell line served as a model for assessing α- and γ-chains interactions through the effect of mutations introduced into the γ-chain. Compared to parental RBL-2H3 cells, the RBL γ-chain deficient cells supported reduced levels of FcεRI expression and mediator release. Transfection of RBL γ-chain deficient cells with wild-type and mutant (T22A and T22S) γ-chain cDNA constructs restored FcεRI expression to levels observed in the parental cell line, but only partially restored mediator release, indicating a defective secretory response.

Collectively this study identifies D194 in FcεRIα as a potential target for developing future anti-allergic drugs that act by inhibiting the FcεRI signalling cascade.

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List of Abbreviations

AM	Acetoxymethyl ester
APC	Antigen presenting cell
BCR	B cell receptor
BMMC	Bone marrow-derived mast cells
BS	Buffered solution
BSS	Balanced salt solution
BSA	Bovine serum albumin
BtK	Bruton's tyrosine kinase
Ca^{+2}	Calcium ion
Cbp	Csk-binding protein
CD	Cluster of differentiation
cDNA	Complementary DNA
CDS	Cell dissociation solution
CRACs	Calcium release activated channels
CsK	COOH-terminal Src kinase
D194	Wild type human FcεRIα subunit
D194N	Human FcεRIα subunit with Asp→Asn
D194L	Human FcεRIα subunit with Asp→Leu
D194T	Human FcεRIα subunit with Asp→Thr

D194S	Human FcεRIα subunit with Asp→Ser
D194R	Human FcεRIα subunit with Asp→Arg
D194I	Human FcεRIα subunit with Asp→Ile
D194V	Human FcεRIα subunit with Asp→Val
DAG	1, 2-diacylglycerol
DCs	Dendritic cells
dH_2O	distilled water
DMEM	Dulbeco's Modified Eagles media
DMSO	Dimethyl Sulphoxide
DNP-HSA	Dinitophenol-human serum albumin
DOK	Downstream of Kinases
DPBS	Dulbeco's phosphate buffered saline
DTT	Di-thiothreitol
EC	Extracellular
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERK	extracellular regulated kinase
FACS	Flourescent activated cell sorting
FCS	Foetal calf serum
FcαRI	High-affinity IgA receptor
FcεRI	High-affinity IgE receptor

FcεRIα	High-affinity IgE receptor alpha subunit
FcεRIβ	High-affinity IgE receptor beta subunit
FcR-γ	Fc gamma subunit
FcγRs	IgG receptor
FcγRI	High-affinity IgG receptor
FcγRII	Low-affinity IgG receptor II
FcγRIII	Low-affinity IgG receptor III
FcγRIIIα	Low-affinity IgG receptor III alpha-subunit
FITC	Fluorescein isothiocyanate
fMLP	n-formyl-Met-Leu-Phe
FS	Forward scatter
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPI	Glycosylphosphatidylinositol
Grb-2	Growth factor receptor-bound protein 2
HRP	Horseradish peroxidise
humanαγγ	Chimera with huFcεRIα EC, rodent γTM and CT domains
huFcεRIα	Human FcεRI alpha subunit
huIgE	Human IgE
ICAM	Intracellular cell adhesion molecules
IFN-γ	Interferon- γ
Ig	Immunoglobulin
IgE/IgM/IgG	Immunoglobulin E/Immunoglobulin M/ immunoglobulin G
IL	Interleukin
IP	Immunoprecipitation
IP_3	Inositol-1, 4, 5-triphosphate

IR	Insulin receptor
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
JNK	Jun amino-terminal kinase
LAB	Linker for activation of B cells
LAT	Linker for activation of T cells
LPS	Lipopolysaccharide
LT	Leukotrine
MAFA	Mast cell function-associated antigen
MFI	Mean fluorescence intensity
MHC	Major histocompatability complex
mAb	Monoclonal antibody
MAPK	p-38 mitogen-activated protein kinase
mIg	Membrane immunoglobulin
mIgE	Mouse IgE
mIgM	Membrane-bound immunoglobulin M
N_2	Nitrogen
NIP-HSA	4-hydroxy-5-iodo-3-nitrophenyl-human serum albumin
NK	Natural Killer
NTAL	Non-T-cell activation linker
PAF	Platelet activating factor
PAMP	Pathogen-associated molecular pattern
PC	Poorly cytokinergic
PH	Pleckstrin homology
P13K	Phosphatidylinositol 3-kinase

PIP_2	Phosphatidylinositol-4, 5-biphosphate
PIP_3	Phosphatidylinositol-1, 4, 5-triphosphate
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PRR	Pathogen recognising receptor
PTK	Protein tyrosine kinase
PVDF	Polyvinyledene difluoride
RBL-2H3	Rat Basophilic Leukaemia sub-line 2H3
RBL-γ-	Rat Basophilic Leukaemia gamma chain deficient mutant sub-line
RBL-γ+	RBL-γ- cell line transfected with wild-type gamma subunit
rFcRγ	Rodent gamma construct
RIPA	Radioimmunoprecipitation assay
SCF	Stem cell factor
SDS	Sodium dodecyl sulphate
1-D SDS-PAGE	One-dimensional SDS-polyacrylamide gel electrophoresis
SH_2/SH_3	Src homology 2/Src homology 3
Shc	SH2-containing proto-oncogene
SHIP	SH2-containing inositol-5'-phosphatase
SHP	SH2-containing protein tyrosine phosphatase
SIT	specific immunotheapy
SLP-76	SH2-containing leukocyte specific protein of 76 kDa
SNAREs	Soluble NSF-attached protein receptors
SOCCs	Store operated calcium channels
SOS	Son of sevenless

SS	Side scatter
Syk	Spleen tyrosine kinase
T22A	RBL-γ- cell line transfected with mutant gamma subunit Thr→Ala
T22S	RBL-γ- cell line transfected with mutant gamma subunit Thr→Ser
TCR	T cell receptor
TE	Trypsin/EDTA
$Th_0/Th_1/Th_2$	T helper subsets
TLR	Toll-like receptor
TM	Transmembrane
TNF	Tumor necrosis factor
WB	Western blot

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INTRODUCTION

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Introduction

1.1: The Allergy "Epidemic"

Type I hypersensitivity or immunoglobulin (Ig) E-mediated allergy is a broad term describing undesirable immune responses that follows the encounter with seemingly non-pathogenic antigens referred to as allergens. The first historical reference to an allergic response dates to 2640B.C and depicts, on a pylon in Luxor, Egypt, the death of a Pharaoh from an anaphylactic shock following a bee sting [http://www.slideshare.net/inemet/anaphylactic-reactions]. Immunoglobulin E and mast cells/basophils are now recognised key players in the allergic response (Williams and Galli, 2000). Immune response of the IgE isotype play an important role in the response to and elimination of parasites from intestines of the host, a function that has been authenticated by various studies in which it has been demonstrated that chronic/heavy helminthic infestation, characterised by high levels of parasite specific IgE, protects against allergy (Yazdanbakhsh and Matricardi, 2004). The Viennese paediatrician Clemens von Pirquet in 1906 coined the term *allergy* from the Greek words *allos* meaning other and *ergon* meaning work after he noticed the fact that some of his patients were sensitive to apparently innocuous entities like dust, pollen and certain foods while the term anaphylaxis was first used by Portier and Richard in 1902 to explain an unexpected effect that occurred during immunization of dogs.

The various ailments associated with allergy are atopic dermatitis, urticaria, allergic rhinitis, asthma and the potentially fatal condition of anaphylaxis (Corry and Kheradmand, 1999). The incidence of allergic diseases is on the rise globally (Pawankar et al., 2008) specially in industrialized countries where 10-30% of the population suffer from diverse allergic symptoms (ISAAC Committee, 1998, Sole et al., 2001) The rise has been rapid and started between 1960-1970 with an ongoing increase witnessed in the 1980s and 90s to levels that have attained almost epidemic proportions such that allergic diseases are being labelled as the epidemic of the 21st

century (Isolauri et al., 2004). The increase witnessed in westernized societies is in the range of 75-100% in cases of asthma (Mannino et al., 2002) and 150% in atopic diseases (Wichmann, 1996). Similar trends are now being observed in the most populous continent Asia that is home to fast emerging economies. According to recent epidemiological data by ISAAC III (International Study of the Asthma and Allergies in Childhood) the incidence of Asthma and allergic rhinitis is also on the rise in low and middle income countries (Bjorksten et al., 2008) with incidence for asthma increasing from 0.8% to 29% and for allergic rhinitis from 5% to 45% of the population in many Asian countries (Pawankar et al., 2008).

The pathology of allergic diseases is not fully understood but it is believed that both genetic and environmental factors have a role to play (Gould et al., 2003). Genetic predisposition is believed to contribute at least to half of the susceptibility to asthma (Duffy et al., 1990). The list of allergens includes house dust mite and cockroach emanations, pollens, and fungal spores (Machado et al., 1996, Helm et al., 2000). Food hypersensitivities which have been documented include cow milk, egg, peanut, soya, wheat, shellfish, tree nuts and fish (Sampson, 1999, Sampson, 2004). Allergic reaction to latex components now commonly referred to as the 'latex-fruit' syndrome are directed against a conserved chitin binding domain in plant host defense proteins with diverse enzymatic functions referred to as the *hevein symphony.* This immune response represents a major problem for health professionals, patients and members of the general public using latex products (Toraason et al., 2000, Agarwal and Gawkrodger, 2002, Filon and Radman, 2006).

Most of these environmental allergens have been present for millennia and current allergy epidemic even in industrialised countries with genetically stable pool such as Japan point to the fact that changes in environmental factors are probably responsible for the current epidemic (Cookson and Moffatt, 1997). Diesel exhaust particles (DEPs) enhance the production of pro-allergenic cytokines from cells present in nasal mucosa and have been held accountable for the increase in airway allergic diseases (Diaz-Sanchez et al., 1996). Chronic respiratory diseases are highly prevalent in workers who are in employment in motor garages where they are exposed to motor vehicle exhaust emission and diesel engine exhausts (Bener et al., 1998). Many components in environmental tobacco smoke [ETS] are chemically similar to DEP, and have been shown to stimulate ongoing IgE synthesis (Smyth et al., 2000). These

chemicals are also implicated in increased airway hypersensitivity and sensitisation to common aero-allergens in young children and held accountable for the increased incidence in allergy and respiratory symptoms (Magnusson and Johansson, 1986, Andrae et al., 1988).

Reunification of Germany provided a case study of the impact of life style on allergic diseases. A study carried out by von Mutius et al (1998) indicated a significant rise in the incidence of hay fever amongst children in East Germany who, following the reunification of the country adapted to a more Westernized way of life. Likewise obesity, a major and ever growing problem in children of industrialized countries, is positively linked with the onset of adult-onset asthma (Camargo et al., 1999). The "Hygiene Hypothesis" that has been much quoted as a probable cause of increased incidence in allergic diseases in affluent Western societies was originally presented by epidemiologist Dr Strachan (1989). The hypothesis stated that there is an inverse relationship between family size and allergic diseases (Strachan, 1989). Strachan proposed that the increase in atopic disorders might be due to a decreased occurrence in early infancy of infections that are commonly transmitted through contact with elder siblings. A consensus about this concept is now evolving and it is postulated that elementary changes in life style have deprived human beings from developing immunoregulatory mechanisms brought about by exposure to certain micro-organisms and viral infections thus contributing to increase in the prevalence of allergic diseases in the last few decades (Pawankar et al., 2008).

1.2: Immune System: An Overview

Immunity is the universal potential of the host to recognize and fight with the aim of eliminating the predatory microbes, which are pathogenic to it and, if allowed to live, will produce noxious effects on the body. It involves both molecular and cellular agents acting in cohesion and involves the coordinated interaction between components of the innate and adaptive immune systems.

Broadly defined, the immune system depends on a combination of the non-specific but immediate acting force of innate system and the more target oriented slowly responding system of the acquired/adaptive immune system with an extent of overlap

present (Basset et al., 2003). The Toll-like receptors (TLRs) expressed by antigen-presenting cells and mast cells recognize and differentiate self from pathogens and bring about the commencement and subsequent steering of adaptive immune responses (Orinska et al., 2005, Kaisho and Akira, 2006). The microbes in their bid to gain entry to the body face first resistance in the shape of skin and mucosal membrane lining the gastrointestinal, respiratory and urogenital tract, which in addition to acting as a physical barrier to the microbes in the external environment, harbour mast cells at a position where they are ideally placed to combat any incoming pathogens (Kitamura et al., 1977, Metcalfe et al., 1997). The mast cells are thus not only on the interface of external and internal environment but form a bridge between both the innate and adaptive immunity due to their established place as a component of the innate system. At the same time, due to the expression of Fc receptors they mediate responses to immunoglobulins, and as a result mediate allergic sensitization. The origin of allergy may be found at this interface and better comprehension of the complex mechanisms related to the role of mast cells in the immune system may lead to developing more efficient intervention strategies to combat the growing menace of allergic diseases.

1.2.1: The Innate System

The innate system is comprised of mechanical, chemical and cellular elements. The mechanical component includes the physical barrier of intact epidermis and mucosa and associated physiological roles such as cilial motility, desquamation and mucus secretion (Basset et al., 2003). The initial and all important task of recognising the pathogen is carried out by cell surface expressed pathogen recognising receptors (PRRs), found on the surface of many cells including epithelial cells, macrophages/monocytes, granulocytes, mast cells and dendiritic cells, that identify a wide array of pathogen associated molecular patterns (PAMPs) (Janeway and Medzhitov, 2002). The PAMPs recognition, which include lipopolysaccharide (LPS), peptidoglycan, lipoproteins and oligosaccharides, leads to activation of the inflammatory response to a wide variety of the pathogenic stimuli (Iwasaki and Medzhitov, 2004). The chemical mediators involved in the innate system can be sub-grouped into pattern recognition molecules, the proteins and peptides that eradicate the micro-organisms and last but not the least depend on cytokines and chemokines generated following activation of innate immune responses that have a crucial

regulatory role in the steering of adaptive immunity (Hedges et al., 1995, Machado et al., 1996, Pitman and Blumberg, 2000, Janeway and Medzhitov, 2002, Wynn, 2009). Mast cells, dendiritic cells, NK cells, macrophages and epithelial cells all play a role in the innate system (Basset et al., 2003).

1.2.2: The Adaptive System

Adaptive immunity, also known as acquired immunity is more specialised than the innate system and builds on the work already performed by its more primitive counterpart. As the name implies a person has to have had a previous exposure to the agent to be able to mount a response against it. The initial encounter with the allergen (immunization) brings about a complex and intricate chain of events leading to production of antibodies by lymhocytes (Owen, 2007). Any subsequent exposure to the same antigen then makes the host capable of combating and eradicating the pathogen through the presence of immunological memory. The adaptive system is very specific and target oriented as it possesses the self/non-self recognition mechanism through the expression of T cell receptor (TCR), B cell receptor (BCR) and Major-histocompatability complexes (MHC) making it a very well organized immune system (Hoebe et al., 2004). The activated T helper cells (Th), through the effects of secreted cytokines and by the binding of CD40 on B cells to CD40 ligand on T cells, promote the production of immunoglobulins by the B cells (Owen, 2007). There are subsets of Th cells, Th1 and Th2, derived from Th0, depending on the cytokines secreted and the target organisms (Gould et al., 2003). Th1 cells secrete IL-2 and interferon -γ in response to challenge by bacteria and viruses, while Th2 cells secrete IL-4, IL-5, IL-6 and IL-10 when challenged by helminth and allergen (Finkelman et al., 1997, Romagnani, 2001). It is Th2 phenotype cells which are responsible for isotype switching of B cells to IgE production which is brought about by cytokines IL-4, IL-13 via signals through CD40 and CD21 (Gauchat et al., 1993, Jeppson et al., 1998). It is interesting to note in this context that activated mast cells express a similar cytokine pattern to Th2 cells and via the expression of CD40L have, in theory, the capability to induce class-switching to the IgE isotype (Machado et al., 1996). Another subset T regulatory cell (Treg) mediate self-tolerance and prevent autoimmune diseases (Steinke and Borish, 2006).

1.2.3: The Immune Cells

Dendiritic Cells

The dendiritic cells are antigen-presenting cells, arising from the bone marrow, and are generally considered to be the link between innate and acquired immunity due to their property of presenting antigens in the tissues and then circulating to regional lymph nodes where they mature (Banchereau and Steinman, 1998, Zitvogel, 2002). These cells initiate the immune response, have PRRs on their surface that recognise PAMPs, and are widely distributed in both lymphoid and non-lymphoid tissues making them ideally located to be at the interface of the host and environment to perform their role (Janeway and Medzhitov, 2002).

NK cells

These cells are also part of the innate system and are derived from the lymphoid cell line. They also have an immunomodulatory role as they activate macrophages by releasing TNFα and IFN-γ and are responsible for killing virally infected and tumour cells during cell-to-cell contact by releasing perforin and granzyme (Middleton et al., 2002).

The Phagocytic Cells-Neutrophils and Macrophages

These are effector cells of the innate immunity that destroy the invading pathogens before they can carry out their deleterious effects, but these cells also initiate the adaptive response by presenting the antigens to T-cells. The first cells to come into action are the resident macrophages but they are rapidly joined by the circulating neutrophils if the need arises (Zhang et al., 2000). Macrophages and neutrophils possess several PRRs that make possible the recognition through direct and indirect means of the opsonised micro-organisms (Janeway and Medzhitov, 2002). The invading pathogen is then internalised in a phagosome, which fuses with endosomes and lysosomes to form phagolysosome leading to killing of micro-organism by using oxidative and non-oxidative mechanisms (Burg and Pillinger, 2001). The recruitment of the neutrophils to the damaged tissue is brought about through the chemotactic action of cytokines such as IL-1β, TNF-α, G-CSF, and chemokines such as fMLP and LPS and substances from the damaged tissue (Philpott et al., 2001). The neutrophils seem to have an immunoregulatory role characterised by the release of certain

chemokines, as well as the release of substantial amounts of TNF and IL-1 (Chaplin, 2003).

1.3: An Overview of Allergic Response

The symptoms of immediate hypersensitivity are caused by the release of preformed and newly synthesized mediators that are released from mast cells and basophils in response to a variety of stimuli (Blank and Rivera, 2004). The best characterized event involves the sensitization of mast cells and basophils by IgE binding to high-affinity IgE receptors; subsequent cell activation follows encounter with cognate allergen representing currently the best defined route leading to the regulated secretion of the pharmacologically active mediators responsible for the symptoms of class I hypersensitivity responses (Nadler et al., 2000). It is however known that mast cells and basophils can be activated by a number of diverse mechanisms, including initial exposure to enzymatically active allergens, which induce the secretion of diverse mediators, including IL-4/13; the importance of these processes is only beginning to emerge. It still remains to be demonstrated if this is in fact the first in a series of steps leading to the synthesis of antibodies of the IgE isotype, although it is generally accepted that mast cells and basophils express similar ligands and cytokine to Th2 cells known to steer B-cells responses to class-switching to the IgE isotype (Dudler et al., 1995, Machado et al., 1996).

The sequence of events involved in the allergy mechanism involves a complex interaction of cells and chemicals. Although the list of allergens is long and varied the commonly encountered allergens are pollen grains, house dust mite, cockroach emanations, mould spores, latex, soya and nuts (Machado et al., 1996, Helm et al., 2000). The current generally accepted mechanism proposes that after exposure these allergens on entry into the body come in contact with the APCs such as basophils and DCs at the mucosal surfaces (Zitvogel, 2002, Wynn, 2009). The antigenic region of these allergens in association with the MHC class II are presented on the cell surface after being internalised and processed by the DCs. The presented peptides then bind to naïve T cells in the lymph nodes leading to Th2-differentiation (Owen, 2007). This brings about release of characteristic Th2 cytokines, IL-4, IL-13 causing B cell IgE class switching and secretion of antigen-specific IgE into the circulatory system

(Platts-Mills, 2001). IgE leaves the circulation and significant amount bind to the high affinity IgE receptors (FcεRI) present on the surface of mast cells, basophils, eosinophils and other effector cells (Wang et al., 1992, Maurer et al., 1994, Gounni et al., 1994, Kinet, 1999). Following this initial priming/sensitisation phase any subsequent exposure to allergens causes cross-linking of adjacent FcεRI receptors present on the surface of mast cells which initiates a downstream signalling cascade culminating in mast cell degranulation causing the rapid exocytosis of pre-formed and stored mediators present in the mast cells granules (Theoharides et al., 2007). This is labeled as the acute phase of the allergy and is characterised by symptoms such as sneezing, coughing, wheezing, itching and tissue swelling that are brought about by action of mediators like histamine on tissues, which causes increased vascular permeability and smooth-muscle contraction (Galli et al., 1999, Wedemeyer and Galli, 2000, Gould et al., 2003). The acute phase is followed by the late-phase that occurs 4-6 hours after the initial response and is brought about by other effector cells including neutrophils, eosinophils, macrophages, lymphocytes and basophils and may last for 1-2 days (Kay et al., 1997, Williams and Galli, 2000). An overview of the allergic response is shown in the **figure 1.1.**

1.4: Mast Cells

There is much evidence for the role of mast cells in allergy and asthma (Bradding et al., 2006) along with their role in different disease and immunological functions such as tissue remodeling, wound healing, pathological fibrosis, arthritis, angiogenesis and body response to neoplasia (Benoist and Mathis, 2002). Mast cells were initially described by Paul Ehrlich in 1878 as a type of granular cell of the connective tissue and the name "Mastzellen" was coined (Riley, 1954). The initial focus of the research was on the intracellular granules of mast cells containing histamine (Riley and West, 1952, Riley, 1954) and the first role of mast cells to come into attention was their involvement with the potentially fatal condition of anaphylaxis (Keller, 1962). Mast cells play a prominent role in allergy (Williams and Galli, 2000) and express high affinity immunoglobulin (IgE) receptors on their cell surface (Metzger, 1992, Turner and Kinet, 1999). It is now established that mast cells are the prime effector cells involved in host defence mechanisms against parasitic worms, bacteria and

intracellular protozoan parasites by acting in synergy with immunoglobulin E (Watanabe et al., 1994, Maurer et al., 1998, Marshall, 2004) and are of utmost importance to host defence and survival during and after a bacterial infection (Echtenacher et al., 1996, Malaviya et al., 1996). Mast cells arise from $CD34^+$ pluripotent stem cells in the bone marrow (Nabel et al., 1981) and are not a component of the connective tissue as was initially believed (Kitamura et al., 1977). They circulate in the blood in immature form and finally reside in the tissues where they mature under the influence of stem cell factor (SCF) and their numbers are regulated by concentrations of local cytokines like IL-4, IL-6, IL-9, IL-10 and TNF (Padawer, 1974). IL-3 is known to stimulate the proliferation and differentiation of mast cells (Lantz et al., 1998).

1.4.1: Mast Cells Activation

Antigen-mediated aggregation of the IgE bound to its high –affinity receptor on mast cells and basophils initiates a downstream signalling cascade leading to exocytosis of the preformed and denovo synthesised mediators (Siraganian, 2003). Mediator release is influenced by cytokines, growth factors and microenvironmental conditions. IL-4 is believed to enhance FcεRI-mediated reactions from human mast cells (Bischoff et al., 1999).The aggregation of FcεRI receptors causes phosphorylation, usually by Lyn (Siraganian, 2003), of tyrosine residues in immunoreceptor tyrosine- based activation motif (ITAM) of both the β and the γ subunits of FcεRI (Reischl et al., 1999, Rivera, 2002, Siraganian, 2003, Galli et al., 2005). The tyrosine phosphorylated ITAMs then act as scaffolds for the binding of the additional signaling molecules with Src homology domain 2 (SH2) like the cytoplasmic protein tyrosine kinase Syk, which binds mainly to the γ subunit of the receptor through its two SH2 domains bringing about conformational change of Syk leading to its activation and autophosphorylation (Costello et al., 1996, Zhang et al., 1996). The activated Syk kinase through direct or indirect means phosphorylates several proteins, including linker for activation of T cells (LAT) (Saitoh et al., 2000), SH2 containing leukocyte-specific protein (SLP-76), Vav, phospholipase C-γ1 (PLC-γ1) and PLC-γ2 (Blank and Rivera, 2004).

The mast cells and basophils on being sensitised by IgE produced by B cells, release a wide array of mediators. These mediators are responsible for immediate

hypersensitivity causing acute phase symptoms. Another protein tyrosine kinase, Fyn, is also constitutively bound to the β chain and following the receptor aggregation causes tyrosine phosphorylation of Grb2-associated binder like protein 2 (Gab2), which then binds the p85 subunit of phosphatidylinositol 3-kinase (PI3K) (Parravicini et al., 2002). The PI3K then catalyses the conversion of phosphatidylinosito-4, 5-biphosphate (PIP_2) to phosphatidylinosito-3, 4, 5-triphosphate (PIP_3). The PIP_3 in the membrane attracts to the membrane many proteins containing pleckstrin homology (PH) domains like BtK, PLC-γ1, PLC-γ2 and phosphoinostide-dependent protein kinase1 (Siraganian, 2003). The tyrosine phosphorylated PLC-γ1 and PLC-γ2 catalyse the hydrolysis of PIP_2 resulting in the generation of inositol-1, 4,5-triphosphate (IP3) and 1, 2-diacylglycerol, these second messengers then bring about release of Ca^{+2} from internal stores by binding of IP3 to specific intracellular receptors (Smith et al., 2001) and activation of protein kinase C (PKC) respectively. After the initial rise of Ca^{+2} there is depletion of the Ca^{+2} stores which necessitates the need for entry from the extracellular medium which is brought about by activation of calcium release activated channels (CRACS also known as store operated Ca channels; SOCCs) on the plasma membranes that allow the response to be maintained (Scharenberg and Kinet, 1998). In order to produce signals for a sustained Ca^{+2} influx the tyrosine phosphorylation and activation of Bruton's tyrosine kinase (BtK), SLP-76, LAT and PLC-γ are mandatory (Siraganian, 2003). These early events are followed subsequently by activation of other enzymes and adaptors including Vav, Shc, Grb2 and SOS, which in turn stimulate small GTPases such as Rac, Ras and Rho. These pathways lead to activation of the extracellular-signal-regulated kinase (ERK), Jun amino-terminal kinase (JNK) and p38 mitogen-activated protein (MAP) kinase pathways, histamine release, phosphorylation of transcription factors that induce the synthesis of new cytokines, and activation of phospholipase A2 ($cPLA_2$) to release arachidonic acid (Siraganian, 2003).

IgE-independent activation can take place through complement receptors or the Toll-like receptor (TLR) and mast cells activators include SCF, complement factors (C3a, C5a), neuropeptides (substance P), adenosine, TLR and scavenger receptors (Dawicki and Marshall, 2007, Brown et al., 2008). A simplified diagrammatic representation of mast cell signalling via FcεRI is given in **figure 1. 2.**

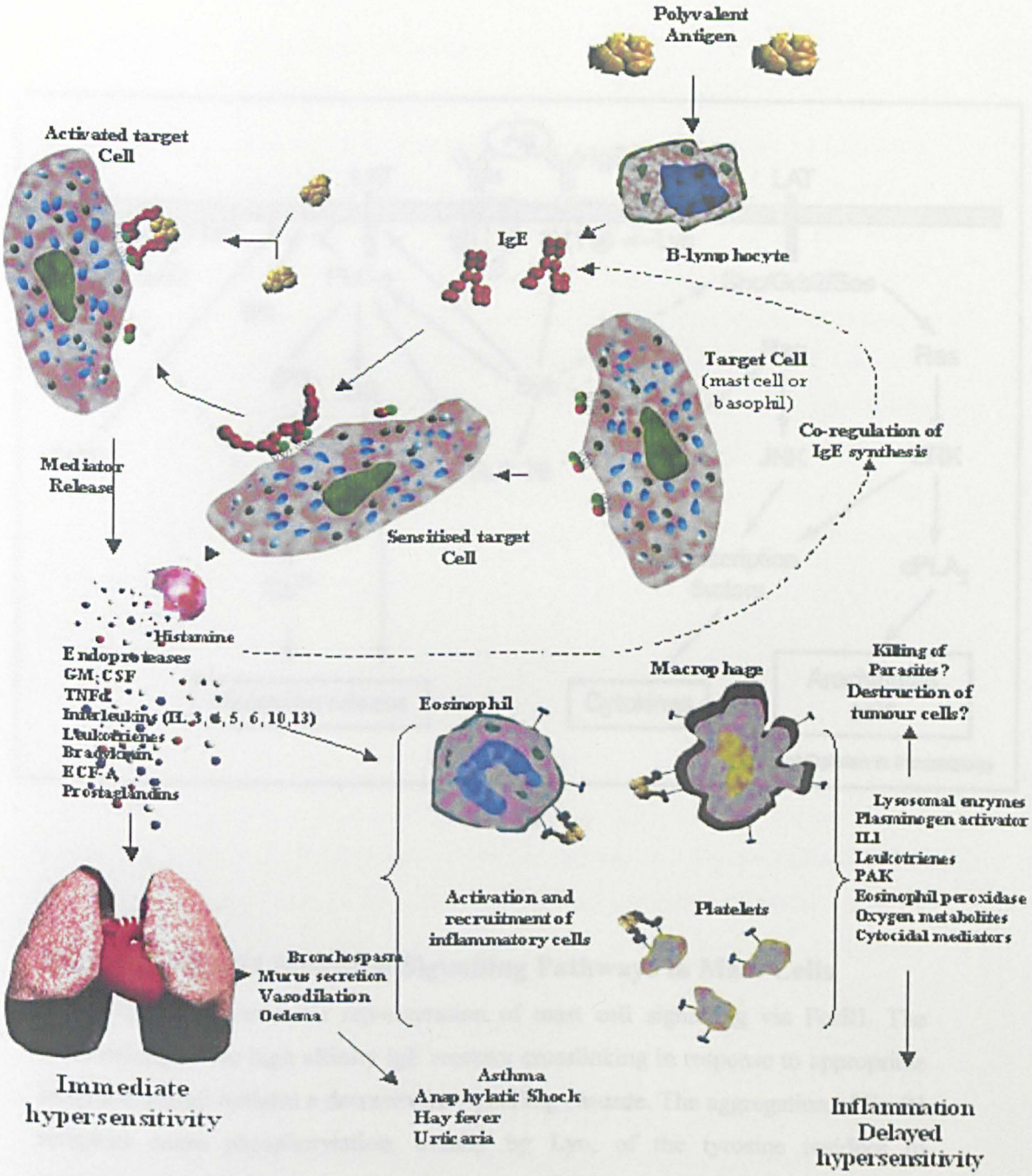


Figure 1.1: Overview of Allergic Response

The mast cells and basophils on being sensitised by IgE, produced by B cells, release a wide array of mediators. These mediators are responsible for immediate hypersensitivity causing acute phase symptoms (Adapted from Cain, 1997).

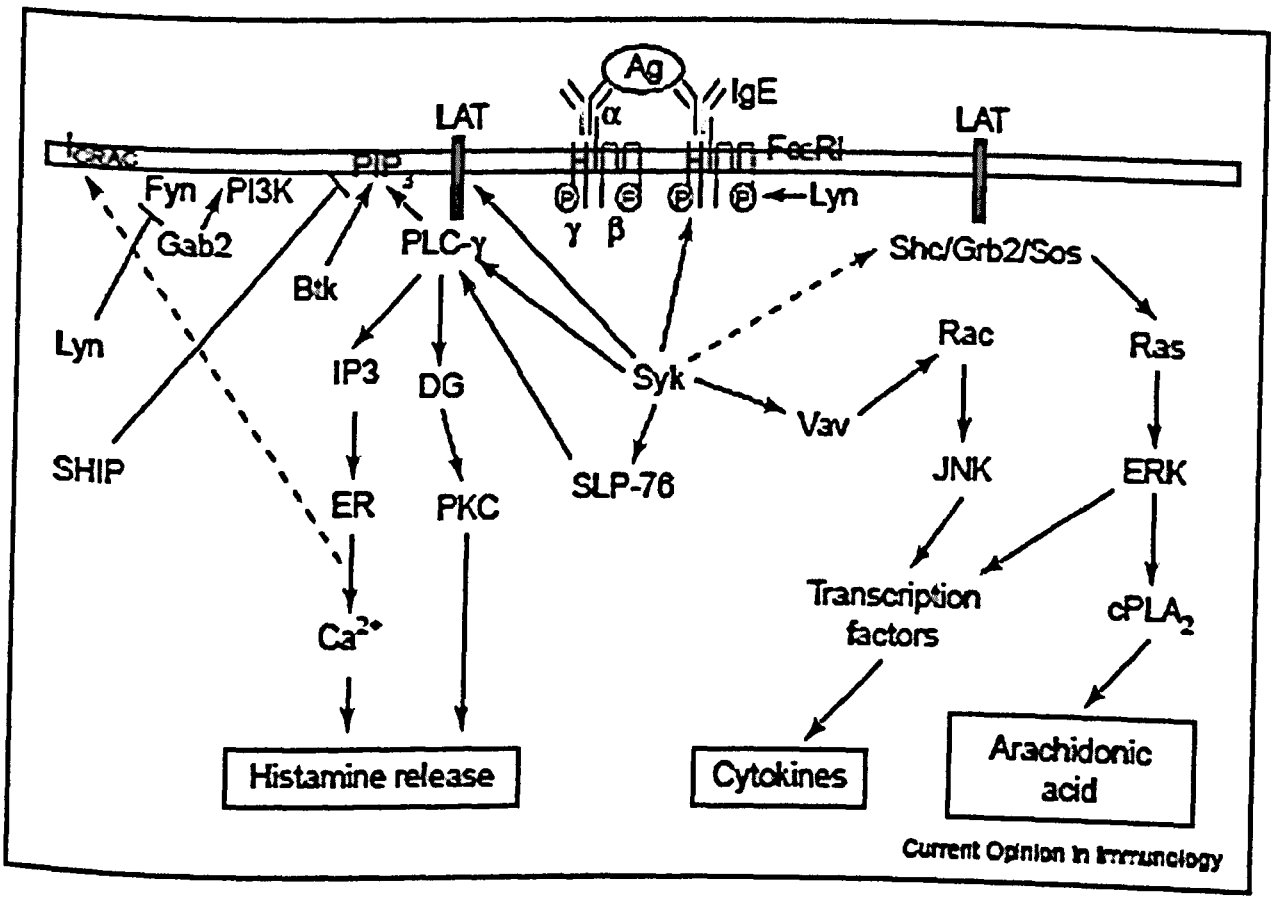


Figure 1.2: FcεRI Mediated Signalling Pathways in Mast Cells

A simplified diagrammatic representation of mast cell signalling via FcεRI. The cross-linking of the high affinity IgE receptor crosslinking in response to appropriate antigenic stimuli initiates a downstream signalling cascade. The aggregation of FcεRI receptors cause phosphorylation, usually by Lyn, of the tyrosine residues in immunoreceptor tyrosine- based activation motif (ITAM) of both the β and the γ subunits of FcεRI. This is followed by recruitment and activation of Syk kinase, multiple adaptor proteins are activated leading to mast cell degranulation, cytokine production and arachidonic acid production (Adapted from Siraganian, 2003).

1.4.2: Mast Cell Degranulation

The fundamental step involved in mast cell response in both innate and acquired immunity is the exocytosis of the contents of its mediators in response to stimulants (Blank and Rivera, 2004). The various symptoms of the atopic phenomena are elicited by the profound quantities of the vesicular contents released in a single stimulatory episode by a process of compound or regulated exocytosis (where the membranes of secretory granules fuse with each other and the plasma membrane) to ensure a maximal biological response which is contrary to what is seen in some other secretory cells (Blank and Rivera, 2004). Mast cells produce a variety of intra and extra-cellular mediators that are produced in response to different stimuli, and they are able to control the kind and the strength of response (Theoharides et al., 2007). Mast cells secretory mediators have key functions and can be divided into three groups (Williams and Galli, 2000)

1) Preformed substances

2) Newly synthesized metabolites

3) Chemokines or Cytokines

Preformed mediators include a biologic amine, typically histamine, proteoglycans, heparin and a wide variety of proteases such as tryptase and chymase (Stevens and Austen, 1989, Huang et al., 1998). Newly generated mediators typically produced during IgE-mediated activation include arachidonic acid metabolites, principally leukotriene C_4 (LTC4), leukotrine B4 (LTB4) and prostaglandin D2 (PGD2) (Murakami et al., 1995) along with platelet-activating factor (Longphre et al., 1996). The leukotrines and prostglandins are responsible for bronchoconstriction and vasodilatation in addition to recruiting neutrophils and activating eosinophils (Henderson et al., 1996, Rothenberg et al., 1999). Most allergens stimulate mast cells degranulation through cross-linking of the IgE sensitised receptors. The local and systemic consequences of mast cell degranulation produce the pleiotropic effects associated with the diverse allergic manifestations (Costa et al., 1997). Furthermore they also regulate the allergic responses thus indicating an immuoregulatory role in addition to its effector cell role (Williams and Galli, 2000).

The wide spectrum of diverse pro-inflammatory mediators released from IgE-activated target also plays an important role in the up-regulation of MHC class II and FcεRI molecules and the potential enhancement of IgE synthesis (Williams and Galli, 2000) by a feedback mechanism that amplifies the response to allergen. The chemotactic effect of mast cells mediators in the recruitment of inflammatory cells like eosinophils, monocytes, neutrophils and platelets is a hallmark of the late phase response, which occurs some 6-12 h after mast cell degranulation (Helm, 1994).

1.5: Immunoglobulins

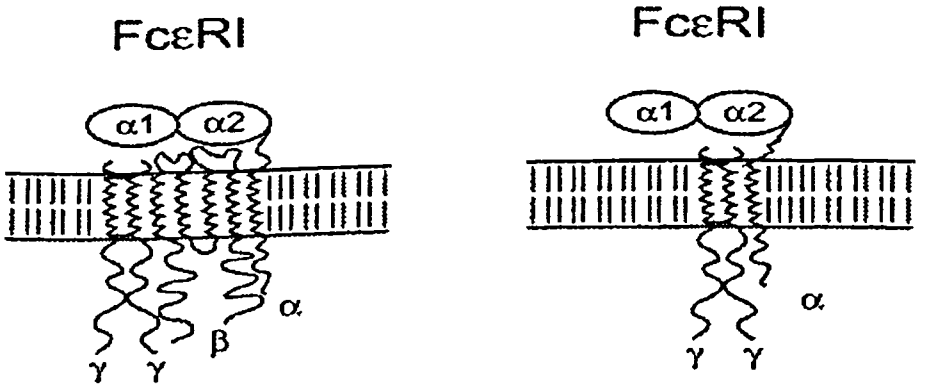
There are nine antibody classes (isotypes) in humans: IgM, IgD, IgG1, IgG2, IgG3, IgG4, IgA1, IgA2 and IgE all having similar structure consisting of heavy (H) and light (L) chains with variable (V) and constant (C) regions made up of Ig domains (Gould et al., 2003). H-chains in IgA, IgD and IgG have 3 CH domains each while in IgM and IgE there are 4. A pair of identical antigen-binding sites is present in the V regions of the L- and H-chains. The Fab region of the antibody is made up of these together with the adjacent CH domain pair (Gould et al., 2003). The Fc region, which contains the FcR binding sites, of the antibody is comprised of the pair of remaining Ig domains.

1.5.1: Immunoglobulin E, IgE

The discovery of a new antibody isotype called IgE was reported in 1967 by Ishizaka and Ishizaka (Ishizaka and Ishizaka, 1967). Immunoglobulin E plays a key role in the early-phase allergic inflammation response but may also have a part to play in the late-phase allergic response (Williams and Galli, 2000) besides being involved in immune response to parasitic infections (Yazdanbakhsh et al., 2001, Maizels and Yazdanbakhsh, 2003). A direct correlation between serum IgE levels and asthma has been established (Burrows et al., 1989; Sunyer et al., 1995). Plasma cells in the mucosa-associated lymphoid tissue are responsible for the production of IgE (Geha et al, 2003). In humans the level of serum IgE is the lowest of all antibodies and is in the range of ~150ng/ml (King et al., 1991). The IgG has three heavy chain domains while IgE molecule is composed of 4 constant heavy chain domains (Cε1 - Cε4) and 1 variable heavy chain domain with the additional domain Cε2 replacing the hinge region (Gould et al., 2003).

1.6: The High Affinity IgE Receptor, FcεRI

The largest class of Fc receptors belongs to the immunoglobulin superfamily and includes IgG specific FcγRI, FcγRII and FcγRIII receptors and IgE specific FcεRI receptor (Kinet, 1999). FcεRI has the highest affinity of all immunoglobulin receptors with a binding constant in the 10^{-9} to 10^{-10} M range for its ligand, IgE (Daeron, 1997). It exists as a tetramer on the surface of human mast cells and basophils consisting of a α-chain, a β-chain and a disulphide-linked dimer of γ-chains, while on Langerhans cells, dendritic cells and monocytes the FcεRI is present as a trimer and is devoid of the β-chain and has only one α-chain and two γ-chains (Turner and Kinet, 1999). However in rodents its expression is limited to the surface of mast cells and basophils and only the tetrameric isoform is present (Kinet, 1999). The two isoforms of FcεRI are shown in **figure 1.3.**



A. B.

Figure 1.3: Isoforms of FcεRI

The two isoforms of FcεRI. The tetramer (αβγγ) shown in A and trimer (αγγ) shown in B (adapted from Gould et al., 2003).

1.6.1: The Ligand –binding Subunit- FcεRIα

The α-chain crosses the cell membrane with a single putative helical segment. The extracellular domain of the α-chain binds IgE with high affinity (Hakimi et al., 1990, Blank et al., 1991, Ra et al., 1993).The α-chain is comprised of two extracellular immunoglobulin-related domains, one transmembrane domain and a short cytoplasmic tail (Kinet et al., 1987, Kochan et al., 1988, Shimizu et al., 1988, Ra et al., 1989). The α-chain is heavily glycosylated and appears as a heterogenous band centered around the 45-kDa marker when resolved by standard SDS-PAGE; however the weight of the α-chain protein core is 27kDa (Kinet, 1999). The α-chain has seven N-linked glycosylation sites which are thought to interact in the ER with the ER folding machine (Letourneur et al., 1995b); however as part of the ER quality control mechanism these glycosylation moieties are trimmed before the subunit can be exported from the ER, failing which the receptor may be retained within the ER (Albrecht et al., 2000). Another system to differentiate between assembled and non-assembled receptors in the ER is the presence of a dilysine ER retention signal in the cytoplasmic domain of the FcεRIα subunit the steric masking of which is done by the cytoplasmic domain of the FcRγ subunit to ensure the exportation of the assembled receptor to the cell surface from the ER (Letourneur et al., 1995a). The human FcεRIα subunit has a considerable homology with human FcγRIII (Ravetch and Kinet, 1991).

1.6.2: The Signal Amplifying Subunit- FcRβ

The FcRβ is also found as a subunit of the low affinity IgG receptor (FcγRIII) (Kurosaki et al., 1992). The β-chain has been computed to span the cell membrane 4 times and it has been proposed that this subunit amplifies the signal transduction tenfolds (Lin et al., 1996, Dombrowicz et al., 1998, Donnadieu et al., 2000a) but is not mandatory for a functional receptor complex (Scharenberg and Kinet, 1997, Kinet, 1999). FcεRIβ also enhances the cell surface expression by early association with the FcεRIα subunit during the process of receptor assembly thus ensuring better receptor exportation and maturation (Donnadieu et al., 2000b). This second amplification effect when added upon the first amplification make the downstream signalling events 12-30 fold greater in tetrameric FcεRI complexes compared to the trimeric FcεRI receptor. The β-chain, like the γ-chain has intracellular tyrosine

activation motifs (ITAMs) that are phosphorylated immediately following receptor cross-linking (Romeo et al., 1992, Samelson and Klausner, 1992, Cambier, 1995, Pawson, 1995) and play a pivotal role in activation of downstream signaling from the receptor complex which culminates in the exocytic release of the mediators of the allergic response.

1.6.3: The Signal Transducing Subunit- FcRγ

FcRγ is found as a homo and hetro-dimer in association with a number of receptors including FcαRI, FcεRI, FcγRI (Ernst et al., 1993), FcγRIII, and T-cell receptor (TCR) (Orloff et al., 1990). The γ-chain is an integral membrane protein consisting of a short 5 amino acid extracellular domain, a 21 amino acid transmembrane domain and a 42 amino acid cytoplasmic domain (Kuster et al., 1990). γ-chains have intracellular tyrosine activation motifs (ITAMs), which is important in initiating the downstream signaling (Romeo et al., 1992, Samelson and Klausner, 1992, Cambier, 1995, Pawson, 1995) . FcRγ exists as a disulphide linked dimer with a disulphide bond between the cystine residues at the N-terminus region (Varin-Blank and Metzger, 1990). It belongs to the same family of molecules as the ζ and η chains of the TCR complex. It has been shown that FcRγ and ζ chain of TCR in their capability to mediate T cell development and function are interchangeable although the native TCRζ subunit is far more efficient (Shores et al., 1997).

1.6.4: Interaction of IgE with FcεRI

Immunoglobulin E in the body is found mostly bound to FcεRI (Geha et al., 2003) and the half-life of FcεRI bound IgE has been measured to be about 14 days in the skin (Tada et al., 1975, Hunt et al., 2005). IgE remains bound to FcεRI even in the absence of antigens; thus the receptor adopts the antigenic specificity of the prevalent IgE repertoire (Garman et al., 2000). The IgE- FcεRI interaction exhibits a 1:1 stoichiometry (Garman et al., 2000). It has been found that monomeric IgE can stimulate mast cell survival through signalling pathways distinct from those initiated by receptor cross-linking (Asai et al., 2001). *In vitro* experiments with monomeric IgE and bone-marrow derived mast cells have demonstrated that a decrease in the concentration of both free and FcεRI -

bound IgE might lead to a decrease in the mast cell numbers (Kalesnikoff et al., 2001, Asai et al., 2001).

Variable regions of heavy and light chains of the immunoglobulin molecules have an allergen-binding site. The Cε3 domain of Fc fragment of IgE is able to bind to two types of immunoglobulin Fc receptors, the high-affinity IgE receptor (FcεRI) and the low affinity IgE receptor (FcεRII) (Vercelli et al., 1989, Nissim et al., 1993, Presta et al., 1994).

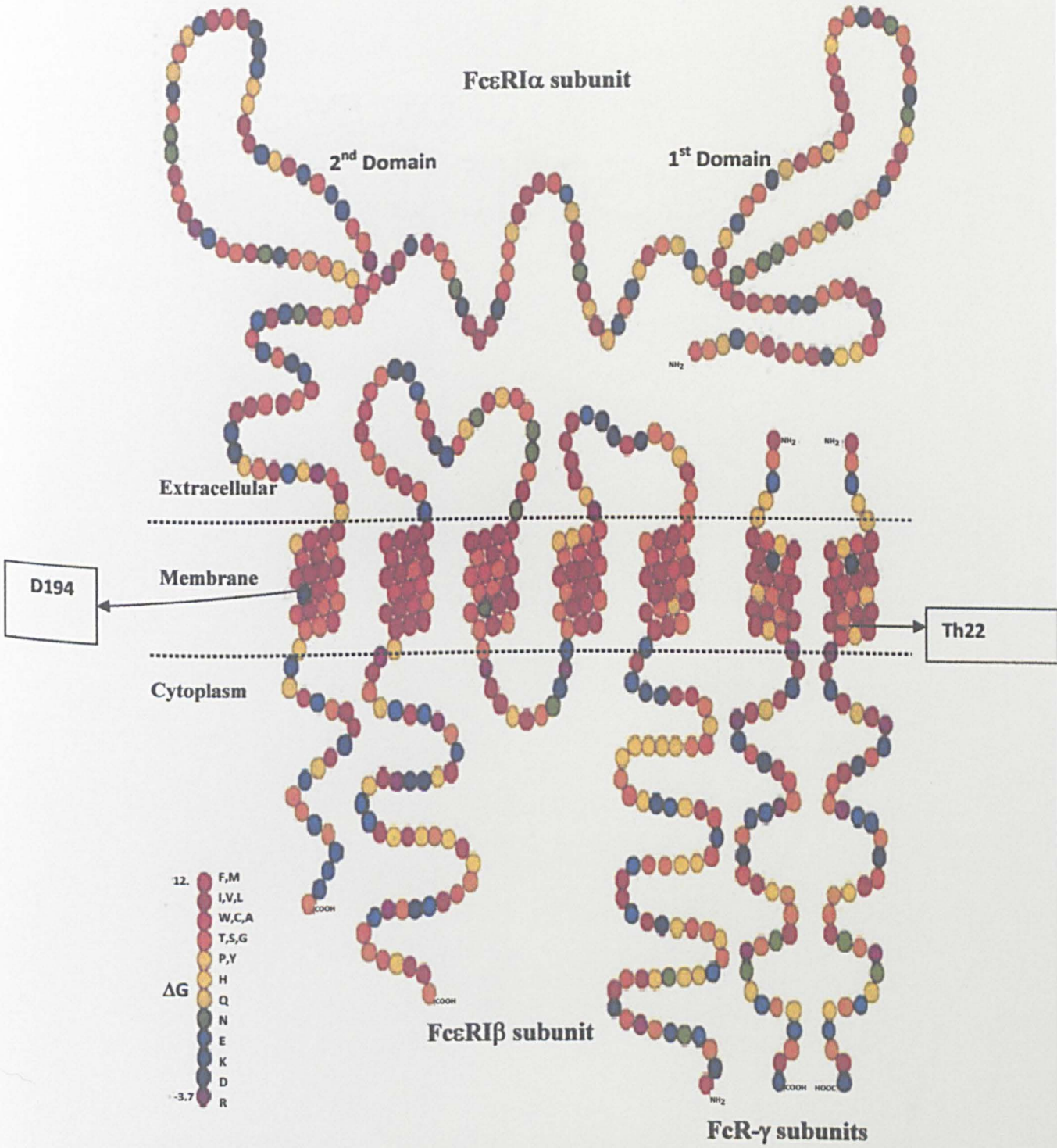


Figure 1.4: Model of Tetrameric High Affinity Receptor

Model of tetrameric high affinity receptor first suggested by Blank et al (1989) and later modified by Kinet and Metzger (1990). The residues have been colour coded

according to their hydrophobicity/hydrophilicity score (see scale). The putative transmembrane domain is predominantly hydrophobic, with the FcεRIα subunit transmembrane domain containing a hydrophilic aspartic acid residue (D194, shown in blue)

1.7: The Role of Conserved Charged Residues in Transmembrane Domains of Immunoreceptors on Receptor Assembly, Expression and Function

The importance of conserved charged residues in the transmembrane domains (TMs) of the immune receptors on receptor subunit assembly, expression and function has been highlighted in various studies over the last two decades.

1.7.1: The Role of Transmembrane Charged Residues of FcεRI

Varin-Blank and Metzger (1990) carried out mutations in α, β, and γ subunits of FcεRI and analysed the effects of these genetically engineered modifications on FcεRI surface expression. The study concluded that transmembrane domains are crucial for the accurate receptor assemblage and transport to the cell surface while the cytoplasmic domains affect the surface expression of the receptors although their role is not mandatory. The COS-7 cell line was used by Varin-Blank and Metzger for transient transfections of the mutated subunits of FcεRI. A conserved charged aspartic acid residue is present in both the α and γ subunits of FcεRI of rat, mouse and human and the ζ chain of TCR of mouse and human (Kuster et al., 1990). This arrangement of a charged hydrophilic residue in a predominantly hydrophobic environment of lipid bilayer is energetically unstable. The mutations introduced in this residue by Varin-Blank and Metzger were D195A in the FcεRIα subunit and D11A in the FcR-γ subunit. They were then co-transfected with the appropriate wild-type rat FcεRI subunit cDNAs. The results indicated a decrease in the cell surface expression with the most adversely affected being the double mutation D195A/D11A. The importance of γ-subunit in the surface expression of the FcεRIα subunit was highlighted when the mutation D11A lead to complete abrogation of the human FcεRIα (wild-type) cell surface expression. The conserved nature coupled with the presence in the

transmembrane region where the subunits of the receptor interact with each other make this residue a potential target for studies to elucidate the mechanisms involved in receptor subunits interactions.

1.7.2: The Role of Transmembrane Charged Residues of IgA Receptor

The transmembrane IgA immunoglobulin receptor (FcαRI, CD89) is expressed alone or in association with FcR-γ subunit on the surface of monocytes, macrophages, neutrophils and eosinophils. Studies carried out on this receptor to understand the relationship of the FcαRI and FcR-γ subunit, by introducing site directed mutations, revealed that the positively- charged arginine residue at position 209 within the FcαRI TM domain is needed for the functional association with the FcR-γ subunit (Morton et al., 1995). Another mutation (Y25F) in the FcR-γ subunit located at the cytoplasmic/transmembrane interface had no affect on the FcεRI-FcR-γ expression but did show pronounced affects on FcαRI-FcR-γ arrangement by substantially decreasing (~10%) the cell surface expression, subunit association and signal transduction capability in the mutant transfected cell line as compared to the wild-type FcαRI-FcR-γ transfected cell line (Wines et al., 2004).

1.7.3: The Role of the Transmembrane Charged Residues of T cell Receptor (TCR)

The TCR is a multi-subunit complex having 8 transmembrane proteins each of which has a charged residue in the transmembrane region (Weiss, 1991). TCR consists of two heterodimeric glycoproteins (TCRα and TCRβ) which together with CD3γ, -δ, -ε, and -ζ form the CD3 complex. The CD3δ, CD3ε, and CDζ subunits have an aspartic acid residue while the TCRα subunit has conserved positively charged lysine/arginine residues, TCRβ subunit has lysine residue and CD3γ has glutamic acid residue. TCR assembly takes place in the ER (Minami et al., 1987) and only intact TCR complexes are efficiently transported to the plasma membrane (Sussman et al., 1988). TCR has been widely studied and it was found that charged TM residues of α and β subunit are needed for TCR subunit interaction. Studies involved transfection of both α or β subunit cDNA in α or β deficient T cell lines which do not express TCR on their cell

surface. One of these studies showed that the α and β subunits do form heterodimers and were unable to associate with CD3 (Alcover et al., 1990). When the lysine residue of β subunit was mutated the cell surface expression of TCR did not take place even when the mutation introduced was of the same charge/structure (Alcover et al., 1990, Morley et al., 1988). A study by Blumberg et al (1990) on TCRα TM residues by carrying out single and double mutations showed abrogation of cell surface expression in case of double mutations (R118G/K123I). The importance of polarity of the transmembrane residues in signal transduction in TCR was highlighted in a study by Fuller-Espie et al (1998) where four polar residues located within the TCRβ CART (conserved antigen receptor transmembrane) motif were replaced by non-polar residues of similar size and shape. Polar to non-polar TM mutations of the conserved tyrosines within the TCRβ subunit greatly reduced the expression level and functional capability of TCR indicating the importance of polar TM residues (Kunjibettu et al., 2001).

1.7.4: The Role of the Transmembrane Charged Residues of B Cell Receptor (BCR)

BCR is composed of a membrane-bound ligand-binding immunoglobulin (mIg) and signal transducing heterodimer, Ig-α/Ig-β. Any mIg that fails to become associated with the Ig-α/Ig-β heterdimer is retained by ER due to the presence of an ER retention signal in the TM domain of mIg. mIg has 26 amino acid α helical transmembrane domains with 13 out of these residues being conserved in 7 out of the 8 isotypes of mIg and 10 of the 26 having a hydroxyl residue. Those residues predominantly present on one side of the α helix were targeted for mutation studies. Various studies (Shaw et al., 1990, Grupp et al., 1993, Sanchez et al., 1993, Stevens et al., 1994) showed that single mutations had no affect on the receptor expression and function but double mutations affect the Ig-α/Ig-β heterodimer association with Y465 and S466 TM residues identified as playing significant function in Ig-α/Ig-β association and receptor expression/function.

1.8: Current Therapeutic Strategies for Treatment of Allergy

Traditionally people have been advised to avoid exposure to the allergens, something that is not always possible. Currently available therapeutic strategies available for combating allergic diseases are inefficient and are frequently associated with debilitating side effects. It is hoped that advances in gene therapy vaccines and drug delivery will provide the basis for the development of more efficient therapeutics using a more target oriented approach that will produce less undesirable side-effects.

1.8.1: Conventional Therapies

The various medications currently available are not effective on their own and are usually used in combinations that provide symptomatic treatment rather than targeting the underlying immunological pathology. The first-line of treatment conventionally has been the administration of oral antihistamines that act by countering the effects of histamine released by the mast cells (Meltzer, 1998). These drugs are however associated with side effects the most significant of which has been sedation, which necessitated the introduction of non-sedating antihistamines. Topical corticosteroids that reduce the inflammation are the most effective treatment for asthma and rhinitis and are extensively being used worldwide. Corticosteroids reduce inflammation by increasing the transcription of anti-inflammatory genes and more decisively suppressing the transcription of inflammatory genes (Pawankar et al., 2008). Another drug, which is time tested in treating the life-threatening situation of anaphylaxis, is epinephrine (Kemp et al., 2008).

Allergen immunotherapy introduced in 1911, targets the specific allergen to which a person is sensitised and has proven to be a potent long-term therapeutic tool capable of reducing the symptoms of allergic rhinitis and asthma, and in children treated for allergic rhinitis decreases the subsequent development of asthma (Barnes, 1999). Demerits associated with this strategy include multiple injections over a period of months and associated risk of anaphylaxis or additional sensitisation (Lewis, 2002). Recently success has been declared by a Cambridge group using this approach for desensitisation to peanut flour (Clark et al., 2009). The drugs used for treating asthma

may be grouped as either relievers or controllers. The relievers or the bronchodilators include β_2 – agonists, theophylline and anticholinergics while the controllers or the anti-inflammatory treatments include corticosteroids, sodium cromoglycate and anti-leukotrines. β_2 –agonists produce bronchodilation by acting directly on the β_2 receptors in smooth muscles lining the airways (Barnes, 1999).

1.8.2: Immunomodulators

An ideal therapeutic strategy for allergic and immunological diseases would be one that can bring about induction of immune tolerance by changing the aberrant immune response and thus provide a long-lasting cure with minimal side effects. For this purpose as the knowledge of pathogenesis of allergic and immunological diseases improve and the underlying molecular mechanisms are elucidated, novel therapeutic agents called immunomodulators are being developed (Casale and Stokes, 2008). The therapies included in this category are Toll like receptor (TLRs) 4 and 9 agonists, immunosimulatory oligodeoxynucleotides, oral and perenterally administered cytokine blockers, and specific cytokine receptor antagonists.

TLRs play an important role in activating the APCs of the immune system; agonists acting on TLRs can alter the allergic phenomena by changing the Th1 and Th2 cytokine balance thus raising concerns about risk/benefit ratio of such broad spectrum therapies. CXR-675 (aqueous formulation of monophosphoryl lipid A) is a TLR4 agonist currently under trials for treating seasonal allergic rhinitis, (Casale et al., 2006) which acts by binding with LPSs and endotoxins present on the cell surface, while TLR9 agonists such as Tolamba act by binding to unmethylated cytosine phosphate guanine (CpG) motifs, most commonly found in bacterial pathogens (Krieg, 2006). In order to develop targeted therapies with lesser side-effects, the Th2 cytokines have emerged as potential targets with strategies targeting single or multiple related cytokines. Therapies against IL-4, IL-5, and IL-13 are at various stages of evaluation. Similarly, oral cytokine (IL-4 and IL-5) synthesis inhibitors are being developed with AVP-13358 showing suppression of IgE, CD23 and Th2 cytokine responses in *ex vivo* and *in vitro* mouse and human assays (Richards et al., 2004). Other strategies that are currently explored include inhibition of Syk kinase, although these may be associated with severe side-effects since syk is an almost ubiquitous

signal transducer (section 1.4.1). Various syk-inhibitors, R112 and R-343, are under evaluation for efficacy and side-effects (Casale and Stokes, 2008).

Another drug category to combat asthma is the leukotrine receptor antagonists group of drugs which has proven to be less effective than the inhaled corticosteroids in the control of asthma (Capra et al., 2006). Cysteinyl-leukotrines (cys-LTs) are potent bronchoconstrictors to counter the effects of which cys-LT_1 receptor antagonists, montelukast and zafirlukast have been developed (Israel et al., 1993).

1.8.3: Mast Cell Therapeutics

Mast cells are now known to play a crucial part in the pathogenesis of allergic diseases by not only inducing an immediate response but also contributing towards the late-phase reaction (Williams and Galli, 2000). As the understanding of the mechanisms underlying mast cell signalling and release of mediators increase, novel therapeutic modalities are being developed. The drugs devised to combat allergic diseases by acting on mast cells as the target can be broadly grouped into those directed at the cell membrane targets (membrane receptors), to intracellular targets (cell signalling, gene expression) or to extracellular targets. These drugs are in various stages of development **(Table 1.1)** and the treatment options might depend on using them in combination as there is variable response in patients and allergic diseases. One drug which has shown promise is passive immunisation with the anti-IgE humanized monoclonal antibody, omalizumab which targets the binding domain of circulating IgE thus blocking its binding to FcεRI on the inflammatory cells leading to the indirect down-regulation of FcεRI expression on the cell membrane (Beck et al., 2004).

Mast cell target	Therapeutic class	Mechanism of action	Stage of development
Cell membrane	Chromones	Potential disruption of Ca^{2+} influx, chloride ion transport and exocytic processes	Clinical use
	β_2 agonists	Increase cytosolic cAMP levels through binding of β2 receptors	Clinical use
	Omalizumab	Monoclonal antibody to free IgE resulting in decreased FcεRI membrane expression	Clinical use
	CCR3 antagonists	Block chemotaxis and degranulation	Clinical trials
	Ca^{2+} and K^+ channel antagonists	Disruption of ion influx with attenuation of degrangulation and chemotaxis	Pre-clinical
	Anti-CD63 antibody	Monoclonal antibody to CD63 which interferes with cellular adhesion to β1 integrins and blocks FcεRI-induced degranulation via impairment of Gab2-PI3k pathway	Pre-clinical
Intracellular	Glucocorticoids	Regulate transcription of numerous inflammatory genes	Clinical use
	Syk kinase inhibitors	Block IgE-FcεRI-mediated downstream signaling (phosphorylation)	Clinical trials
	MAPK inhibitors	Block phosphorylation of multiple intracellular proteins (including transcription factors) that are involved in cellular proliferation, differentiation, survival and chronic inflammation	Clinical trials
	PDE4 inhibitors	Block hydrolysis of cAMP to 5'AMP	Clinical trials
Extracellular	5-LO inhibitor	Blocks the conversion of arachidonic acid to LTA_4 which subsequently prevents CysLT formation	Clinical use
	Tryptase inhibitors	Block the protease activity of tryptase	Pre-clinical
	CysLTR1 antagonists	Block the binding to and effects of CysLT on target cells	Clinical use
	H_{1-4} receptor antagonists	Block the binding to and effects of histamine on target cells	H_1/H_2: Clinical use H_3: Clinical trials H_4: Pre-clinical
	PAR-2 antagonists	Block PAR-2 receptor signaling following activation by proteases (e.g., tryptase)	Pre-clinical
	DP and CRTH-2 receptor antagonists	Block the binding to and effects of PGD_2 on target cells	Pre-clinical

*Representative strategies in each target class are presented.

MAPK, mitogen-activated protein kinases; PI3K, phosphoinositide-3 kinase; PDE, phosphodiesterase; 5-LO, 5-lipoxygenase; PAR-2, proteinase-activated receptor 2; CRTH-2, chemoattractant receptor homologue on T helper type 2 cells; DP, D prostanoid.

Table 1.1: Mast Cell and Mast-product Directed Therapeutics

The drugs devised to combat allergic diseases by acting on mast cells as the target can be broadly grouped into those directed at the cell membrane targets (membrane receptors), to intracellular targets (cell signalling, gene expression) or to extracellular targets (Adapted from Brown et al., 2008).

1.9: Aim of Study

Our group is exploring the relationships between the structure of the FcεRI and its ability to mediate transmembrane signalling. The methodology employed was to carry out site-directed mutations in the alpha and gamma subunits of the FcεRI. When compared to other multisubunit membrane receptors the FcεRI is characterised by weak subunit interactions (Kinet et al., 1985) so that even minor modifications of one or more of the subunits can lead to significant overall changes. Immunoreceptors have conserved transmembrane motifs that are believed to play a crucial role in receptor assembly, expression and function.

Transmembrane domains of FcεRIα and FcγRIIIα subunits have an invariant eight amino acid motif (LFAVDTGL), which has been shown to be conserved in mice, rats and humans. This predominantly hydrophobic motif has a hydrophilic aspartic acid residue (D194) that in theory is energetically unstable (Ravetch and Kinet 1991). The aim of the present study was to examine the role of the D194 residue within the TM domain of the huFcεRIα subunit on cell surface expression and FcεRI -mediated signaling. Rat Basophilic Cells (RBL-2H3.1) originating from rats treated with β-ethylamine were chosen for the study due to their identical phenotype to the immature human mast cells (Eccleston et al., 1973). The RBLs are functionally homologous to human mast cells in FcεRI cell surface expression, granular content and mediator release and represent an accepted model system to study mast cell/basophil physiology. The ligand-binding domain of the rodent FcεRI complex does not recognise primate or human IgE. Previous studies have shown that in RBL-2H3 cells the transfected huFcεRIα subunit forms a functional chimeric rat/human receptor complex with endogenous FcR-γ and FcR-β subunits. These rat/human chimeric receptors can be specifically activated via a human IgE (huIgE) mediated cross-linking stimulus to mediate cell secretion (Gilfillan et al., 1992, Wilson et al., 1993) and conclusions to be drawn from the outcome of these experiments are more relevant to the assessment of mast cell signaling pathways than earlier studies based on COS cell transfection (Varin-Blank and Metzger 1990). The outcome of preliminary mutagenesis studies by Iodice (2006) led to the proposal of a model structure for FcεRI by Professor P.Artimyuk (UoS), which proposed an interaction between the D194 residue of the alpha subunit and Thr22 residue of the gamma chain as shown in **figure 1.6** (Iodice, 2006). This model differed significantly from the one proposed

from the one proposed previously by (Farber and Sears, 1991) which suggested an interaction between positively charged K29 (FcR-γ) which corresponds to K30 in FcR-γ subunit, with negatively charged D203 (rat FcγRIIIα) homologous to D194 in huFcεRIα. In the model proposed by our group there is a 2:1 stoichiometry between the FcR-γ and FcεRIα subunits rather than a 1:1 association predicted in the earlier model (Farber and Sears, 1991). The other difference between the two models predicted that K29, found in a relatively hydrophilic sequence RLKIQV, is located in the cytoplasmic rather than the TM region, as proposed by Farber and Sears (1991). Thus our model considered only the interaction between the minimal essential receptor subunits of FceRIα and γ involved in FcεRI-mediated signalling.

In order to assess the proposed model and to elucidate the FcεRIα and FcR-γ subunits interactions it was necessary that mutations be carried out in the FcR-γ subunits. For this purpose a subline of RBL-2H3, which was reported to be defective in the expression of FcεRI, RBL γ-chain deficient cell line (RBL-γ-) (*section 2.2),* a gift from Professor Draber, was used to make a distinction between cells responses mediated by the endogenous FcRγ from those of FcεRI. The RBL γ-chain deficient cell line was prepared (Draberova and Draber, 1995) using chemical mutagenesis suppressing γ-chain expression. The γ subunit, is essential for the cell surface expression of FcεRI and FcγRIII (Kinet, 1992) and the resultant γ-chain deficient cell line expresses a lower level of the IgE receptor. Following transfection of γ-chain deficient RBLs with an expression plasmid encoding the rodent gamma construct demonstration of cell surface expression of the receptor complex could be demonstrated in the γ-chain transfected cells. The cells could be sensitized with mouse IgE and challenged with antigen to assess the release of mediators (Bocek et al., 1995). This suggested that the RBL γ-chain deficient cell line could be employed as a host for the transfection of native and mutant γ-chain constructs for the assessment of a structure/function relationship in FcεRIα/γ interaction. This investigation formed the basis for the second part of the project since transfection of gene construct encoding native and mutant forms of FcεRIγ should facilitate, through site-directed mutagenesis, an investigation into the role of Thr22 in supporting surface expression and IgE mediated, antigen induced cell activation.

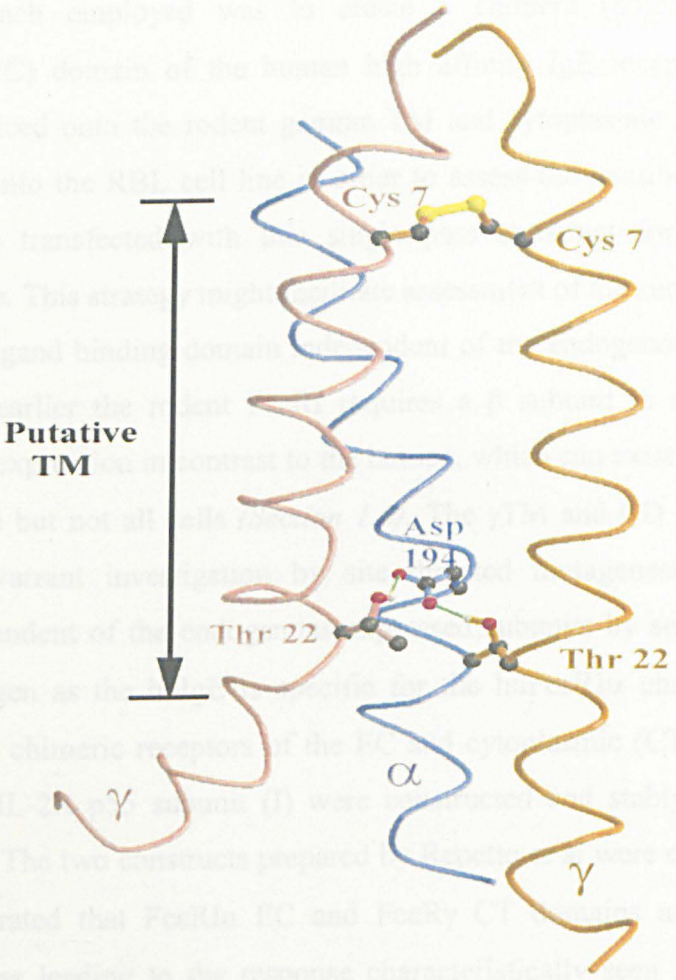


Figure1.6: Modeling of Transmembrane Helices

The transmembrane regions of both the FcεRIα and FcR-γ subunits were identified from NCBI annotations and confirmed by hydropathy plots (Kyte and Doolittle, 1982). The proposed model envisages two FcR-γ subunits linked at Cys7 residue by disulphide bond and a central located single FcεRIα helix all with inter-helical angles of approximately -25°. The aim was to expose most of the hydrophobic side chains on the side to permit a favorable interaction with lipid tail groups in the membrane. The model positioned D194 of the FcεRIα subunit in the hydrogen bonding distance from the Thr22 residues present in the both FcR-γ subunits (indicated by arrows). The model was subjected to energy minimization using the X-PLOR program (Brunger et al., 1987) in order to improve stereochemistry and to optimize contacts from the original model (adapted from Iodice, 2006).

Another approach employed was to create a chimera (humanαγγ) using the extracellular (EC) domain of the human high affinity IgE receptor alpha subunit (huFcεRIα) spliced onto the rodent gamma TM and cytoplasmic domain (CD) and transfect them into the RBL cell line in order to assess the possibility of selectively activating cells transfected with this single pass construct for antigen induced mediator release. This strategy might facilitate assessment of the surface expression of the huFcεRIα ligand binding domain independent of the endogenous rodent FcεRIα. As mentioned earlier the rodent FcεRI requires a β subunit in addition to two γ subunits for its expression in contrast to the human, which can exist as a trimer on the surface of some but not all cells *(Section 1.6)*. The γTM and CD contain important residues that warrant investigation by site directed mutagenesis which can be activated independent of the endogenous expressed subunits by stimulating through huIgE and antigen as the huIgE is specific for the huFcεRIα chain. Repetto et al (1996) prepared chimeric receptors of the EC and cytoplasmic (CT) domains of the FcεRI and the IL-2R p55 subunit (I) were constructed and stably transfected into RBL-2H3 cells. The two constructs prepared by Repetto et al were α/γ/γ and I/γ/γ and results demonstrated that FcεRIα EC and FcεRγ CT domains are mandatory for signalling process leading to the response characteristically seen after the receptor aggregation (Repetto et al., 1996).

This ongoing study should provide significant information regarding the importance of conserved polar amino acids in the TM domain of multi-subunit cell surface receptors. Identification of molecular interactions that occur at contact sites between TM domains of the subunits of the high-affinity IgE receptor by mutational analysis may reveal targets for the development of therapeutic agents aimed specifically at inhibition of FcεRI-mediated cell signaling. The identification of TM amino acid residues implicated in the down stream signalling may reveal useful targets in the treatment of the IgE-mediated hypersensitivity through the introduction of a peptide sequence which contains the amino acid which has the ability to inhibit transmembrane interaction in receptor complexes thus interrupting the downstream signalling cascade at an early stage. This approach has been successfully applied to the development of "molecular wedge" anti-cancer drugs, by developing TM-derived peptides that inhibit *neu* oncogene receptor aggregation thus retarding growth of neu-transformed cells in monolayers and tumors in nude mice (Lofts et al., 1993). This

approach was also used to modulate the T-cell receptor signaling and may have wider applications for the development of selective inhibitors of T cell-mediated diseases. Manolios et al (1997) showed that a specific synthetic peptide (core peptide) , based on a conserved motif present in the TM region of TCRα subunit which contained positively-charged residues interacting with similarly negatively-charged residues of the CD3 complex (Clevers and Ferrier, 1998), entered and suppressed T cell function. This novel therapeutic agent has shown promise in inhibiting T cell functions *in vitro* in T cell hybridoma (2B4.11) and in reducing clinical signs of three T cell mediated diseases in *in vivo* studies in animal models of immunoreceptor-mediated pathologies (Manolios et al., 1997). These core peptides are not only anchor proteins but appear to function by inhibiting downstream signalling by blocking receptor subunit interactions in the membrane. The most interesting aspects of these peptides is their ability to internalize to many cell types without any complications (Huynh et al., 2003).

The current study aimed at a better comprehension of mast cell and IgE receptor complex signalling to pave the way for development of efficient proactive preventive measures and therapeutic tools to combat allergic disease thus relieving the sufferings of millions of human beings world wide.

CHAPTER 2

MATERIALS AND METHODS

Chapter 2

Materials and Methods

2.1: Materials and Equipment

All chemicals were purchased from SIGMA unless stated otherwise. Deionised water was used as solvent.
All solutions were formulated as described by Sambrook (2001).

2.1.1 Cell Culture Reagents

1.2 ml Cryogenic Vials	Nalge Nunc Inc.
50 ml Polypropylene tubes	Greiner Bio-One
100 mm tissue culture dishes	Iwaki
CDS	Sigma-Aldrich
DMEM	Sigma-Aldrich
DPBS	Sigma-Aldrich
FCS	Autogen Bioclear
Geneticin (G-418 sulphate)	Invitrogen
Pencillin/streptomycin solution	Sigma-Aldrich
Trypsin/EDTA	Sigma-Aldrich
Universal tubes	Bibby Sterilin

2.1.2: Protein Manipulation

Acrylamide/Bis (30%)	Sigma-Aldrich
Bradford protein assay reagent	Bio-Rad
Bovine serum albumin (BSA, Fraction V)	Fischer Scientific
Developer/fixer solution	Kodak

Dried semi –skimmed milk	Somerfield
Enhanced Chemiluminescence detection kit	Amersham Biosciences
Full Range molecular weight marker	Fermentas
Low Range molecular weight marker	Amersham Biosciences
Nitrocellulose membrane (HybondC+)	Amersham Biosciences
Protease Inhibitor Cocktail Tablets	Roche
Protein A agarose, fast flow	Chemicon
TEMED (N'N'N'N'-tetra methyl ethylenediamine)	Bio-Rad
X-ray film	Kodak

2.1.3: Antibodies Used

Anti-phosphotyrosine(Clone 4G10,mouse)	Upstate
DNP-specific mouse IgE (mIgE)	SPE-7 supernatant developed at The Antibody Resource Centre,University Of Sheffield
Goat anti-human IgE biotinylated	Vector Laboratories
Goat anti-mouse IgE FITC-conjugated	Bethyl Laboratories
HumanIgE (Chimeric NIP-specific)	Serotec
Mouse anti-syk-[01]	Abcam
NIP-specific human IgE (huIgE)	JW8 developed at The Antibody Resource Centre, University Of Sheffield
Rabbit anti FcR-γ	Upstate
Rabbit anti-syk	Santa Cruz Biotechnology
Streptavidin R-phycoerythrin	Sigma-Aldrich
Swine anti-rabbit HRP-conjugated	Dakocytomation

2.1.4: Molecular Biology Reagents

Agar (Bacto)	Difco
Agarose (SeaKem)	FMC
Ampicillin	Roche
Calf Alkaline Phosphatase	Autogen Bioclear
DNA Markers (1 Kb)	Fermentas
Plasmid Midi Kit	Qiagen
METAFECTENE™ PRO	Biontex
Restriction enzymes	Promega
T4 DNA Ligase	Promega
Tryptone	Unipath
DNA gel extraction kit	Bio-Rad
Plasmid miniprep kit	Qiagen
Yeast extract	Difco

2.1.5: Buffers

Dulbecco's phosphate buffered saline (DPBS) was purchased from Sigma and sterilization was done by autoclaving.

Tissue culture Trypsin/EDTA solution (0.5% trypsin, 0.2% EDTA) was prepared by diluting the Trypsin-EDTA (TE) 10x stock (Sigma-Aldrich).

Tissue culture DMEM was supplemented with 10% FCS. Media used for transfected cell lines was further supplemented with Geneticin (G-418) 400μg/ml, was initially reconstituted in 20ml of DMEM and then added to FCS supplemented media via a 0.2μm filter. 5ml of 100u/ml penicillin and 100μg/ml streptomycin solution (Sigma-Aldrich) was added to both types of media.

NIP-HSA was prepared according to manufacturer's protocol.

2.1.6: Equipment

37° C Incubator	LEEC
37° C Incubator (Galaxy R CO_2)	SLS
Agarose gel electrophoresis equipment	Bio-Rad
Balances (AE200, PJ3000)	Mettler
96- Well plate reader (Milenia Kinetics Analyser)	Diagnostic Product Corporated
Centrifuge (Minispin, <2ml)	Eppendorf
Centrifuge (Centaur II, <50ml)	MSE
Cryomed liquid nitrogen container	Forma Scientific
FACSVantage flow cytometer	BD Biosciences
FACSCalibur flow cytometer	BD Biosciences
FACSMoFlo flow cytometer	Dakocytomation
FACSCyan ADP cytometer	Dakocytomation
FACSort flow cytometer	Dakocytomation
Electrophoresis (Mini Protean II)	Bio-Rad
Electroporator-Gene pulse	Bio-Rad
Fluorchem™ Imaging System	Alpha Innotech Corporation
Haemocytometer	Weber Scientific
Heat block	Grant
Microwave	Goodmans
Laminar Flow cabinets	BioMat
pH Meter (Toledo 320)	Mettler
Pipettes (p10, p20, p200, p1000)	Gilson
Pipettes multichannel	Anachem limited
Wheel Rotator	Stuart Scientific
Spectrophotometer (PU 8625)	Philips
UV Transilluminator	UVP
Vortexer (whirlmixer)	Fisions
Water Bath	Clifton/ Nickle electrode

2.2: Cell Lines

Rat Basophilic Leukaemia (RBL) cell line RBL-2H3.1 employed in the study was derived from rats treated with β –chlorethylamine that developed basophilic leukaemia (Eccleston et al., 1973). The cell line has been extensively used in high affinity IgE receptor studies due to its identical phenotype to immature human mast cells. The RBLs have similarities to immature human mast cells in FcεRI cell surface expression, granular content and mediator release and thus represent an easily maintained mast cell-like model for the study of IgE-mediated, antigen induced mediator secretion (Blank and Varin-Blank, 2004).

The other cell line used was RBL gamma chain deficient, a subline of RBL-2H3, which was defective in the expression of FcεRI and was a gift from Professor Draber of University of Prague. RBL γ-chain deficient cell line was isolated from RBL-2H3 cell chemically mutagenised by 5 successive cycles of negative immunomagnetic separation followed by cloning of FcεRI deficient cells using limiting dilution (Draberova and Draber, 1995) causing it to have an FcεRI, which is devoid of the γ subunit, a subunit which is essential for the cell surface expression of FcεRI and FcγRIII (Kinet, 1992).

2.3: Mammalian Cell Culture Techniques

2.3.1: Culturing RBL Cell Line

RBL-2H3.1 cells were grown at 37° C in a humidified atmosphere of 5% CO_2, as monolayer cultures in 100/145mm plates in DMEM supplemented with 10% FCS. The transfected cell lines were grown in media supplemented with Geneticin (G-418). The cells on reaching a confluency of 80-90% were harvested by trypsinisation. The media was aspirated and cells washed in DPBS to remove FCS. Trypsin/EDTA was added to each plate and incubated at 37° C for 5 minutes. Cells were lifted from the plate by gentle washing with a P1000 Gilson and added to an equal volume of media containing FCS (inactivating the trypsin). Cells were recovered by centrifugation (180g for 3 minutes) and re-suspended in 1ml of the appropriate media. Aliquots according to the size of tissue culture plates were added to the appropriate volume of media allowing confluency 2 days later.

2.3.2: Cryogenic Preservation

For long-term storage of cell lines, a sub confluent monolayer of cells was harvested by trypsinisation. Proceeding recovery of cells by centrifugation, the pellet was resuspended in FCS (90%) and transferred to a cryogenic storage vial. After the addition of DMSO (10%), cells were placed in vapour phase of liquid nitrogen in a modified holder for the nitrogen storage bank for 1 hour before being catalogued and placed in liquid nitrogen.

2.3.3: Thawing of Cells

After removal from the liquid nitrogen storage bank the cells were re-suspended in 20ml of appropriate media pre-warmed to 37° C. Cells were recovered by centrifugation (180g for 3 minutes) and re-suspended in 1ml of the appropriate media. Aliquots according to the size of tissue culture plates were added to the appropriate volume of media and left overnight. The following day the cells were checked for adhesion and media was replaced.

2.4: β –Hexosaminidase Assay: Measurement of Secretory Output from RBL Cell Lines in Response to an Immunological Stimulus

Reagents:

Buffered Solution (BS): (pH7.4)	120mM NaCl, 5mM KCl, 25mM PIPES, .04mM $MgCl_2$, 1mM $CaCl_2$
Substrate:	p-nitrophenyl N-acetyl β-D-glucosaminide Stock solution (50mM) in DMSO

Protocol:

Cell lines were harvested by trypsinisation as described previously (*section 2.3.1*). Viable cells numbers were counted on a haemocytometer ($1x10^{-4}$ volume). Cells were

resuspended at a cell density of $0.5x10^6$ /ml and sensitized with DNP-specific mIgE (SPE-7 1/500) or NIP-specific huIgE (JW8 1/500). Aliquots (100µl) were plated out in triplicate on 96-well plates and incubated at 37°C for 16 hours. All solutions from this point were pre-warmed to 37°C. Next day, 96 well plates were examined for cell confluency before the media was removed and cells washed twice with DPBS. DNP-HSA and NIP-HSA dilutions (0.1-10000ng/ml) and Triton -X100 (1%) were prepared in BS. BS (100µl) was added to the first set of three wells (blank), followed by 100µl of Triton-X100 (total) to the last set of triplicate wells, and addition of DNP-HSA/NIP-HSA dilutions to the remaining wells as appropriate. Following incubation for 20-30 minutes at 37°C, 50µl of supernatant from each well was transferred to an identically labelled 96-well plate. Substrate solution was diluted in 0.2M citrate buffer (pH4.5) to a final concentration of 2mM. 50µl of substrate solution was added to each well containing supernatant prior to 2-3 hours incubation at 37°C allowing time for the reaction to take place. The reaction was terminated by the addition of 150µl 1M Tris-HCl (pH 9.0). Plates were analyzed using an ELISA reader set at 405nm. Analysis of plates allowed percentage release for each DNP-HSA / NIP-HSA dilution to be calculated using the following equation.

$$\frac{\text{(Average of test triplicate)- (average of blank triplicates) x 100}}{\text{(Average of total triplicates)}}$$

2.5: Cell Lysate Preparation for 1D- PAGE

2.5.1: Sample Preparation

Reagents:

RIPA Lysis buffer 150mM NaCl
1% Igepal CA-630 (Nonidet P-40)
0.5% Sodium deoxycholate
0.1% SDS, 50mM Tris-HCl (pH 8)

All lysis buffers were supplemented with protease inhibitor cocktail tablets (Roche), 10-100 µl PMSF and 1mM Sodium orthovanadate.

Protocol

Cells were cultured as monolayer on 145 mm plates and sensitized for 16 hours with either DNP specific mIgE (SPE-7, 1/500) or NIP specific huIgE (JW8), 1/500) at 37°C. Solutions and universal tubes were all pre-warmed to 37°C. The following day cells were washed with pre-warmed PBS and harvested in pre-warmed CDS. This was followed by recovery by centrifugation (180g for 3 minutes). The cell pellet obtained was resuspended in 1 ml of pre warmed BS. Cell numbers were determined and re-suspension was carried out at 5 x 10^6/ml. The cells were divided equally into universal tubes dependent on the number of samples required. The appropriate sample was activated for 2 minutes with the corresponding cross-linking agent (100ng/ml, optimal concentration determined from β –Hexosaminidase assay). The reaction was halted by addition of 10ml of ice cold PBS to each universal tube and the tubes were then kept on ice for 2 minutes. The cells were recovered by centrifugation (180g for 3 minutes) followed by re-suspension in 1 ml of DPBS and centrifugation (180g for 3 minutes) at room temperature. The pellets were resuspended in 1ml RIPA Lysis Buffer (to which sodium orthovanadate and 10-100µl PMSF was added) transferred to cooled micro centrifuge tubes, vortexed and left on rotating for 60 minutes at 4°C. From this point onwards all procedures were carried out at 4°C. The tubes were then centrifuged (10000g for 15 minutes) and cell lysate obtained transferred to new micro centrifuge tubes and assayed for protein content employing Bradford's method.

2.5.2: Bradford's Method for Protein Estimation

The need for carrying out protein quantification prior to immunoprecipitation is to ensure uniform protein concentrations in all samples under study. The principle of Bradford Assay is that binding of Coomassie Blue G-250 dye in the Bradford reagent to protein causes a shift in absorption from 465 to 595 nm. A standard curve was prepared using BSA. 900µl of Bradford Reagent (1/4 dilution, Bio Rad), 95µl dH_2O and 5µl of cell lysate were mixed in a 1.5ml cuvette to achieve a final volume of 1ml. After a lapse of 5 minutes readings were taken on spectrophotometer set at 595nm.

2.5.3: Immunoprecipitation

Protein A Agarose beads (Chemicon) were used in all immunoprecipitations. All pipette tips were cut off when working with protein A to avoid bead degradation. All steps were performed at 4°C unless otherwise stated.

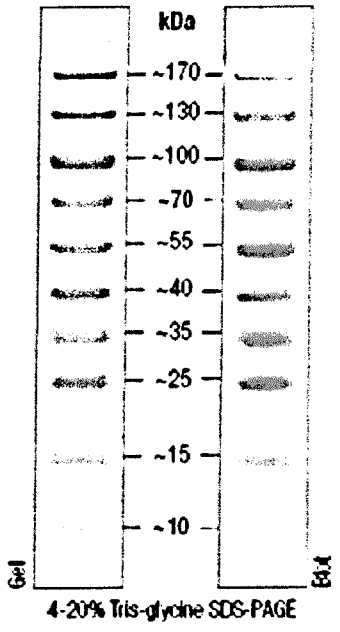
Protocol:

Protein A was prepared following the manufacturer's instructions (Chemicon). Cell lysate was incubated with protein A (100µl) of 50% slurry for 30 minutes (end-to-end rotation). This process is known as pre clearing which reduces any non-specific binding by proteins within the sample, which may interfere with later analysis. Lysates were centrifuged (10000g for 15 minutes) and the supernatant was transferred to new micro-centrifuge tubes being careful not to disrupt protein A pellet. Protein estimation of sample was carried out using the Bradford protein assay allowing sample normalisation. At this point, 15-25µl of cell lysate was taken from the samples to be used in anti-phosphotyrosine immunoprecipitation for the later purpose of FcR-γ quantification. An equal amount of appropriate antibody was added to each sample following the manufacturer's instructions. Lysates were incubated overnight with end-to-end rotation to allow antigen-antibody complex formation. The following day, protein A (100µl) was added to each sample as appropriate prior to 2-4 hour incubation with end-to-end rotation allowing immune complex capture. Immunoprecipitates were pulsed in a centrifuge (10000g), the supernatant removed and washed three times with appropriate ice-cold lysis buffer. After the last washing all of the supernatant was removed from the immunoprecipitates using a Gelsaver pipette microtip. Immunoprecipitates were resuspended in 60µl of Laemmli's 1-D SDS-PAGE loading buffer (50mM Tris-HCl, pH 6.8, 2% SDS, 10% Glycerol, 100mM DTT) and boiled for 5 minutes. Samples were centrifuged (10000g for 5 seconds) and resolved on a 12.5-17% SDS-PAGE gel.

2.5.4: One-dimensional Polyacrylamide Gel Electrophoresis

Proteins were resolved by polyacrylamide gel electrophoresis using the Mini-Protean II electrophoresis system (Bio-Rad). Acrylamide gels (12.5-17%) were made using a multi-gel casting chamber following the manufacturer's instructions. Discontinuous acrylamide

gels were used consisting of a stacking gel (5% acrylamide in Tris-HCl pH 6.8) allowing uniform entry of sample into the gel followed by a separating gel (12.5 –17% acrylamide in Tris-HCl pH 8.8) for the resolution of proteins according to molecular weight. Following the addition of 60µl of 1–D SDS sample buffer, samples were boiled for 5 minutes and were pulsed in a centrifuge (10000g). The samples were then allowed to cool to room temperature prior to loading in parallel with a molecular marker (Fermentas) to allow estimation of relative molecular weights of the resolved proteins. Electrophoresis was performed in a Tris-Glycine buffer (25mM Tris, 250mM Glycine pH 8.3, 0.1% SDS). Proteins were resolved at 50V for 20 minutes to allow proteins to enter the stacking gel followed by 100-120V until the dye front reached the end of separating gel. On the completion of electrophoresis, gels were used for Western transfer to PVDF membrane.



Protein Ladder (10-250kDa) (Fermentas)

2.5.5: Western Transfer of Proteins to PVDF Membrane

Electrophoretic transfer of proteins to PVDF (Amersham Biosciences) was performed using the Mini Trans-Blot system (Bio-Rad) according to the manufacturer's instructions

in a Tris-Glycine buffer 25mM Tris, 250mM Glycine pH 8.3, 20% methanol). The PVDF membrane required a pre-soaking step in methanol for 1 minute followed by washing in distilled water for 5 minutes before transfer buffer equilibration for 10 minutes. The assembly consisted of the gel and membrane in direct contact, sandwiched between two sheets of filter paper and sponge on either side and held together in a transfer cassette. The cassette was placed in the Trans-Blot System checking for correct orientation. Electrophoretic transfer was carried overnight at 30V (4°C).

2.5.6: Immunodetection of Transferred Proteins

Membranes were removed from the Mini Trans Blot system and placed in universal tubes/50ml polypropylene tubes depending on the size of membrane (protein side facing the lumen). All steps were carried out on a roller platform mixer (Denley Spiramix) to ensure even distribution of solution across the membrane and minimize antibody usage. Depending on the primary antibody used blocking of the non-specific binding sites on the membrane was carried out by immersing the membrane either in 3% Bovine serum albumin (anti-phosphotyrosine, clone 4G10) or 5% dried semi-skimmed milk (all other antibodies) in DPBS-Tween (1xDPBS supplemented with 0.1% tween-20, Sigma-Aldrich) for 60-90 minutes at room temperature (alternatively it may be left overnight in blocking solution in refrigerator at 2-8°C). The prepared blocking solutions were kept aside for antibody dilutions. The membranes were rinsed three times in DPBS-Tween. Membranes were incubated for 45-60 minutes at room temperature with the appropriate primary antibody diluted in appropriate blocking solution. Following primary antibody incubation the membranes were rinsed three times and washed in DPBS-Tween in four steps of 1x15minutes and 3 x 05minutes. Dilution of the secondary horseradish-peroxidase (HRP) antibodies was made in appropriate blocking solution and the membranes were incubated for 45-60 minutes. Membranes were washed as described previously and prepared for protein detection using Enhanced Chemiluminescence (ECL) according to manufacturer's instructions.

2.5.7: Detection of Proteins with Enhanced Chemiluminescence (ECL)

Membranes were drained of excess DPBS-Tween and incubated with equal volumes (enough to cover the membrane) of both ECL reagents for 1 minute at room temperature. Membranes were placed in a photographic cassette (protein side up) and covered with cling-film and taken to dark room. A sheet of autoradiography film was placed on top of the membrane in the cassette and exposure of film for varying lengths of times dependent on signal strength was carried out followed by developing and fixing of the film (KODAK).

2.6: Assessing FcεRI Expression in Transfected Cell lines by Flow Cytometry

Reagents:

Wash buffer	DPBS suplemented with 1% FCS (4°C)
Primary antibody	Mouse IgE (mIgE) (UoS)/ human IgE (Serotec)
Secondary antibody	Biotinylated goat anti human IgE (Vector Laboratories)
Flurochrome antibody	Streptavidin R-phycoerythrin (Sigma-Aldrich)
	Goat anti-mouse IgE FITC-conjugated (Bethyl Laboratories)

Protocol:

Transfected cell lines were grown in 100mm tissue culture plates to cell confluency for the days of FACS analysis. Cells were washed with pre-warmed PBS and harvested in pre-warmed CDS. This was followed by recovery by centrifugation (180g for 3minutes). The cell pellet obtained was washed twice in 10ml wash buffer and resuspended at a density of $5X10^6$ cells/assay in ice-cold wash buffer. 50µl of cell suspension was added to non-sterile polypropylene FACS tube as un-labelled negative control and placed on ice till cell analysis. Another 50µl cell suspension was added to appropriately labelled non-sterile polypropylene FACS tube as labelled control. The appropriate 1° antibody (mIgE / huIgE ,1µg) was added to 100µl cell suspension in appropriately labelled non-sterile polypropylene FACS tube labelled as sample and incubated on ice for 30 minutes. After

the 30 minutes incubation period the polypropylene FACS tubes containing the labelled control and the sample were washed two times with ice cold wash buffer before finally resuspending the pellet in 1ml cold wash buffer. Biotinylated goat anti human IgE (1/400 dilution in wash buffer) / goat anti-mouse IgE FITC-conjugated (1/500) was added and mixed to each tube and left on ice for 30 minutes. The cells were recovered by centrifugation and washing as described above. Streptavidin R-Phycoerythin antibody (1/50 dilution in wash buffer) was added (RBL transfected cells) and mixed to both tubes (labelled control and sample) and again incubated on ice for 30 minutes. Two final washes were carried out to remove excess Streptavidin R-Phycoerythin / goat anti-mouse IgE FITC-conjugated antibody as described above and the cells were resuspended in 200µl wash buffer and kept on ice till the analysis. Samples were analysed using a FASort or a FACS™ CYAN ADP flowcytometer (Dakocytomation).

2.7: FACS Cell Sorting of Transfected Cell Lines by Flow Cytometry

Reagents same as above.

Transfected cell lines were grown in 100/145mm tissue culture plates to cell confluency (~12X10^{6}cells/plate) for the days of FACS sort. Cells were washed with pre-warmed PBS and harvested in pre-warmed CDS. This was followed by recovery by centrifugation (180g for 3minutes). The cell pellet obtained was washed two times in 10ml wash buffer and resuspended in 1 ml ice-cold wash buffer. Cell suspension (50µl) was added to non-sterile polypropylene FACS tube as unlabelled negative control and was placed on ice till cell sorting. Another 50µl cell suspension was added to non-sterile polypropylene FACS tube as labelled negative control and was placed on ice. Human IgE / mIgE (10µg) was added and mixed to the remaining cell suspension (sample) which was incubated on ice for 30 minutes. Labelled control and sample were centrifuged (180g for 3minutes) at 4°C; the supernatant was removed and two washes with ice cold wash buffer were carried out before finally resuspending the pellet in 1ml cold wash buffer. Biotinylated goat anti human IgE (1/400 dilution in wash buffer) / goat anti-mouse IgE FITC-conjugated (1/500) was added and mixed to each tube and left on ice for 30 minutes. The cells were

recovered by centrifugation and washed as described above. Streptavidin R-Phycoerythin antibody (1/50 dilution in wash buffer) was added (RBL transfected cells) and mixed to both the tubes (labelled control and sample) and were kept on ice for 30 minutes. Final two washes were carried out as described above to remove excess Streptavidin R-Phycoerythin / goat anti-mouse IgE FITC-conjugated antibody and cells were resuspended in 200-500µl of wash buffer and were kept on ice till the time of cell sorting. Samples were FACS cell sorted using a FACSAria or a FACS MoFlo flowcytometer (Dakocytomation). The sorted cells were collected in the appropriate media and plated in 100mm plates in the appropiate media (supplemented with Penicillin 100U/ml and streptomycin 100µg/ml to avoid contamination) as described in *section 2.3.1.*

2.8: Assesment of Intracellular Calcium Mobilisation

Intracellular Ca^{2+} mobilization brought about by antigenic stimulus (NIP/DNP) was assessed using the Ca^{2+} indicator Fluo-3AM , 4-(6-Acetoxymethoxy-2,7-dichloro-3-oxo-9-xanthenyl)-4'-methyl-2,2'(ethylenedioxy) dianiline-N',N',N',N'- tetraacetic acid tetrakis (acetoxymethyl) ester. Developed by Minta et al (1989), Fluo 3 remains non-fluorescent until bound with Ca^{2+} resulting in a 40-fold increase in mean fluorescence. Analysis was carried out in the visible light range with excitation at ~488nm and emission at~ 525nm. Acetoxylmethyl ester was added to give an uncharged molecule that can diffuse across the cell membrane allowing entry into the cell cytoplasm. Acetoxymethyl ester is hydrolysed by action of cytoplasmic esterase yielding Fluo 3 within cell (Tsien, 1981).

Reagents:

Balanced Salt Solution (BSS)	148mM NaCl, 49mM KCL, 63mM D-sorbitol, 2.63mM K_2HPO_4, 1mM KH_2PO_4, 10.1mM HEPES
Fluo-3-AM	Sigma-Aldrich, 500 µM stock in DMSO
Ionomycin	Sigma-Aldrich, 1mg/ml stock in DMSO

Protocol:

Cells were cultured as a monolayer on 145mm plate and passively sensitized for 16 hours with either DNP-specific mIgE (SPE-&, 1/500) or NIP-specific huIgE (JW8, 1/500) at 37°C. The cells were washed in DPBS and harvested with CDS and centrifuged (180g for 3 minutes). Cells were counted and resuspended in BSS at $1x10^6$/ml. For the purpose of background reading 0.5 ml of each cell suspension was taken. This was followed by addition of Fluo 3-AM (5μM) and incubation at room temperature for 30 minutes was carried out in the dark. Cells were pelleted and resuspended in DPBS (containing no additional $CaCl_2$ or $MgCl_2$) at a density of $2x10^6$/ml. IgE sensitized cells loaded with Fluo-3AM were analyzed using a FACSort flow cytometer preset for Fluo-3AM studies. Data was recorded in the form of a density plot of mean fluorescence against time. After an initial background reading of 30-50 seconds, samples were activated with the appropriate cross-linking agent (NIP/DNP 100ng/ml). In the event where no calcium mobilization was witnessed ionomycin (10μM) (a calcium ionophore) was used as a positive control to ensure that the sample has been loaded with Fluo-3AM.

2.9: Molecular Biology Methods

2.9.1: Bacterial Strain

All cloning work was carried out using the XL 1-blue *E.coli* strain (Stratagene).

Genotype – *rec*A1 *end* A1 *gyr*A96 *thi*-1 *hsd*R17 supE44 *rel*A1 *lac* [F'*pro* AB *lac*I^q ZΔM15 *Tn* 10 (Tet^1)].

2.9.2: *E.Coli* Growth Media

Bacterial cultures were grown in LB-broth or LB-agar as required supplemented with the antibiotic (Ampicillin 10μg/ml).

LB Broth- MQ water, Tryptone (10g/L), Yeast extract (5g/l), Sodium Chloride (10g/L).

LB Agar: LB Broth; Bacteriological Agar (15g/L).

2.9.3: Vectors

pEE6 (Celltech)

This vector was used previously to generate the RBLhuFcεRIα transfected cell line *(Section 1.11)* (Wilson et al., 1993). The vector confers resistance to ampicillin and neomycin, allowing selection of transformants in E.Coli and mammalian cells using G418.

pUC18

pUC18 is a 2686bp vector. The vector has a β-lactamase resistance gene conferring ampicillin resistance and lacZ operon, which allows selection of the successfully ligated clones.

2.9.4: Preparation of Competent Bacterial Cells

Buffers

TFB1- 30mM KCH_3COO, 10mM $CaCl_2$, 50mM $MnCl_2$, 100mM $RbCl_2$, 15% Glycerol pH 5.8 with CH_3COOH

TFB2- 10mM PIPES, 75mM $CaCl_2$, 50mM $MnCl_2$, 10mM $RbCl_2$, 15% Glycerol. pH 6.5 with KOH

Protocol:

A single colony from a freshly streaked LB plate was cultured overnight in 2.5ml LB medium at 37°C with shaking (approximately 225rpm). The following day the entire culture was used to inoculate 250ml LB medium containing 20mM $MgSO_4$ in a 1L flask. Cells were cultured at 37°C with shaking until the OD was between 0.4 and 0.6 (upto 6 hours). Cells were centrifuged (4500g for 5 minutes) at 4°C and the pellet was gently resuspended in 100ml ice-cold TFB1. After incubation on ice for 5 minutes at 4°C, the cells were centrifuged as before and gently resuspended in 10ml TFB2. After incubation on ice for 15-60 minutes the cells were divided into aliquots (200μl) and quick-frozen in a dry ice / isopropanol bath for storage at -80°C.

2.9.5: Transformation of Competent Bacterial Cells

Competent cells were thawed slowly on ice. Approximately 10ng plasmid DNA was added to 200µl cells and mixed by swirling with a pipette tip. After incubation on ice for 30 minutes, cells were heated in a water bath at 42°C for 45-60 seconds then cooled on ice for 2 minutes. LB medium (3ml) was added and the cells were incubated for 45 minutes with shaking (approximately 150rpm) at 37°C. 50- 200µl of mix was spread on plates containing the selection antibiotic (ampicillin). The plates were incubated overnight at 37°C and examined for colony growth the next day.

2.9.6: Isolation of Plasmid DNA

2.9.6.1: QIAprep Spin Miniprep Kit

The QIAprep miniprep kit was employed when small amount of DNA was required. The kit contained all the required solutions and the protocol provided with the kit was followed. Cells were isolated from a 3ml overnight culture by centrifugation. The kit is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica in the presence of high salt. The procedure consists of following three basic steps

- Preparation and clearing of a bacterial lysate
- Adsorption of DNA onto the QIAprep membrane
- Washing and elution of plasmid DNA

Plasmid yield with the QIAprep miniprep system was variable and dependent on plasmid copy number per cell, the individual insert in the plasmid, factors that affect growth of the bacterial culture, the elution volume, and the elution incubation time. A 1.5ml overnight culture yielded between 5 and 15µg of plasmid DNA.

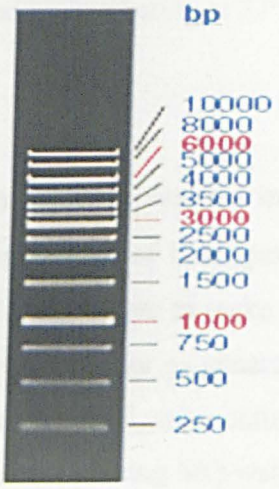
2.9.6.2: QIAGEN Plasmid Midi Kit

When large quantities of high purity plasmid DNA were required for mammalian cell transfection the QIAGEN plasmid Midi Kit was used allowing upto 100-200µg of DNA to be isolated. This kit is also based on the modified alkaline lysis procedure, followed by binding of plasmid DNA to QIAGEN Anion-Exchange Resin under appropriate low-salt

and pH conditions. Cells were isolated from a 50ml overnight culture by centrifugation. RNA, proteins, dyes, and low-molecular weight impurities were removed by a medium-salt wash. Plasmid DNA was eluted in a high salt buffer and then concentrated and desalted by isopropanol precipitation and subsequently washed with 70% ethanol. The DNA was eluted in a microcentrifuge with 100-1000µl water or TE buffer as specified in the protocol provided with the kit.

2.9.7: Agarose Gel Electrophoresis

TAE agarose gel electrophoresis was employed to visualise isolated plasmids, restriction enzyme digests and PCR products. A 6x loading buffer (MBI) was added to the DNA before loading on 1% agarose gel containing 0.5µg/ml ethidium bromide. Electrophoresis was performed using a Biorad subcell at 100V, 80mA for 1 hour. A molecular weight standard of 1Kb ladder (Fermentas) was loaded onto the gel. The DNA was visualised and photographed under UV light on the Fluorchem™ 8000 imaging system.



1Kb DNA Ladder (Fermentas)

2.9.8: DNA Manipulation

2.9.8.1: Restriction Enzyme Digests

Restriction enzymes digests were typically carried out in a 20µl volume, with 2µl 10x buffer and 10units of enzymes (1µl). The quantity of DNA used was dependent on the application (typically 0.5-1.0µg for test digest and up to 5µg for cloning) and the remaining volume made up to 20µl with sterile MQ water. The temperature and time for digestion depended on the specific restriction enzyme but was commonly 37°C for 1-2hr. The optimal 10x buffer for the specific enzymes was used according to the manufacturer's recommendation.

2.9.8.2: Dephosphorylation

To prevent religations without insertion of the desired fragment, 5' phosphate ends of pEE6 vector were removed prior to ligation using calf intestinal phosphatase. Digestion of pEE6 was performed to provide linearised plasmid for ligations. This was followed by adding 1µl alkaline phosphatase, 2µl 10x phosphatase buffer and 17µl sterile MQ water to the 20µl digest mixture followed by incubation at 37°C for 1 hour.

2.9.8.3: Ligation

Ligation of DNA fragment was typically carried out in 30µl total volume. The reaction mixture contained 1µl T4 DNA ligase, 3µl of 10x ligase buffer, varying ratios of 1:2 or 1:3 of vector (pEE6) to insert DNA and water to make up the volume. Generally a ratio of 1:2 or 1:3 of vector to insert was aimed for as generally a 2-3x molar excess of insert DNA gives optimal ligation. A plasmid re-ligation control was always set up along side whereby an identical reaction was set up having MQ water in place of DNA. The reaction was incubated at 16°C overnight.

2.9.8.4: DNA Purification from Agarose Gel

DNA gel extraction from agarose gel was carried out by using Quantum Prep™ Freeze N Squeeze DNA gel extraction spin columns kit (BIO-RAD). This kit purifies via filtration in a spin column format. The DNA band was excised from an agarose gel, and the gel slice cut into small pieces and placed in filter cup. The cup plus gel piece was put in a -20°C freezer for 5 minutes, then removed and immediately centrifuged (13,000g for 3 minutes) at room temperature. The agarose debris was retained within the filter cup and the bottom of the tube contained the recovered DNA.

2.9.8.5: Site Directed Mutagenesis

In vitro site-directed mutagenesis was carried out using Stratagene QuickChange® Site-Directed Mutagenesis Kit which allows site specific mutation in a double-stranded plasmid. This is a rapid four step procedure generating mutants with more than 80% efficiency. Miniprep plasmid DNA was used to make point mutations. QuickChange® Site-Directed Mutagenesis Kit was performed using *PfuTurbo*® DNA polymerase and a temperature cycler. The procedure utilizes a supercoiled double-stranded DNA (dsDNA) vector (pUC18) with the insert of interest and two synthetic oligonucleotide primers containing the desired mutation. The primers each complementary to opposite strands of vector are extended during temperature cycling by *PfuTurbo*® DNA polymerase. This generates a mutated plasmid containing staggered nicks. This is followed by treating the product with Dpn I. The *Dpn* I endonuclease is specific for methylated and hemimethylated DNA and is used to digest the parental DNA tempelate and to select for mutation-containing synthesised DNA. DNA isolated from almost all *E. Coli* strains is dam methylated and therefore susceptible to *Dpn* I digestion. The nicked vector DNA containing the desired mutations is then transformed into XLI-Blue supercompetent cells (Stratagen).

2.9.8.6: DNA Sequencing

The DNA was sequenced by Cogenics and then analysed using ALIGN, CHROMA and CLONE computer programmes to compare the sequence with genomic huFcεRIα subunit. The primers used for the sequencing were M13-48REV and M13For-40 to read the DNA from both ends. Clones with additional or no mutations were rejected.

M13-48REV GGGATAACAATTTCACACAG

M13For-40 GTTTTCCCAGTCACGAC

2.10: Transfection Mammalian Cells

Stable transfection in the mammalian cells was obtained by using the technique of electroporation. Cells were grown to 50-60% confluency and harvested as already described then washed with DMEM containing no FCS or antibiotics, to remove FCS so as to prevent sparking during electroporation. The cells were counted and resuspended in DMEM to give 10^7 cells/0.8ml. The cell suspension was added to electroporation cuvette (0.4cm electrode gap) in 0.8ml aliquots. Plasmid DNA prepared by using the Qiagen midiprep kit (*section 2.9.6.1*) was mixed with the cells, and a control prepared using 50µl MQ water in place of DNA. The cuvettes were then incubated on ice for 10 minutes. Each cuvette was given a single pulse of 250V, 960µF using a Gene Pulser (Bio-Rad) then incubated on ice for further 10 minutes. The cells were then plated out after resuspending in 20ml of appropriate fresh complete medium. After 24-48 hours recovery period the medium was aspirated and selection medium was introduced. The cells were maintained with change of medium being carried out every 2-3 days. Cells in the control died by 7-10 days and the successful transfected cells were grown as normal and selected using flowcytometry (*section 2.7*).

Another technique employed for carrying out transient transfection in RBL-2H3.1 was by using the commercially available METAFECTENE™ PRO transfection reagent as part

of collaboration with the manufacturer (Biontex). This reagent uses the Biontex' RMA-Technology (Repulsive Membrane Acidolysis) with new toxicity optimisation module (TOP-technology), which regulates the lipolex release of genetic material. The protocol provided by the manufacturer was followed.

CHAPTER 3

Mutagenesis of Human High Affinity IgE Receptor Alpha Chain subunit

CHAPTER 3

Mutagenesis of Human High Affinity IgE Receptor Alpha Chain subunit

3.1: Conserved Motif-FcεRIα

Previous studies have demonstrated the presence of an invariant eight amino acid motif (LFAVDTGL) within the transmembrane domain of FcεRIα and FcγRIIIα subunits, shown to be conserved in many species including rat, mice and humans. The conserved motif contains a polar aspartate residue at position 194 within a predominantly non-polar setting (Ravetch and Kinet, 1991) *(Section 1.11)*.

3.1.1: Residues Targeted for Site Directed Mutagenesis

Protein activity can be transformed by using the protein engineering technique of site directed mutagenesis thus providing the researchers with an insight into the structure function relationship leading to a better understanding of the cellular responses. This technique was employed to assess the functional consequences of site-specific mutagenesis replacing the D194 residue of human FcεRIα subunit (huFcεRIα) to obtain an improved understanding of the role of this residue within the transmembrane domain in FcεRIα-mediated signalling. The mutations introduced previously included asparagine (D194N), leucine (D194L), alanine (D194A), glutamic acid (D194E), and lysine (D194K) (Iodice, 2006). The RBL-2H3.1 cells transfected with mutant huFcεRIα constructs D194E, D194A, and D194K failed to support cell surface expression (Iodice, 2006) while the wild type (D194) and mutant variant D194N expressed the huFcεRIα subunit at the cell surface in association with the endogenous FcR-β and FcR-γ subunits (Iodice, 2006). The transfected cell lines D194 and D194N demonstrated FcεRI aggregation, tyrosine phosphorylation of FcR-γ and Syk kinase, intracellular Ca^{+2} mobilisation and mediator release in response to antigen-mediated activation, analogous to that observed in parental RBL-2H3.1 cell line (Iodice, 2006). However the RBL-2H3.1 cell line transfected with mutant D194L gene construct, although supporting cell surface

expression, showed absence of FcεRI-mediated signalling in response to antigenic stimulus, but the pattern of cell surface expression was suggestive of constitutive receptor pre-clustering irrespective of antigenic stimuli (Iodice, 2006).

In order to gain a more comprehensive understanding of the role of the aspartate residue 5 new mutations were carried out in the present study which included replacing the D194 residue with polar residues threonine (D194T), serine (D194S), arginine (D194R) and non-polar residues valine (D194V) and isoleucine (D194I). The idea was to replace D194 with amino acids of different sizes and polarity and assess the effect of these mutations on the huFcεRIα expression and FcεRIα-mediated signalling.

The construct verification of the mutations carried out is shown in **figure3.1.a and 3.1.b.**

3.1.2 Primer design

Primers were designed to carry out single point mutation in the D194 domain with the aid of computer software. An additional silent mutation in the form of Stu I was also incorporated to aid in assessing the presence of mutation with the help of restriction digestion with Stu I and running on TAE agarose gel. The sequence of the primer was necessitated by the fixed position of the domain in which the point mutation was to be carried out. Primers were based on approximately 20bp sequence around the mutation. The mutagenic primers show the altered bases in bold and the altered codons are underlined while the silent mutation is shown in bold green. The primers are shown as below.

D194T (Threonine)

5′ 5′ G GTG ATT CTG TTT GCT GTG ACC ACA GGC CTA TTT ATC TCA ACT CAG CAG 3′
3 5′ CTG CTG AGT TGA GAT AAA TAG GCC TGT GGT CAC AGC AAA CAG AAT CAC C 3′

D194V (Valine)

5′ 5′ G GTG ATT CTG TTT GCT GTG GTC ACA GGC CTA TTT ATC TCA ACT CAG CAG 3′
3′ 5′ CTG CTG AGT TGA GAT AAA TAG GCC TGT GAC CAC AGC AAA CAG AAT CAC C 3′

D194I (Isoleucine)

5′ 5′ G GTG ATT CTG TTT GCT GTG ATC ACA GGC CTA TTT ATC TCA ACT CAG CAG 3′

3′ 5′ CTG CTG AGT TGA GAT AAA TAG CCC TGT GAT CAC AGC AAA CAG AAT CAC C 3′

Silent mutation- Stu I 5′ 5′ AGG CCT 3′

3′ 5′TCC GGA 3′

The mutations encoding the replacement of Asp with threonine and valine were successful but problems were encountered with the isoleucine mutation. This necessitated the design of a new primer and successful mutation was introduced by altering the bases.

D194I (Isoleucine new primer)

5′ 5′ G GTG ATT CTG TTT GCT GTG ATT ACA GGC CTA TTT ATC TCA ACT CAG CAG 3′

3′ 5′ CTG CTG AGT TGA GAT AAA TAG CCC TGT AAT CAC AGC AAA CAG AAT CAC C 3′

huFcεRIα TTTTTTATCCCATTGTTGGTGGTGATTCTGTTTGCTGTG**GAC**ACAGGATTATTTATC

F F I P L L V V I L F A V D T G L F I

D194 ***Aspartic acid*** *Polar* *Negatively-charged*

TTTTTTATCCCATTGTTGGTGGTGATTCTGTTTGCTGTG**GAC**ACAGGATTATTTATC

F F I P L L V V I L F A V D T G L F I

D194N ***Asparagine*** *Polar* *Non-Charged*

TTTTTTATCCCATTGTTGGTGGTGATTCTGTTTGCTGTGA**AAC**ACAGGATTATTTATC

F F I P L L V V I L F A V N T G L F I

D194L ***Leucine*** *Non-Polar* *Non-Charged*

TTTTTTATCCCATTGTTGGTGGTGATTCTGTTTGCTGTG**GTC**ACAGGATTATTTATC

F F I P L L V V I L F A V L T G L F I

Figure 3.1a-Sequence Verification of transmembrane domains of Wild type (D194) and mutant (D194N and D194L) huFcεRIα constructs.

These variants were generated in a previous study (Iodice, 2006).

The constructs were sequenced by Lark (Cogenics) Technologies Ltd. The sequence of transmembrane domain of huFcεRIα subunit was obtained from www.expasy.org. Translated amino acids are shown beneath each sequence. Green shaded area indicates the invariant motif with the mutations in bold underlined and in different colours: D194 (red), D194N (pink) and D194L (yellow).

D194T Threonine *Polar Non-Charged*

TTTTTTATCCCATTGTTGGTGGTGATTCTGTTTGCTGTG**ACC**ACAGGATTATTTATC
F F I P L L V V I L F A V **T** T G L F I

D194V Valine *Non-Polar Non-Charged*

TTTTTTATCCCATTGTTGGTGGTGATTCTGTTTGCTGTG**GTC**ACAGGATTATTTATC
F F I P L L V V I L F A V **V** T G L F I

D194I Isoleucine *Non-Polar Non-Charged*

TTTTTTATCCCATTGTTGGTGGTGATTCTGTTTGCTGTG**ATT**ACAGGATTATTTATC
F F I P L L V V I L F A V **I** T G L F I

D194R Arginine *Polar Charged*

TTTTTTATCCCATTGTTGGTGGTGATTCTGTTTGCTGTG**CGT**ACAGGATTATTTATC
F F I P L L V V I L F A V **R** T G L F I

D194S Serine *Polar Non-Charged*

TTTTTTATCCCATTGTTGGTGGTGATTCTGTTTGCTGTG**TCC**ACAGGATTATTTATC
F F I P L L V V I L F A V **S** T G L F I

Figure 3.1b-Sequence Verification of transmembrane domains of mutants (D194T, D194I, D194V, D194R, and D194S) huFcεRIα constructs. These variants were generated in the current study.

The constructs were sequenced by Lark (Cogenics) Technologies Ltd. Translated amino acids are shown beneath each sequence. Green shaded area indicates the invariant motif with the mutations in bold underlined and in different colours: D194T (orange), D194V (blue), D194I (lavender), D194R (plum) and D194S (grey).

3.2: Vectors

3.2.1: pUC18

The cloning vector used for carrying out site directed mutagenesis and subsequent sequencing of the mutation was pUC18 **(figure3.2)** which is a relatively small, high copy number vector having β-lactamase gene conferring ampicillin resistance which allows selection of successfully ligated clones by growing them on LB media to which ampicillin has been added.

3.2.2: Mammalian expression vector

pEE6 **(figure 3.3)** has been used previously by our research group for expression of the wild type and mutant huFcεRIα subunits in the RBL mammalian cell line. The vector comprises the gene conferring resistance to ampicillin and neomycin thus allowing selection of successfully ligated clones using G418.

3.3: Cloning into pUC18

The huFcεRIα construct was introduced into the pEE6 expression vector. It was necessary to subclone the construct into pUC18 cloning vector to carry out subsequent site-directed mutagenesis and sequencing. The pEE6 vector with huFcεRIα subunit DNA construct and pUC18 vector were digested using the EcoRI and HindIII restriction enzymes (*Section 2.9.8.1*). The pUC18 vector was dephosphorylated to prevent re-ligation of the digested linearised vector (*Section 2.9.8.2*). The digested vector pUC18 and pEE6 with huFcεRIα were resolved in parallel on 1% TAE agarose gel (*Section 2.9.7*). The representative results are shown in the **figure 3.4a.** The desired bands i.e. *794bp (huFcεRIα)* and 2686bp *(pUC18) w*ere excised from the agarose gel and purified using Quantum Prep™ Freeze N Squeeze DNA gel extraction spin columns kit (Bio-Rad) *(section 2.9.8.4)*. The purified bands of pUC18 and huFcεRIα were ligated together (*Section 2.9.8.3*) The ligation reactions were set up overnight at 16°C, including a negative control (water), to rule out any re-ligations. Ligation reactions were subsequently transformed into XLI-Blue supercompetent cells (*Section 2.9.5*). The transformed cells were plated out onto LB ampicillin plates for selection and incubated overnight at 37°C. Next day the plates were examined for colony, numbers of which was

highly variable but were below 50. The variability in the number of colonies could be due to multiple factors namely competence of cells used for transformation, the quality of purified DNA, quantity of DNA agarose bands excised and the ratio of the vector to DNA insert.

Six colonies were picked from the plate into 3ml LB media and incubated overnight on shaker at 250rpms in 37°C room. Next day Qiagen DNA miniprep kit was employed for small scale DNA extraction (*Section 2.9.6.1*). Test digestion of 10µl miniprep plasmid DNA was carried out using the EcoRI and Hind III restriction enzymes (*Section 2.9.8.1*) followed by resolving on TAE gel to confirm the presence of the desired fragment **(figure 3.4b).**

3.4: Site-Directed Mutagenesis

Quickchange Site- Directed Mutagenesis Kit (Stratagene) was employed to introduce the point mutations (*Section 2.9.8.5*). The nicked vector DNA, containing the respective desired mutations, was than transformed into XLI-Blue supercompetent cells (*Section 2.9.5*). The transformed cells were plated out onto LB ampicillin plates for selection and incubated overnight at 37°C. Six colonies were picked from the plate into 3ml LB media and incubated overnight on shaker at 250rpms in 37°C room. Next day Qiagen DNA miniprep kit was employed for small scale DNA extraction (*Section 2.9.6.1*). Test digestion of 10µl miniprep plasmid DNA was carried out using the Stu I, EcoRI and Hind III restriction enzymes (*Section 2.9.8.1*) followed by resolving on TAE gel to confirm the presence of the desired mutation **(figure 3.5).** Miniprep plasmid DNA samples were sent for sequencing to check the integrity of the mutations.

3.5: Sequencing

The DNA was sequenced by Cogenics and the results were then analysed using ALIGN, CHROMA and CLONE computer programmes to compare the sequence with genomic huFcεRIα subunit. The primers used for the sequencing were M13-48REV and M13For-40 to read the DNA from both ends. Clones with additional or no mutations were rejected.

M13-48REV CGGATAACAATTTCACACAG

M13For-40 GTTTTCCCAGTCACGAC

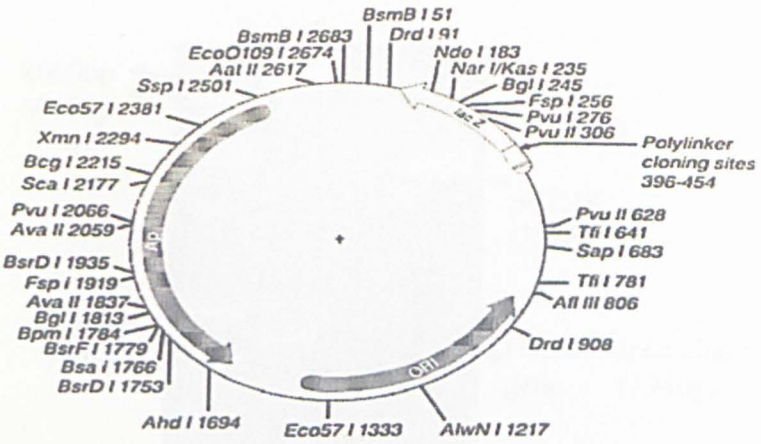


Figure 3.2 Bacterial expression vector pUC18

This vector was used previously to introduce site directed mutations in the huFcεRIα subunit. pUC18 is a 2686bp vector. The vector has a β-lactamase resistance gene conferring ampicillin resistance and lacZ operon that allows selection of the successfully ligated clones through blue and white screening procedure. The successful ligations appear as white *E.Coli* colonies grown in ampicillin-enriched agar plates. Adapted from: http://web.bio.utk.edu/peterson/BCMB%20515/pUC18map.htm.

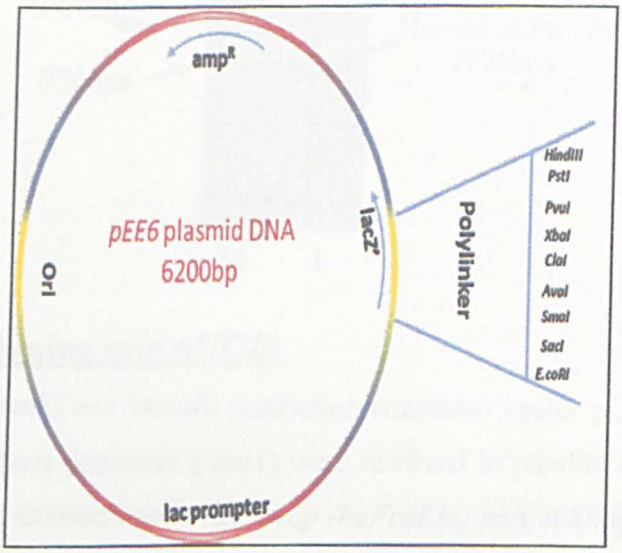


Figure 3.3 Mammalian Expression Vector pEE6

pEE6 is a 6200bp expression vector. It has the bacterial ampicillin resistance gene (ampR) and polylinker site for the insertion of the regulatory gene which allows gene expression. Modified from Stephens and Cockett (1989).

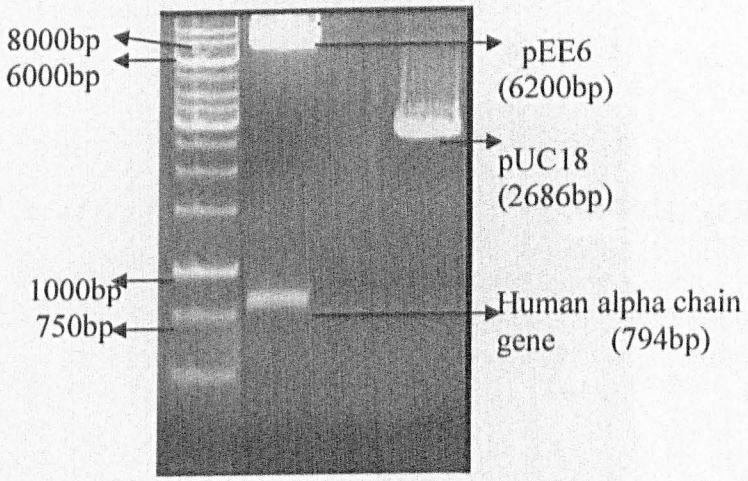


Figure 3.4a M 1 2

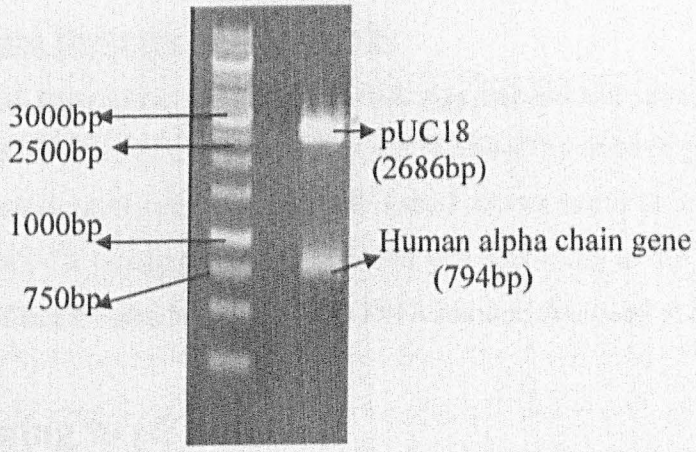


Figure 3.4b M 1

Figure3.4: Cloning into pUC18

The digested (EcoRI and HindIII restriction enzymes) vector pUC18 (Lane2) and pEE6 with huFcεRIα gene construct (lane1) were resolved in parallel on 1% TAE agarose gel (figure3.4a). The desired bands i.e.*794bp (huFcεRIα)* and 2686bp *(pUC18)* were excised from the agarose gel and purified using Quantum Prep™ Freeze N Squeeze DNA gel extraction spin columns kit (Bio-Rad) *(section 2.9.8.4)*. The purified bands of pUC18 and huFcεRIα were ligated together (*Section 2.9.8.3*). In figure 3.4b pUC18 with *huFcεRIα* clone digest show the correct appearance of two bands 2686bp pUC18 and 794bp *huFcεRIα* in lane 1 while lane M is the 1Kbp DNA marker (*Section2.9.7*).

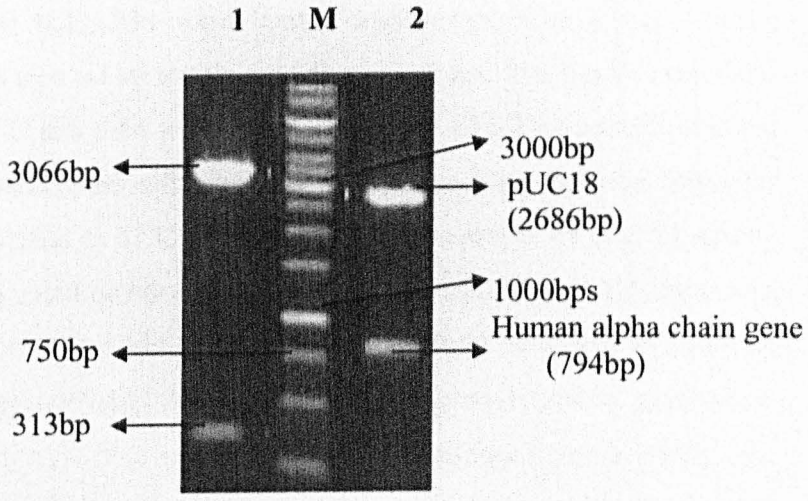


Figure 3.5: Site Directed Mutagenesis

Test digestion of 10µl miniprep plasmid DNA was carried out using the Stu I (lane1) andEcoRI /Hind III (lane 2) restriction enzymes followed by resolving on 1%TAE gel to confirm the presence of the desired mutation. Lane1 shows bands at 313bps and 3066bps after digestion of PCR product with Stu I, which was according to the incorporated silent restriction site of Stu I. Lane M, is the 1Kbp DNA marker *(Section2.9.7)*.

3.6: Subcloning to pEE6

After sequencing the clones, with the successful mutation, were employed to subclone the mutant cDNA construct into expression vector pEE6 for subsequent transfection into mammalian cells (RBLs).

The pUC18 vector with mutant huFcεRIα subunit DNA construct and pEE6 vector were digested using the EcoRI and HindIII restriction enzymes *(section 2.9.8.1)*. The pEE6 vector was dephosphorylated *(section 2.9.8.2)* to prevent re-ligation of the digested linearised vector. The digested vector pEE6 and pUC18 with mutant huFcεRIα were resolved in parallel on 1% TAE agarose gel *(section 2.9.8.4)*.The representative results are shown in the **figure 3.6a**. The desired bands i.e.*794bp (huFcεRIα) and 6200bp (pEE6) w*ere excised from the agarose gel and purified using the Quantum Prep™ Freeze

N Squeeze DNA gel extraction spin columns kit (Bio-Rad) *(section 2.9.8.4)*. The purified bands of pEE6 and mutant huFcεRIα were ligated together *(section 2.9.8.3)* and a negative (water) control was also set up to rule out any re-ligations. The ligation reactions were set up overnight at 16°C and then were transformed into XLI-Blue supercompetent cells *(section 2.9.5)*. The transformed cells were plated out onto LB ampicillin plates for selection and incubated overnight at 37°C. Next day the plates were examined for colony numbers, which were highly variable but were below 50. Six recombinant colonies were picked from the plate into 3ml LB media and incubated overnight on shaker at 250rpms in 37°C room. Next day Qiagen/Wizard DNA miniprep kit was employed for small scale DNA extraction *(section 2.9.6.1)*. Test digestion of 10µl miniprep plasmid DNA was carried out using the EcoRI and Hind III restriction enzymes *(section 2.9.8.1)* followed by resolving on TAE gel to confirm the presence of the desired fragments **(figure 3.6b)**. The miniprep plasmid DNA was transformed into XLI-Blue competent cells *(section 2.9.5)*. The transformed cells were plated out onto LB ampicillin plates for selection and incubated overnight at 37°C. Next day the plates were examined for recombinant colonies. Two colonies were picked from the plate into 5ml LB media in universals and incubated for 8 hours in 37°C room with continuous shaking at 250rpms. After the passage of 8hours the contents of the universals were shifted to 50ml LB media in 250ml conical flasks for overnight incubation in 37°C room with continuous shaking at 250rpms. Next day QIAGEN DNA midiprep kit was employed for large scale DNA extraction *(section 2.9.6.2)*. Test digestion of 02µl midiprep plasmid DNA was carried out using the EcoRI and Hind III restriction enzymes *(section 2.9.8)* followed by resolving on TAE gel to confirm the presence of the desired fragments **(figure 3.7)**.

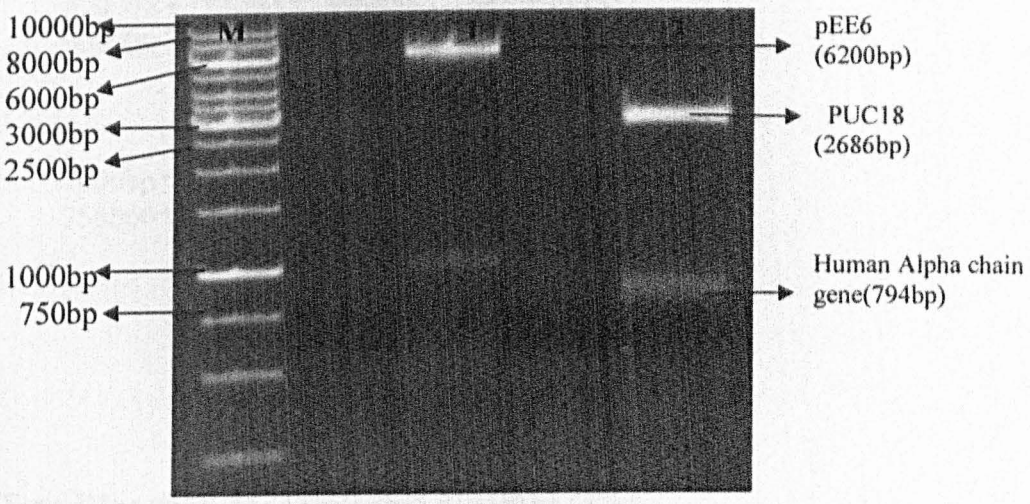


Figure 3.6a

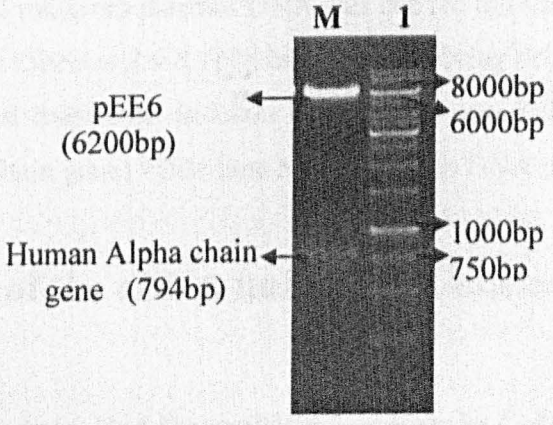


Figure3.6b

Figure 3.6: Subcloning into pEE6

The digested vectors pEE6 and pUC18 with huFcεRIα were resolved in parallel on 1% TAE agarose gel (3.6a). The desired bands i.e. *794bp (huFcεRIα) and 6200bp (pEE6)* were excised from the agarose gel and purified using the Quantum Prep™ Freeze N Squeeze DNA gel extraction spin columns kit (Bio-Rad) *(section 2.9.8.4)*. The purified bands of pEE6 and mutant huFcεRIα were ligated together (*Section 2.9.8.3*). In figure 3.6b pEE6 with *huFcεRIα* clone digest show the correct appearance of two bands 6200bp pEE6 and 794bp *huFcεRIα* in lane 1 while lane M is the 1Kbp DNA marker (*Section2.9.7*).

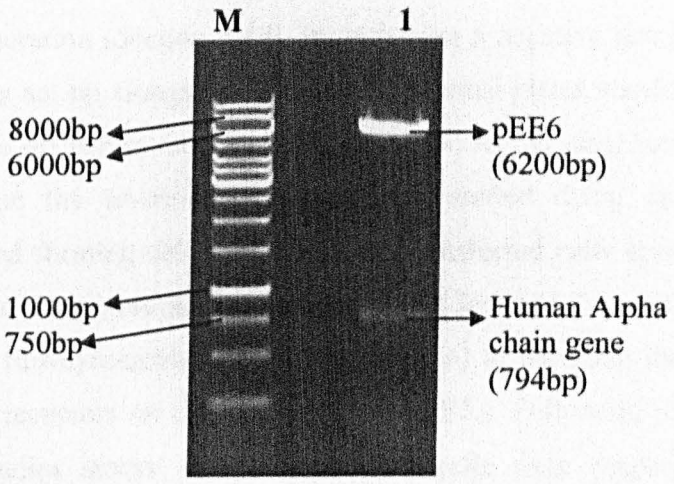


Figure 3.7 Test Digestion of Midiprep Plasmid DNA

Test digestion of 02 µl midiprep plasmid DNA was carried out using the EcoRI and Hind III restriction enzymes (*Section 2.9.8.1*) followed by resolving on TAE gel to confirm the presence of the desired fragments. In Lane 1 two bands are visible 6200bp (pEE6) and 794bp (human alpha chain gene) while lane M is the 1Kbp DNA marker (*Section2.9.7*).

3.7: Expression of the cDNA huFcεRIα Constructs

3.7.1: Transfection into Rat Basophilic Leukemia Cell line (RBL-2H3.1)

The RBL-2H3.1 cell line was chosen as the host for gene constructs coding the mutant huFcεRIα subunit constructs (D194T, D194I, D194V, D194S and D194R). It has been shown previously that transfection of huFcεRIα CDNA construct into RBL-2H3 resulted in expression of huFcεRIα by forming a functional chimeric rat/human receptor complex with endogenous FcR-β and FcR-γ subunits *(Section 1.11)* (Gilfillan et al., 1992, Wilson et al., 1993). These stably transfected RBL-2H3.1 cells with huFcεRIα provided a model system to elucidate the mast cell signalling by investigating the huFcεRI –mediated cell signalling using NIP-specific huIgE *(Section 2.4)*.

Initially transient transfection using METAFECTENE PRO (*Section 2.10*) was attempted but failed to provide viable cell lines. Subsequently stable transfections were carried out

out using electroporation (*Section 2.10*). In each case a negative control using water in place of DNA was set up alongside. The cells in control plates started dying by end of first week and died off before the end of second week. At the same time in the plates set up for transfection the un-transfected cells also started dying and the successful transfectants started forming colonies. When the transfected cells appeared crowded or attained confluence they were spread out and split (*Section2.3.1*). The cells were then used to carry out flowcytometric studies (*Section 2.6*) to ascertain the presence of the desired huFcεRIα receptors on the cell surface of RBLs. Following repeated rounds of flowcytometric studies stocks of the transfected cells were prepared by cryogenic preservation (*Section 2.3.2*). The cells with the highest levels of cell surface expression were selected to create frozen stocks or employed for release assays *(Section 2.4)*, flowcytometric and intracellular calcium mobilisation studies *(Section 2.8)*.

CHAPTER 4

Identification of huFcεRIα Expression and Assessment of Mediator Release via the Transfected Receptor

Chapter 4

Identification of huFcεRIα Expression and Assessment of Mediator Release via the Transfected Receptor

4.1 Introduction

The RBL-2H3.1 cells transfected with the wild type and mutant huFcεRIα cDNA constructs (D194T, D194I, D194V, D194S and D194R) *(Section3.7.1)* were assessed for the cell surface expression of the huFcεRIα subunit and cellular responses following a receptor-mediated cross-linking stimulus by measuring phosphorylation of the γ-subunit and Syk kinase, intracellular Ca^{2+} mobilisation, and the exocytic release of mediators. These rat/human chimeric receptor complexes can be specifically activated via a human IgE (huIgE) mediated cross-linking stimulus to mediate cell secretion (Gilfillan et al., 1992) leading to release of histamine, inositol phosphate production, intracellular Ca^{2+} mobilisation, and tyrosine phosphorylation of endogenous FcεRIβ/ FcεRI-γ subunits in response to anti human IgE cross-linking. Similar studies were carried out in our laboratory by Wilson et al (1993) and a stably transfected cell line with huFcεRIα (D194) was obtained which on being sensitised with the human IgE (JW8) and challenged by model antigen NIP-HSA confirmed the formation of a functional receptor complex as seen by 5HT (5-hydroxy tryptamine) release (Coleman, 1988). Transfection of RBLs with huFcεRIα allowed the independent manipulation of the transfected receptor subunit with human IgE, which does not recognize the endogenous rodent receptor (Eccleston et al., 1973).

4.1.1 RBL-2H3 cell line

The research into understanding of the immunological processes is often difficult due to multiple factors involving isolation and primary culturing of the degranulating cells like mast cells and basophils (Passante et al., 2009). Tissue mast cells and blood basophils

obtained from peripheral blood progenitors require elaborate and lengthy protocols for primary culturing and differentiation (Saito et al., 2006). Peritoneal mast cells are comparatively easier to obtain by peritoneal lavage of small animals like rats but tend to loose their response to antigenic stimuli during the purification process (Coutts et al., 1980) and are difficult to maintain in primary culture for long periods of time (Horigome et al., 1994). The rat cell line, RBL-2H3, which can be maintained in continuous culture, has served as an established model system for the study of secretory events due to their ease of culture, and property of adherence to plastic surfaces thus making available in short time of a great number of monoclonal cells by simple cell culture techniques (Blank and Varin-Blank, 2004). RBL-2H3 cell line was developed in 1973 by inducing leukaemia in rats fed with the chemical carcinogen, β-chlorethylamine (Eccleston et al., 1973). Since then the cell line has been extensively used for studying IgE-FcεRI interactions, as it bears FcεRI receptors, (Ortega et al., 1988) and receptor cross-linking activates signalling pathways that culminate in degranulation of multiple mediators (histamine, 5-HT, β-hexosaminidase) almost identical to those observed in primary mast cells and basophils (Barsumian et al., 1981, Funaba et al., 2003). This cell line has also been employed in assessment studies for mast cell stabilisers (Ikawati et al., 2001). The RBL model is expected to continue to serve in future as the model system for studying the biological characteristics of mast cells together with an additional potential role in clinical research (Blank and Varin-Blank, 2004).

4.2: Results

4.2.1: Flow Cytometric Analysis of Transfected Mutant huFcεRIα Receptor Subunits

Flow cytometry is a technique which provides information about various parameters like cell size, granularity and degree of flouresence associated with cells under investigation. Flow cytometry is a technique that combines optic, fluidic and electronic systems. On exposure to the laser system cells scatter the light, which is then picked up by multiple sensors located at various angles. The light scattered in the forward direction (Forward scatter, FS) provides information about the cell size whilst light scattered in a side

direction (Side Scatter, SC) is proportional to the relative granularity of the cell while separate fluorescence channels detect any fluorescence associated with the cell. Populations of huFcεRIα expressing cells were sorted on the basis of granularity, size and fluorescence and collected and used for preparing stocks of cells with the aim of using them to assess the cells capacity to release mediators or support calcium mobilisation. RBL-2H3 cells transfected with the mutant huFcεRIα cDNA constructs were assessed for clonal stability and highest huFcεRIα expressing cells were selected by employing flow cytometry *(Section 2.6)*. The transfected cell lines D194 (wild-type) D194T, D194V, D194I, D194R and D194S were subjected to repeated rounds of FACS cell sorting *(Section 2.7)* and analysis. Cells were labelled in series with a combination of human IgE (JW8), biotinylated anti-human IgE and streptavidin R-phycoerthrin antibodies. Data from the FACS analysis of the transfected cell lines is shown in **figures 4.1 and 4.2.**

The data clearly demonstrate the presence of D194 (wild type used as reference control) and D194T receptors on the cell surface of respective transfected cell line (**figure 4.1b and d respectively**). The cell population histogram (**figure 4.1b and d**) shows the shift to right compared to the unlabelled control (**figure 4.1a and c**) indicating the expression of the transfected receptor (human FcεRIα subunit) on the cell surface of the transfected RBL-2H3 cells.

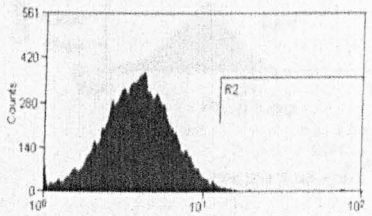
In **figure 4.1f** no shift to the right is visible as compared to the unlabelled control (**figure 4.1e**) indicating the absence of the D194I receptors on the cell surface of transfected cell line.

Similarly in **figure 4.1h** no shift to the right was witnessed as compared to the unlabelled control in **figure 4.1g** thus indicating the failure of D194V receptors to reach the cell surface of RBL-2H3 cell line following transfection.

In **figure 4.2b** again no shift to the right was observed compared to the unlabelled control in **figure 4.2a** thus demonstrating the absence of D194R receptor expression on the cell surface of transfected RBL-2H3 cell line

In **figure 4.2b** again no shift to the right was observed compared to the unlabelled control in **figure 4.2a** thus demonstrating the absence of D194R receptor expression on the cell surface of transfected RBL-2H3 cell line

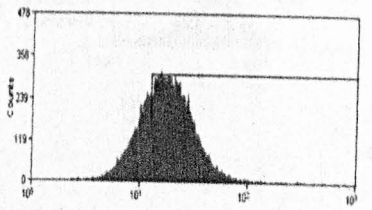
Similarly In **figure 4.2d** no shift to the right was observed as compared to the unlabelled control in **figure 4.2c** indicating a failure of the transfected cell line to support cell surface expression of genes encoding huFcεRIα D194S.



Region	% Hist
Total	100.00
R2	1.33

D194 control

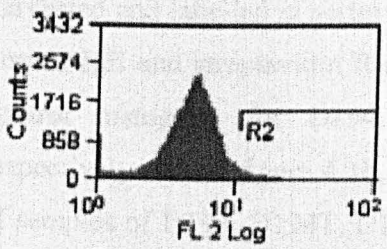
Figure 4.1a



Region	% Hist
Total	100.00
R2	73.84

D194 Sample

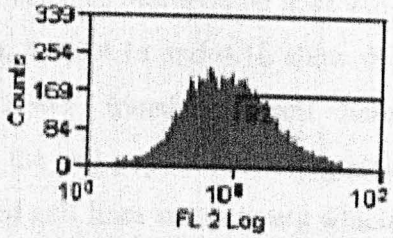
Figure4.1b



Region	% Hist
Total	100.00
R2	4.14

D194T control

Figure 4.1c



Region	% Hist
Total	100.00
R2	41.74

D194T

Figure 4.1d

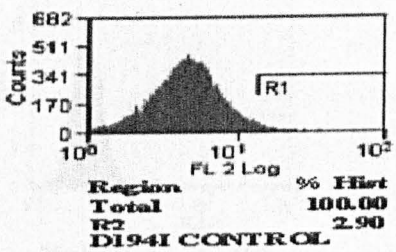


Figure 4.1e

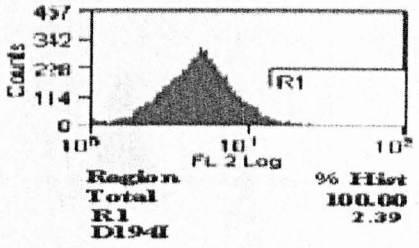


Figure 4.1f

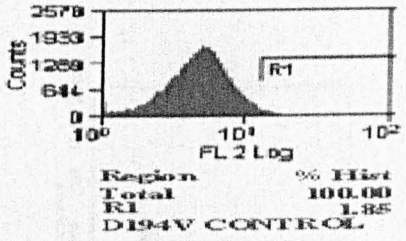


Figure 4.1g

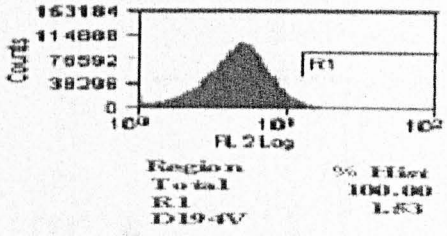
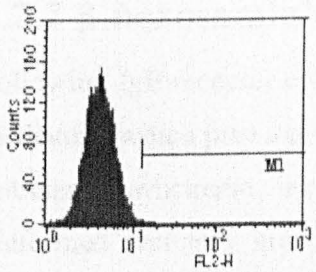


Figure 4.1h

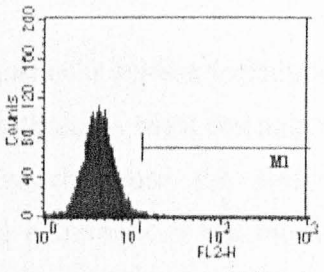
Figure 4.1: Assesment of cell Surface Expression in D194 (wild type), D194T, D194V and D194I Transfected Cell Lines by Flow Cytometry

RBL-2H3 cells transfected with the wild and mutant huFcεRIα cDNA constructs were harvested and labelled in series with a combination of human IgE, biotinylated goat anti-human IgE and streptavidin R-phycoerthrin. Figures 4.1a, 4.1c, 4.1e and 4.1h show the control histogram for D194, D194T, D194I and D194V transfected cell lines respectively. While figure 4.1b, 4.1d, 4.1f and 4.1h show the cell population histograms of samples of D194, D194T, D194I and D194V transfected cell lines respectively which were assessed for huFcεRIα receptor expression.



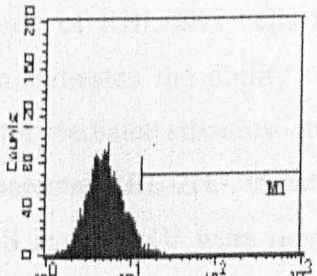
Marker	% Gated
All	100.00
M1	0.18

Figure 4.2a:D194R Control



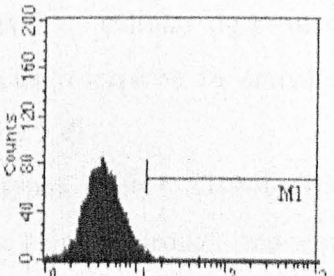
Marker	% Gated
All	100.00
M1	0.15

Figure 4.2b:D194R



Marker	% Gated
All	100.00
M1	1.53

Figure 4.2c:D194S Control



Marker	% Gated
All	100.00
M1	0.55

Figure 4.2d:D194S

Figure 4.2: Assesment of Cell surface Expression in D194S and D194R Transfected Cell Lines by Flowcytometry RBL-2H3 cells transfected with the mutant huFcεRIα cDNA constructs were harvested and labelled in series with a combination of human IgE, biotinylated goat anti-human IgE and streptavidin R-phycoerthrin. Figures 4.2a and 4.2c show the control histogram for D194Rand D194S transfected cell lines respectively. Figure 4.2b and 4.2d show the cell population histograms of samples of D194R and D194S transfected cell lines respectively which were assessed for huFcεRIα receptor expression.

4.2.2 β-hexosaminidase Release Assays

Following IgE receptor cross-linking by cognate antigen mast cells release inflammatory mediators, which play a pivotal role in the allergic disease pathology. Mast cell activation initiates downstream intracellular signaling cascades, which cause the fusion of preformed secretory granules with plasma membrane and culminate in the release of pharmacologically active mediators into the local environment. In view of their similar granular content to mast cells RBL-2H3 cells are commonly chosen as a reliable and convenient model to study IgE mediated degranulation of mast cells, although in other aspects they have similarities to basophils rather than other histamine-releasing cell types (Passante et al., 2009). This provides a tool to monitor the presence or absence of response of RBL-2H3 cells to antigenic stimulus and level of cellular degranulation, which indicates the ability of the parental, and transfected receptors to convert the receptor mediated stimulus into a secretory response (*Section 2.4)*.

The parental RBL-2H3, transfected cell lines D194 (wild-type), D194T, D194I, D194R, D194S and D194V were monitored for β-hexosaminidase release through endogenous and huFcεRIα transfected receptors by challenging them with mIgE specific DNP-HSA and human IgE specific NIP-HSA model antigens respectively (Gilfillan et al., 1992, Wilson et al., 1993, Aketani et al., 2001, Dearman et al., 2005). The response through the endogenous mouse FcεRIα in parental RBL-2H3 and all the transfected cell lines (D194, D194T, D194I, D194V, D194S and D194R) demonstrates release in response to mIgE specific DNP-HSA **(figure 4.3)**. D194T cell line exhibited release in response to human IgE specific NIP-HSA **(figure 4.3)**. The parental RBL-2H3, D194I, D194R, D194S and D194V transfected cell lines failed to release above background readings in response to human IgE specific NIP-HSA **(figure 4.3)** as the endogenous rodent FcεRIα specifically binds and responds only to rodent but not primate IgE antibodies (Eccleston et al., 1973). On being stimulated by monoclonal or polyclonal antibodies the RBLs exhibits a marked prozone effect (bell shaped dose-response curve) (Dearman et al., 2005) similar to the response in freshly isolated peritoneal murine mast cells (Coleman, 1988) and human basophils (Hoffmann et al., 1999).

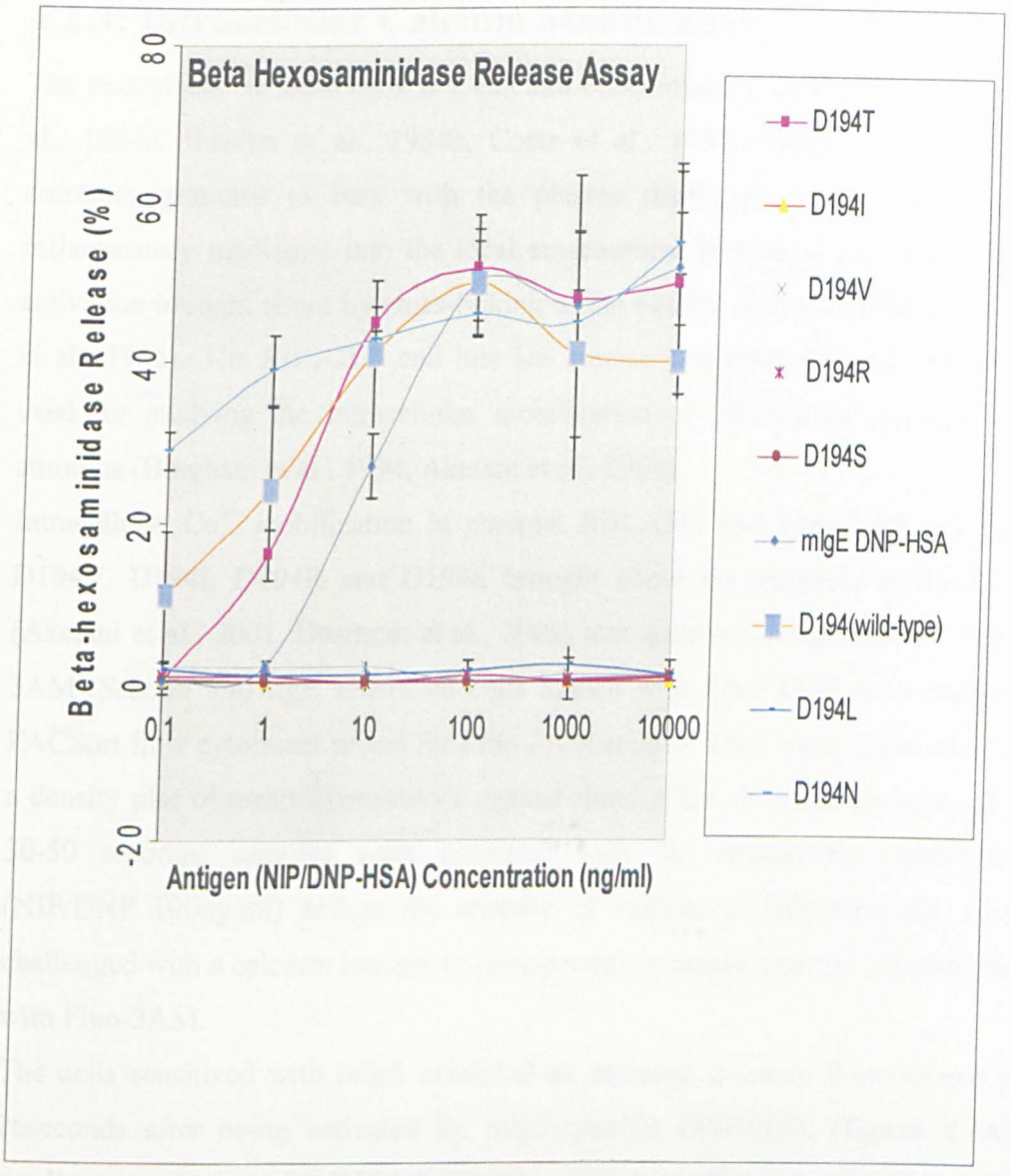


Figure 4.3: Release of β-hexosaminidase through FcεRIα transfected receptors in RBL-2H3 and RBL-2H3 transfected cell lines D194 (wild-type), D194T, D194R, D194I, D194S and D194V cell lines in response to antigenic stimulus

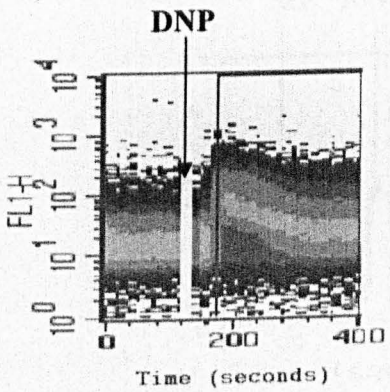
Cells were cultured, harvested, re-suspended at $0.5x10^6$/ml in appropriate media with DNP-specific mIgE (SPE-7, 1/500) / NIP-specific huIgE (SPE, 1/500) and plated into 96 well plates. After 16h, cells were washed and activated with DNP-HSA / NIP-HSA cross-linking agent (0.1-10000ng/ml) for 20 minutes prior to incubation with β-hexosaminidase substrate for 2 hours.

4.2.3: Intracellular Calcium Mobilisation

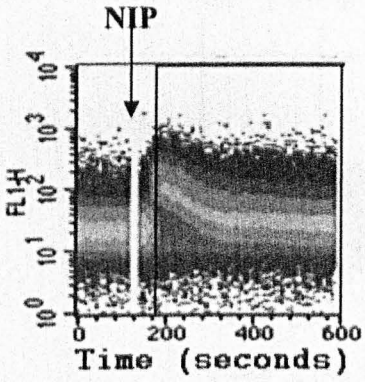
The exocytosis in mast cells is a calcium concentration- dependent process (Beaven et al., 1984a, Beaven et al., 1984b, Costa et al., 1997) which causes the intracellular secretory granules to fuse with the plasma membrane and release the preformed inflammatory mediators into the local environment in response to IgE FcεRI-mediated activation brought about by cross-linking of the FcεRIs with multivalent agents (Metzger et al., 1986). The RBL-2H3 cell line has similar properties to mast cells and has been used for studying the intracellular mobilization of calcium in response to antigenic stimulus (Bingham et al., 1994, Aketani et al., 2001).

Intracellular Ca^{2+} mobilization in parental RBL-2H3 and tranfected cell lines D194T, D194V, D194I, D194R and D194S brought about by antigenic stimulus (NIP/DNP) (Aketani et al., 2001, Dearman et al., 2005) was assessed using the Ca^{2+} indicator Fluo-3AM *(Section 2.8)*. IgE sensitized cells loaded with Fluo-3AM were analysed using a FACSort flow cytometer preset for Fluo-3AM studies. Data were recorded in the form of a density plot of mean fluorescence against time. After an initial background reading of 30-50 seconds, samples were activated with the appropriate cross-linking agent (NIP/DNP 100ng/ml) and in the absence of calcium mobilization the samples were challenged with a calcium ionophore (ionomycin) to ensure that the cells had been loaded with Fluo-3AM.

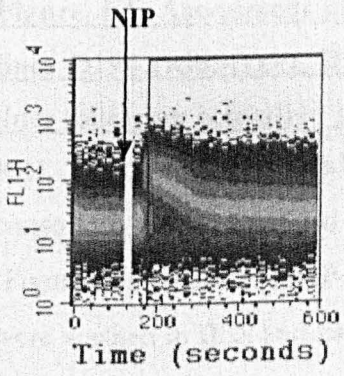
The cells sensitized with mIgE exhibited an increase in mean fluorescence peaking at 26seconds after being activated by mIgE-specific DNP-HSA **(figure 4.4A)**. Similar results were obtained for D194 (wild-type used as a reference control) and D194T cell line when sensitized with huIgE and activated by huIgE-specific NIP-HSA **(figure 4.4B and C respectively)**. However in case of D194I, D194V, D194R and D194S cell lines, no calcium mobilization was observed when the cells were sensitised with huIgE and activated by huIgE-specific NIP-HSA **(figures 4.4 D, E, F and G respectively)**. The same cell lines (D194I, D194V, D194R and D194S) that failed to elicit calcium mobilization with huIgE-specific NIP-HSA were activated by ionomycin (calcium ionophore) and exhibited an immediate increase in mean fluorescence which was maintained until a slow decrease occurred due to a depletion of intracellular Ca^{2+} **(figures 4.4D, E, F and G respectively).**



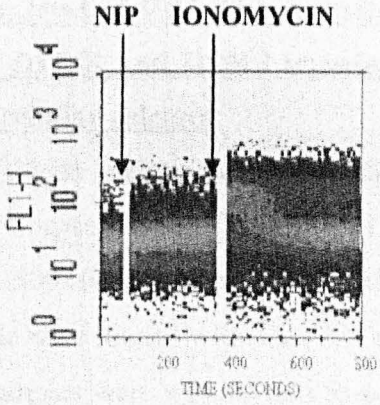
A: Control



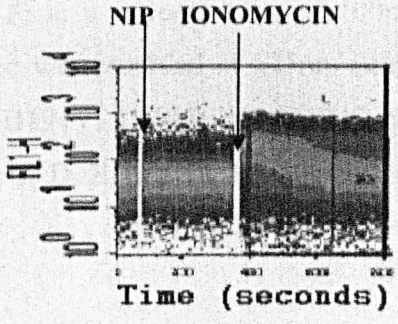
B. D194 (wild –type) (Reference control)



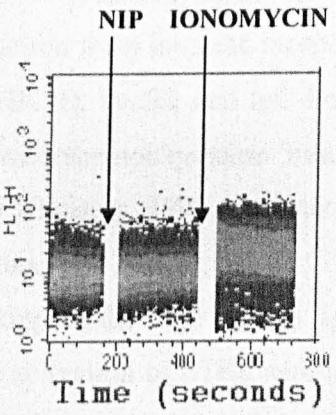
C. D194T



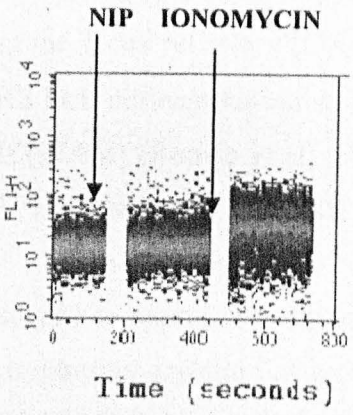
D. D194I



E. D194V



F: D194R



G: D194S

Figure 4.4: Assessment of Intracellular calcium level of RBL-2H3 transfected cell lines D194 (wild-type), D194T, D194V, D194I, D194R and D194S transfected cell lines following activation in the absence of extracellular calcium

RBL-2H3 cells transfected with the wild-type (D194) and mutant huFcεRIα cDNA constructs were sensitised with (Figures A) DNP-specific mIgE (SPE-7, 1/500) or (Figures B, C, D, E, F) NIP-specific huIgE (JW8, 1/500) for 16 hours. IgE sensitised cells were washed in BSS and harvested with CDS. Cells were loaded with Fluo 3-AM (5μM) for 30 minutes at room temperature in the dark. Samples were washed and re-suspended at $2x10^6$ /ml in DPBS (without $CaCl_2$ or $MgCl_2$). After an initial background reading the cells were activated with appropriate cross-linking agent DNP (Figures A) and NIP (Figures B, C, D, E, and F) at a concentration of 100ng/ml. Following excitation at 488nm emitted fluorescence was recorded at 525nm using a FACSort flow cytometer (BD biosciences). D194I, D194V, D194R and D194S cells were then activated with ionomycin (10μM, Figures C, D, E, and F). Results are representative of three separate experiments.

4.2.4: FcR-γ and Syk Kinase Phosphorylation

The activation of protein tyrosine kinases (PTKs) (Reth, 1989) is a mandatory step in signal transduction from immune receptors (IRs) including the T cell receptor (TCR), B-cell receptor (BCR), FcεRI, and IgG-Fc receptors. The IRS lack intrinsic kinase activity but have immunoreceptor tyrosine- based activation motif (ITAM) (Romeo et al., 1992, Samelson and Klausner, 1992, Cambier, 1995, Pawson, 1995) which play a pivotal role in cell activation.

The cross-linking of the high affinity IgE receptor in response to appropriate antigenic stimuli causes activation of PTKs within seconds leading to phosophorylation of various substrates including IR subunits (Benhamou et al., 1990, Paolini et al., 1991). The aggregation of FcεRI receptors causes phosphorylation, usually by Lyn, of the tyrosine residues in ITAMs of both the β and the γ subunits of FcεRI (Reischl et al., 1999, Rivera, 2002, Siraganian, 2003, Galli et al., 2005). Current evidence suggests that tyrosine phosphorylated ITAMs then act as scaffolds for the binding of the additional signaling molecules with Src homology domain 2 (SH2) like the cytoplasmic protein tyrosine kinase Syk, which binds mainly to the γ subunit of the receptor through its two SH2 domains. This is followed by conformational change of Syk leading to its activation and autophosphorylation *(Section 1.4.1)*.

Syk family has two members syk and ZAP-70 which are essential for lymphocyte development and signal transduction via IRs (Turner et al., 2000, Latour and Veillette, 2001). Syk kinase has 8 tyrosine residues and is responsible for signal transduction in FcεRI (Siraganian, 2003) and BCR (Dal Porto et al., 2004). Syk kinase expression has been demonstrated in RBL-2H3 cell line and is a rapid process preceeding calcium mobilization, tyrosine phosphorylation being detected within 1 minute after receptor stimulation (Benhamou et al., 1990). Other studies with RBL-2H3 cell line are suggestive of preferential binding of Syk SH2 domains with FcR- γ ITAMs and activation of Syk kinase proceeding tyrosine phosphorylation of the FcR- γ (Shiue et al., 1995). Studies with RBL-2H3 Syk-deficient variant (TB1A2 cells) and Syk deficient mast cells demonstrated the presence of β and γ chain phosphorylation but downstream events are abrogated (Costello et al., 1996, Zhang et al., 1996). There was reinstatement of intracellular signaling and degranulation on reconstitution of Syk kinase expression in

TB1A2 cell line and Syk deficient mast cells (Costello et al., 1996). Syk kinase inhibitors have been shown to inhibit signaling and degranulation by selectively inhibiting FcεRI-mediated activation (Oliver et al., 1994, Moriya et al., 1997).

Studies by Paoilini et al (1991) demonstrated a rapid (< 30sec) *in vivo* phosphorylation of FcR- γ (tyrosine and threonine) and FcR- β subunits (tyrosine and serine) following engagement of FcεRI receptor. FcR- γ subunit belongs to the same family as TCRζ subunit and in their capability to mediate T cell development and function are interchangeable although the native TCRζ subunit is far more efficient (Shores et al., 1997) *(Section 1.6.3)*. Further studies (Paolini et al., 1995) using ζ-deficient T cell line demonstrated the requirement of FcR- γ ITAMs in FcεRI-mediated signalling. Studies where mutations were introduced in the tyrosine residues of β and γ chains ITAMs abolished signalling (Letourneur and Klausner, 1991, Jouvin et al., 1994, Lin et al., 1996). Similarly it was found that the downstream signaling cascade can be initiated by attaching ITAM sequence of γ-chain to unrelated extracellular domains (Letourneur and Klausner, 1991, Romeo and Seed, 1991). However it is the ITAM sequence of γ-chain, which alone can initiate downstream signaling in FcεRI (Letourneur and Klausner, 1991, Romeo and Seed, 1991) while β-chain serves as amplifier of the γ-chain signaling (Lin et al., 1996, Dombrowicz et al., 1998).

4.2.4.1: Immunoprecipitation Studies Results

The tyrosine phosphorylation status of FcR- γ and Syk kinase was investigated by immunoprecipitation studies *(Section 2.5.3)*. The parental RBL-2H3.1 and the RBL.2H3.1 cells transfected with the mutant huFcεRIα cDNA constructs D194T cell lines exhibited tyrosine phosphorylation of FcR- γ subunit at ~12-15kDa (Bingham et al., 1994, Iodice, 2006) and Syk kinase at ~72kDa (Hutchcroft et al., 1992, Iodice, 2006) once they were sensitised with huIgE and activated by huIgE-specific NIP-HSA antigen system. The level of tyrosine phosphorylation was reduced in case of D194T cell line as compared to the parental RBL-2H3.1 cell line probably due to lower huFcεRIα receptor numbers as also seen in other previously transfected cell lines (D194, D194N and D194L) which expressed huFcεRIα receptors (Iodice, 2006).

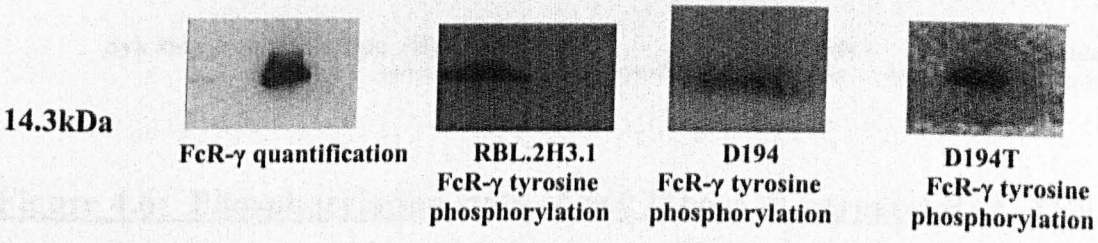


Figure 4.5: Phosphorylation state of FcR-γ in parental RBL-2H3.1 and RBL-2H3.1 cells transfected with the mutant huFcεRIα cDNA constructs (D194 (wild-type), D194T)

Parental RBL-2H3.1 and RBL-2H3.1 cells transfected with the mutant huFcεRIα cDNA constructs were cultured and sensitised overnight with DNP-specific mIgE (SPE-7, 1/500) or NIP-specific huIgE (JW8, 1/500) respectively. Cells were cultured, harvested, resuspended at $5x10^6$/ml in appropriate media and activated with appropriate cross-linking agent (100ng/ml) for 2 minutes. Cells were lysed using RIPA buffer followed by immunoprecipitation using a mouse monoclonal anti-phosphotyrosine antibody (clone 4G10, Millipore). Immunoprecipitates were resolved by SDS-PAGE (12.5% acrylamide). Electrophoretic transfer of proteins to PVDF (Amersham Biosciences) was carried out overnight at 30V (4°C). Western blots were probed with rabbit polyclonal FcR-γ (Millipore) antibody and protein bands were visualised using ECL.

FcR-γ quantification was carried out on aliquot removed from samples prior to immunoprecipitation and probed with rabbit polyclonal FcR-γ (Millipore) antibody.

Results are representative of at least 5 separate experiments.

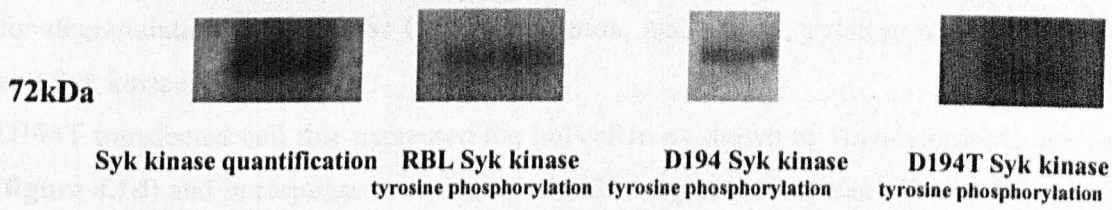


Figure 4.6: Phosphorylation state of Syk kinase in parental RBL-2H3.1 and RBL-2H3.1 cells transfected with the mutant huFcεRIα cDNA constructs (D194 (wild-type) and D194T

Parental RBL-2H3.1 and RBL-2H3.1 cells transfected with the mutant huFcεRIα cDNA constructs were cultured and sensitised overnight with DNP-specific mIgE (SPE-7, 1/500) or NIP-specific huIgE (JW8, 1/500) respectively. Cells were cultured, harvested, resuspended at $5x10^6$/ml in appropriate media and activated with appropriate cross-linking agent (100ng/ml) for 2 minutes. Cells were lysed using RIPA buffer followed by immunoprecipitation using a rabbit polyclonal anti-Syk antibody (Santa Cruz). Immunoprecipitates were resolved by SDS-PAGE (12.5% acrylamide). Electrophoretic transfer of proteins to PVDF (Amersham Biosciences) was carried out overnight at 30V (4°C). Western blots were probed with mouse monoclonal anti-Syk (Abcam) and mouse monoclonal anti-phosphotyrosine antibody (clone 4G10, Millipore) antibodies. Protein bands were visualised using ECL.

Syk kinase quantification was carried out on aliquot removed from samples prior to immunoprecipitation.

4.3: Summary of Results

The RBL.2H3.1 cells transfected with the mutant huFcεRIα cDNA constructs (D194T, D194I, D194V, D194S and D194R) *(Section3.7.1)* were assessed for the cell surface expression of the huFcεRIα subunit and the response to the antigenic stimulus by looking

for degranulation, intracellular Ca^{2+} mobilisation, and phosphorylation of the γ-subunit and Syk kinase.

D194T transfected cell line expressed the huFcεRIα as shown by flowcytometric studies **(figure 4.1d)** and in response to antigenic stimulus degranulation was affected as seen by β-hexosaminidase release assay **(figure 4.3)** and intracellular calcium mobilisation **(figure 4.5d).** The immunoprecipitation studies on D194T cell line showed phosphorylation of γ-chain **(figure 4.6)** and Syk kinase **(figure 4.7)** on being stimulated. These results indicate the presence of functional huFcεRIα subunit in D194T transfected cell line working in synergy with the endogenous rodent β and γ chains. These results are comparable to those seen following transfection of RBL-2H3.1 cell line with huFcεRIα (D194)(Wilson et al., 1993) and mutant huFcεRIα cDNA constructs (D194N) (Iodice, 2006).

D194I, D194V, D194S and D194R transfected cell lines did not express the huFcεRIα receptor **(figures 4.1f,h and 4.2b,c)** and no degranulation in response to antigenic stimulus as seen in β-hexosaminidase release assay **(figure 4.3)** and intracellular calcium mobilisation **(figure 4.5c,d, e and f).** Similar results were obtained when RBL-2H3.1 cell line was transfected with mutant huFcεRIα cDNA constructs (D194E, D194K and D194A) (Iodice, 2006). This data indicates that mutations introduced at the position D194 have significant effects on receptor expression and downstream signalling thus highlighting the importance of this residue.

CHAPTER 5

High Affinity IgE-Fc Receptor α and γ Subunit Interactions

Chapter 5
High Affinity IgE Receptor α and γ Subunit Interactions

5.1: Introduction

The high affinity IgE receptor (FcεRI) belongs to the immunoglobulin superfamily (Metzger 1992; Kinet 1999) and as the name suggests possess the highest affinity of all immunoglobulin receptors with a binding constant in the 10^9 to 10^{10} M^{-1} range for its ligand, IgE (Metzger 1992; Daeron 1997; Kinet 1999) compared to $6.3x10^7 M^{-1}$ in the low affinity receptor, FcεRII also referred to as CD23 (Spiegelberg, 1984). CD23 is distributed on several different cell types namely B lymphocytes, macrophages, eosinophils, platelets, some T cells, and follicular dendiritic cells and has diverse roles including antigen presentation and regulation of IgE synthesis (Delespesse et al., 1991, Delespesse et al., 1992, Bonnefoy et al., 1997). FcεRI exists as a tetramer on the surface of mast cells and basophils *(Section 2.6)*. The tetramer comprises the ligand binding subunit or α-chain, a β-chain and a disulphide-linked γ-chain dimer. In contrast, in Langerhans cells, dendiritic cells and monocytes FcεRI is present as a trimer and is devoid of the β-chain comprising only one α-chain and two γ-chains (Turner and Kinet 1999). However in rodents only the tetrameric isoform is present and the β-chain is essential to facilitate cell surface expression of the receptor complex (Kinet, 1999).

Since the minimal signalling complex depends on FcεRIα and FcR-γ subunits interaction, it was necessary that mutations designed to shed light on α and γ- chain-mediated signalling be carried out in the FcεR-γ subunits on the same lines as those carried out in the α-subunit *(Chapter 4)*.

5.2: Human αγγ Construct

The subunits (α, β, and γ) of FcεRI are composed of three domains: extracellular (EC), transmembrane (TM) and cytoplasmic (CT) domains (Ravetch and Kinet, 1991, Beaven and Metzger, 1993). The EC domain FcεRIα subunit is responsible for

ligand binding through its high affinity IgE Fc binding domain (Kuster et al., 1990). The cytoplasmic domain of the γ-subunit of FcεRI is responsible for steric masking of dilysine ER retention signal in the cytoplasmic domain of the FcεRIα subunit to ensure the exportation of the assembled receptor to the cell surface from the ER (Miller et al., 1989, Letourneur et al., 1995a). In order to investigate the role of γ TM and CD in inter subunit interactions it was necessary that site directed mutagenesis studies be carried out in these two domains . However these mutated γ dimers need activation independent of the endogenous expressed subunits for which an extracellular label, EC domain of huFcεRIα, was chosen. This allowed the ligand binding domain of the receptor complex to be stimulated through huIgE and antigen as the endogenus rodent FcεRIα specifically binds and responds to the murine, but not primate IgE antibody (Eccleston et al., 1973).

A chimeric construct composed of the extracellular (EC) domain of the human high affinity IgE receptor alpha subunit (huFcεRIα) spliced onto the rodent gamma TM and cytoplasmic domain (CD) was generated. In the present study this chimera was transfected into the RBL-2H3 cell line, a cell line which has already been shown to form a functional rat/human chimeric receptor complex on being transfected with huFcεRIα (Gilfillan et al., 1992, Wilson et al., 1993).

The earliest studies aimed at the identification of a structure function relationship in FcεRI used non-mast cell lines like COS7, CHO and P815 (Kuster et al., 1990, Varin-Blank and Metzger, 1990, Miller et al., 1990, Blank et al., 1991) which were transfected with receptor subunits. These cell lines do not produce granules containing mast cell mediators and as a consequence only the effects of various FcεRI domains on cell surface expression could be analysed in these studies. It was found that the rat FcεRIα requires both the β, and γ subunits for its expression but the huFcεRIα could be transiently expressed even in the absence of the β subunit in COS 7 cell line. Although deletion of CDs decreases the efficiency of membrane insertion, these were not mandatory for receptor surface expression (Varin-Blank and Metzger, 1990). Various studies have been conducted using chimera made up of the CD of the γ subunit FcεRI or TCRζ and the EC domain of CD4 (Romeo and Seed, 1991) or Tac, the interleukin 2 receptor(Letourneur and Klausner, 1991). In the study by Letourneur et al (1991) a chimeric receptor consisting of EC domain of α chain of the interleukin receptor (Tac) and CT domain of either ζ or γ when expressed in T cells or RBLs

could be activated leading to release of interleukin 2 in T cells and serotonin in RBLs. A chimera made up of EC and TM domains of CD8 to the CT domain of the ζ chain was prepared and used in a study by Irving et al (1991) suggested that CD8 can activate the appropriate signal transduction pathway in the absence of CD3. Chimeric proteins containing EC and TM domains of Tac receptor and CT domains of either ζ or γ suggested that FcεRI-γ is responsible for some but not all the changes in downstream signalling that are observed following receptor cross-linking (Eiseman and Bolen, 1992). In a study by Wilson et al (1995) where RBLs were transfected with chimera made up of EC domain of Tac joined to the CT domains of γ and β subunits (TTβ and TTγ) the level of signalling activities differed between the two chimeras with TTγ exhibiting a full range of signalling activity to no signalling response in TTβ. Repetto et al (1996) constructed chimeric receptors of the EC and CT domains of the FcεRI and the IL-2R p55 subunit (I) and stably transfected them into RBL-2H3 cells. The two constructs prepared by Repetto et al were α/γ/γ and I/γ/γ and results demonstrated that FcεRIα EC and FcεRγ CT domains are mandatory for signalling process (Repetto et al., 1996).

5.2.1: Results

The already established huFcεRIα/γ/γ construct was present in pUC18 cloning vector. Three 10μl miniprep plasmid DNA samples were sent for sequencing to check the authenticity of the construct. Cogenics sequenced the DNA and results were then analysed using ALIGN, CHROMA and CLONE computer programmes. The primers used for the sequencing were M13-48REV and M13For-40 to read the DNA from both ends. The huFcεRIα/γ/γ construct was found to contain two unwanted mutations. These spurious mutations were corrected using site-directed mutagenesis.

5.2.1.1: Primer Design

Two primers were designed to carry out single point mutation with the aid of computer software to correct the unwanted mutations in the huFcεRIα/γ/γ construct. The sequence of the primers was necessitated by the position of the mutations which needed to be corrected. The primers are shown on the next page with the altered bases in bold and the altered codons are underlined.

Correction 1

5′ 5′CACAATGGC**AGC**CTTTCAGAAGAGAC 3′

3′ 5′GTCTCTTCTGAAAG**GCT**GCCATTGTG 3′

Correction 2

5′ 5′TGCCCTA**GGA**GAGCCGCAGCTCTGCTATATC 3′

3′ 5′GATATAGCAGAGCTGCGGCTC**TCC**TAGGGCA 3′

5.2.1.2: Site-Directed Mutagenesis

Quickchange Site- Directed Mutagenesis Kit (Stratagene) was employed to carry out the point mutations (*Section 2.9.8.5*) one at a time. The nicked vector DNA containing the respective desired mutations was than transformed into XLI-Blue supercompetent cells (*Section 2.9.5*). The transformed cells were plated out onto LB ampicillin plates for selection and incubated overnight at 37°C. Six colonies were picked from the plate into 3ml LB media and incubated overnight on shaker at 250rpm in the 37°C room. Next day Qiagen DNA miniprep kit was employed for small scale DNA extraction (*Section 2.9.6.1*). Test digestion of 10µl miniprep plasmid DNA was carried out using the EcoRI and Hind III restriction enzymes (*Section 2.9.8.1*) followed by resolving on TAE gel **(figure 5.1)**. Miniprep plasmid DNA samples were sent for sequencing to Cogenics to check for the correction of first mutation. On confirmation of the correction the same technique as mentioned above was employed to correct the second mutation. Miniprep plasmid DNA samples were again sent to Cogenics for sequencing to check the correction of the second mutation and absence of any other unwanted mutation.

5.2.1.3: Subcloning to pEE6

After sequencing, the clones, with the corrected mutations, were used to subclone the cDNA construct (huFcεRIα/γ/γ) into expression vector pEE6 for subsequent transfection into mammalian cells (RBLs). Vectors pEE6 and pUC18 with huFcεRIα/γ/γ were digested using the EcoRI and Hind III restriction enzymes (*Section 2.9.8.1*). The digested vector pEE6 and pUC18 with huFcεRIα/γ/γ were

resolved in parallel on 1% TAE agarose gel *(section 2.9.8.4)*. The desired bands i.e.1117*bp (*huFcεRIα/γ/γ) and *6200bp (pEE6) w*ere excised from the agarose gel and purified using the Quantum Prep™ Freeze N Squeeze DNA gel extraction spin columns kit (Bio-Rad) *(section 2.9.8.4)*. The purified bands of pEE6 and huFcεRIα/γ/γ were ligated together *(section 2.9.8.3)* and a negative (water) control was also set up to rule out any religations. The same procedure as described previously in section 3.6 was employed. The ligation reactions were transformed into XLI-Blue supercompetent cells *(section 2.9.5)*. Qiagen/Wizard DNA miniprep kit was employed for small scale DNA extraction *(section 2.9.6.1)*. Test digestion of 10μl miniprep plasmid DNA was carried out using the EcoRI and Hind III restriction enzymes *(section 2.9.8.1)* followed by resolving on TAE gel to confirm the presence of the desired fragment. The miniprep plasmid DNA was transformed into XLI-Blue competent cells (*Section 2.9.5)*. QIAGEN Plasmid Midi Kit was employed for large scale DNA extraction *(section 2.9.6.2)*. Test digestion of 10μl midiprep plasmid DNA was carried out using the EcoRI and Hind III restriction enzymes *(section 2.9.8.1)* followed by resolving on TAE gel to confirm the presence of the desired fragment **(figure 5.2)**.

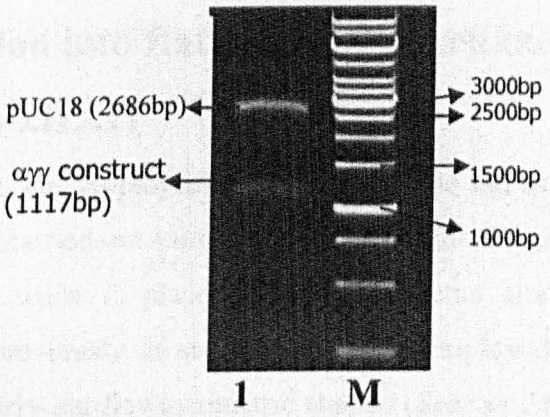


Figure 5.1 Test Digestion of Miniprep Plasmid DNA

Test digestion of 10µl miniprep plasmid DNA was carried out using the EcoRI and Hind III restriction enzymes (*Section 2.9.8.1*) followed by resolving on TAE gel to confirm the presence of the desired fragment. In Lane 1 two bands are visible 2686bp (pUC18) and 1117bp (huFcεRIα/γ/γ construct) while lane M is the 1Kbp DNA marker (*Section2.9.7*).

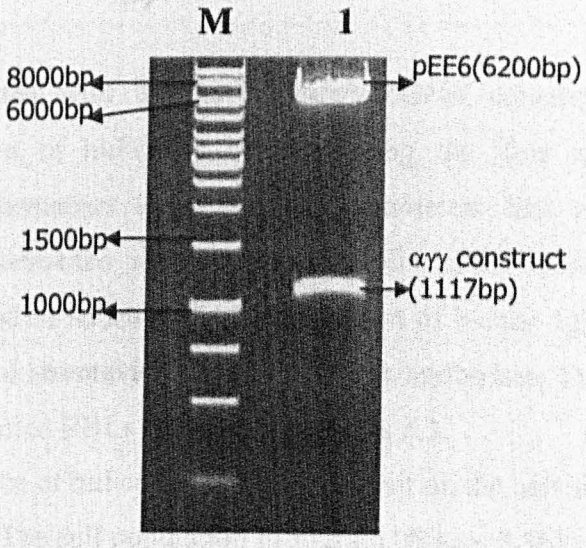


Figure 5.2 Test Digestion of Midiprep Plasmid DNA

Test digestion of 10µl midiprep plasmid DNA was carried out using the EcoRI and Hind III restriction enzymes (*Section 2.9.8.1*) followed by resolving on TAE gel to confirm the presence of the desired fragment. In Lane 1 two bands are visible 6200bp (pEE6) and 1117bp (huFcεRIα/γ/γ construct) while lane M is the 1Kbp DNA marker (*Section2.9.7*).

5.2.1.4: Transfection into Rat Basophilic Leukaemia Cell line (RBL-2H3.1)

The RBL-2H3.1 cell line was employed as the host for the huFcεRIα/γ/γ construct. Stable transfections were carried out using electroporation (*Section2.10*). In each case a negative control using water in place of DNA was setup alongside. The same procedure as described previously in section 3.7.1 was employed. The transfected cells were then used to carry out flowcytometric studies (*Section 2.6*) to ascertain the presence of the desired huFcεRIα receptors on the cell surface of RBLs. Following flowcytometric studies stocks of the transfected cells were prepared by cryogenic preservation (*Section 2.3.2)* and employed for release assays and intacellular calcium mobilisation studies *(Section 2.8*).

5.2.1.5: Flow Cytometric Analysis of Transfected RBL-2H3 Cells for huFcεRIα Receptor Subunits

The RBLs cell lines transfected with the huFcεRIα/γ/γ cDNA constructs were assessed for surface expression of huFcεRIα by employing the flow cytometry technique which provides information about various parameters like cell size, granularity and degree of flouresence associated with cells under investigation *(Section 2.6).* Cells were labelled in series with a combination of human IgE (JW8), biotinylated anti-human IgE and streptavidin R-phycoerthrin antibodies. Data from the FACS analysis of the transfected RBLs is shown in **figures 5.3.**

The data demonstrates the absence of huFcεRIα receptor subunit on the cell surface of transfected RBLs (**figure 5.3b**). The cell population histogram (**figure 5.3b**) shows no shift to right compared to the unlabelled control (**figure 5.3a**) indicating the absence of the transfected receptors (human FcεRIα subunit) on the cell surface of the transfected RBL-2H3 cells.

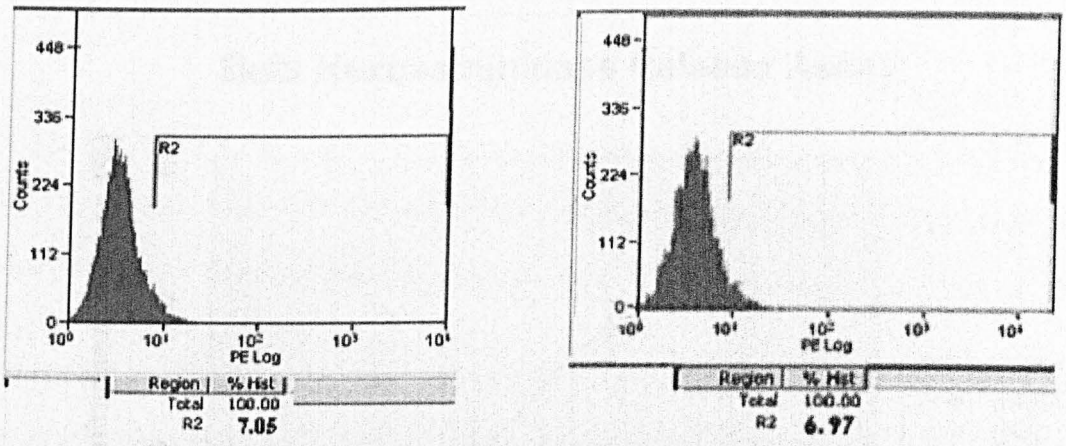


Figure 5.3: Assessment of cell surface expression of huFcεRIα transfected receptors in RBL-2H3 cells transfected with huFcεRIα/γ/γ construct by flowcytometry

RBL-2H3 cells transfected with the huFcεRIα/γ/γ cDNA constructs were harvested labelled in series with a combination of human IgE, biotinylated goat anti-human IgE and streptavidin R-phycoerthrin. Figures 5.3a shows the control histogram while figure 5.3b shows the cell population histograms of sample of huFcεRIα/γ/γ transfected cell lines which were assessed for huFcεRIα receptor expression.

5.2.1.6: β-hexosaminidase Release Assays

The RBL-2H3 transfected cell line with the huFcεRIα/γ/γ cDNA constructs was monitored for β-hexosaminidase release through endogenous and huFcεRIα transfected receptors by challenging them with mIgE specific DNP-HSA and human IgE specific NIP-HSA model antigens respectively *(*as described previously in *Section 4.2.2)* (Aketani et al., 2001, Dearman et al., 2005). The RBL-2H3 transfected cell line with the huFcεRIα/γ/γ cDNA constructs demonstrates release in response to mIgE specific DNP-HSA **(figure 5.4)**. The RBLs transfected with huFcεRIα/γ/γ cDNA constructs when sensitised with human IgE and subsequently challenged with human IgE specific NIP-HSA model antigen failed to release above background readings **(figure 5.4)**.

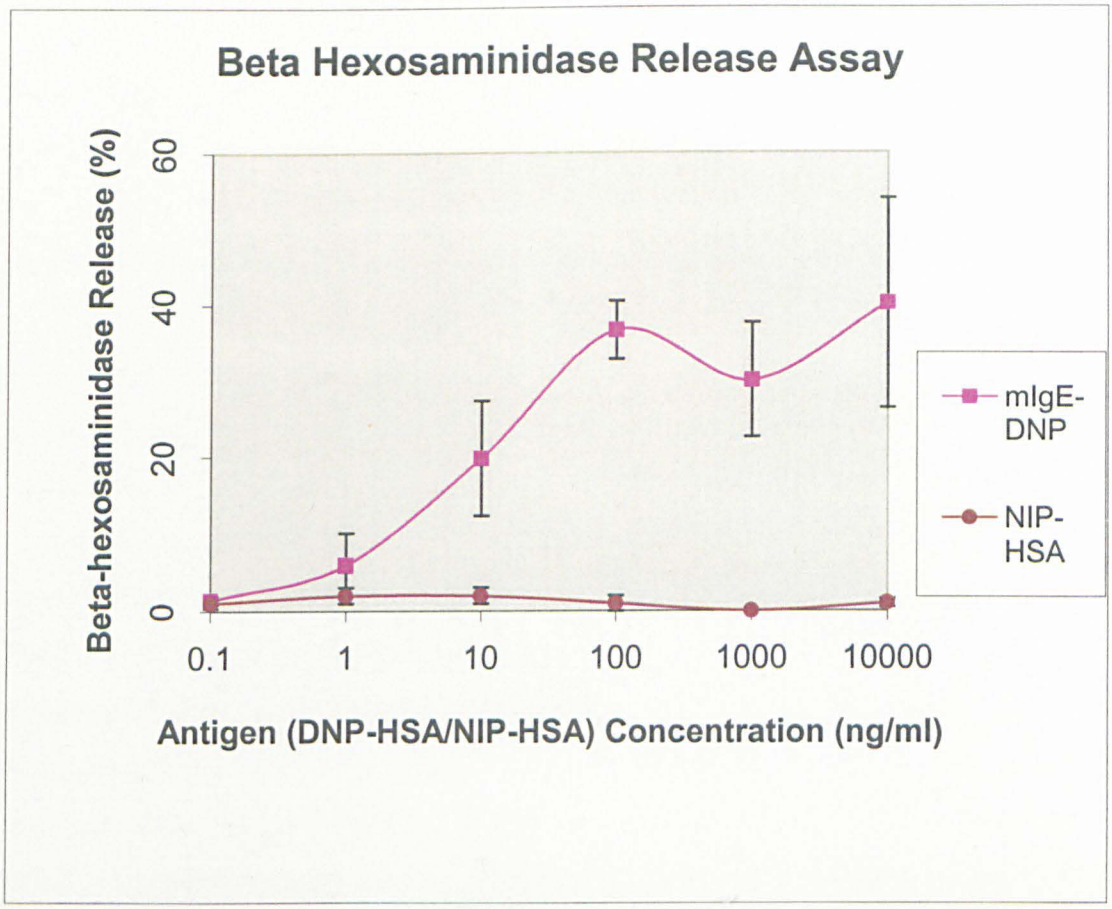


Figure 5.4: Release of β-hexosaminidase through rodent FcεRIα and huFcεRIα transfected receptors in RBL-2H3 transfected with human huFcεRIα/γ/γ construct in response to antigenic stimulus

Cells were cultured, harvested, resuspended at $0.5x10^6$/ml in appropriate media with NIP-specific huIgE (SPE, 1/500)/ DNP-specific mIgE and plated into 96 well plates. Next days, cells were washed and activated with DNP-HSA/NIP-HSA cross-linking agent (0.1-10000ng/ml) for 20 minutes prior to incubation with β-hexosaminidase substrate for 2 hours. β-hexosaminidase release was assessed as described in *section 2.4*. Data is presented as mean ± S.D. from three separate experiments performed in triplicate.

5.2.1.7: Intracellular Calcium Mobilisation

Intracellular Ca^{2+} mobilization in RBL-2H3 transfected with huFcεRIα/γ/γ cDNA constructs brought about by antigenic stimulus (NIP/DNP) was assessed using the Ca^{2+} indicator Fluo-3AM (*Section 2.8)* (Aketani et al., 2001, Dearman et al., 2005). IgE sensitized cells loaded with Fluo-3AM were analysed using a FACSort flow cytometer preset for Fluo-3AM studies. Data was recorded in the form of a density plot of mean fluorescence against time. After an initial background reading of 30-50 seconds, samples were activated with the appropriate cross-linking agent (NIP/DNP 100ng/ml) and in the event of no calcium mobilization the samples were challenged with a calcium ionophore (ionomycin) as a positive control to ensure that the cells have been loaded with Fluo-3AM.

The RBL-2H3 transfected with huFcεRIα/γ/γ cDNA constructs sensitized with mIgE exhibited an increase in mean fluorescence peaking at 26seconds after being activated by mIgE-specific DNP-HSA (**figure 5.5a**) and no calcium mobilization was observed on being sensitized with huIgE and activated by huIgE-specific NIP-HSA **(figures 5.5b)**. The same transfected RBLs on failure to elicit calcium mobilization with huIgE-specific NIP-HSA were activated by ionomycin as a positive control and exhibited an immediate increase in mean fluorescence which was maintained until a slow decrease occurred relating to a depletion of intracellular Ca^{2+} **(figures 5.5b).**

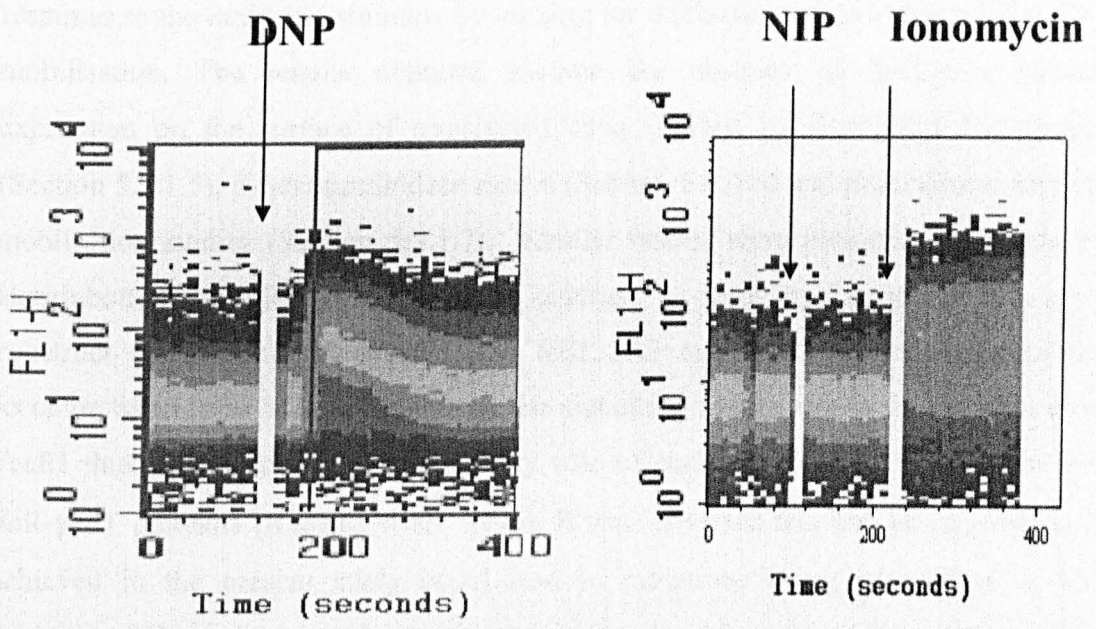


Figure 5.5: Assessment of Intracellular calcium level of RBLs transfected with huFcεRIα/γ/γ cDNA constructs following activation in the absence of extracellular calcium

RBL-2H3 cells transfected with the huFcεRIα/γ/γ cDNA constructs were sensitised with (Figures A) DNP-specific mIgE (SPE-7, 1/500) or (Figure B) NIP-specific huIgE (JW8, 1/500) for 16 hours. IgE sensitised cells were washed in BSS and harvested with CDS. Cells were loaded with Fluo 3-AM (5μM) for 30 minutes at room temperature in the dark. Samples were washed and resuspended at $2x10^6$/ml in DPBS (without $CaCl_2$ or $MgCl_2$). After an initial background reading the cells were activated with appropriate cross-linking agent DNP (Figures A) and NIP (Figures B) at a concentration of 100ng/ml. Following excitation at 488nm emitted fluorescence was recorded at 525nm using a FACSort flow cytometer (BD biosciences). The cells were then activated with ionomycin (10μM, Figure B). Results are representative of three separate experiments.

5.2.1.8: Discussion

The RBLs cell lines transfected with the huFcεRIα/γ/γ cDNA constructs (Section 5.2.1.4) were assessed for the cell surface expression of the huFcεRIα subunit and the

response to the antigenic stimulus by looking for degranulation and intracellular Ca^{2+} mobilisation. The results obtained showed the absence of huFcεRIα subunit expression on the surface of transfected cells as seen by flowcytometric studies (Section 5.2.1.5), β-hexosaminidase assays (Section 5.2.1.6) and intracellular calcium mobilsation studies (Section 5.2.1.7). Similar results were obtained previously by Higginbottom in 1996 (PhD thesis). In contrast, a study by Repetto et al a α/γ/γ construct when stably transfected into RBL-2H3 cells lead to expression of the receptor which was able to exhibit all the signalling events similar to the native rat FcεRI thus demonstrating the mandatory role of both the FcεRIα EC domain and FcR-γ CT domains (Repetto et al., 1996). It was expected that similar expression if achieved in the present study could lead to mutations being introduced in TM domains of the γ that would provide insight into the intersubunit interactions. The huFcεRIα/γ/γ cDNA construct had the problem of presence of spurious point mutations, which needed correction. Similar problems were encountered in the generation and transfection of α/γ/γ cDNA constructs into RBL cells in a study by Higginbottom (1996). It is therefore surprising that the results published by Repetto and collaborators (1996) could not be reproduced in the present and previous study conducted by Higginbottom (1996). In the study by Repetto et al VIS expression vector (unavailable), which possesses a visna virus promoter for constitutive expression, was used for transfection into COS and RBL cell lines (Repetto et al., 1996), While in the study by Higginbottom pMAMneo vector, which is an inducible vector, was employed for transfection of α/γ/γ cDNA constructs into RBL cells (Higginbottom, 1996). In the present study constitutive expression pEE6 vector, which has been sucessfully used for the expression of wild-type and mutant huFcεRIα constructs into RBL-2H3 cell line, was used and the reason for the non-expression of huFcεRIα/γ/γ cDNA constructs remains uncertain.

The human-rodent chimeric receptors are assembled differently than the endogenous rodent receptors as seen in study in which COS 7 cells were transfected with human/rat chimeric complexes (Varin-Blank and Metzger, 1990).

5.3: RBL Gamma Chain Deficient Cell Line

The RBL γ-chain deficient cell line was generated by Draberova and Draber (1995) using chemical mutagenesis, which was reported to have resulted in a variant cell RBL cell line deficient in γ subunit expression, and it is known that the γ subunit is essential for the cell surface expression of FcεRI and FcγRIII (Kinet, 1992). The originators of the RBL γ-chain deficient cell line transfected these cells with the rodent gamma construct (rFcεRγ) to enable the surface expression of FcεRI, which was then challenged with antigen to assess the release of mediators after sensitising the transfected cells with mouse IgE and a successful outcome was published by Petr Bocek et al (1995). This work formed the basis for the next part of the project which was to use the site-directed mutation technique to introduce mutations into TM residue at position 22 (Thr22) of the gamma subunit and to assess their effect on the surface expression by carrying out stable transfection in the RBL γ-chain deficient cell line. Two mutations were generated and introduced in place of threonine residue. One was alanine (T22A) and second was serine (T22S). The intention was to assess the effect of replacement of threonine with an almost similar-sized non-polar alanine and slightly smaller-sized polar serine residue on signal transduction.

5.3.1: Subcloning to pEE6

The already established wild-type and mutant rFcεRγ constructs (T22A and T22S) were present in the pUC18 cloning vector **(figure 5.6)**. Sequencing (Cogenics) of the wild-type and mutant (T22A & T22S) rFcεRγ construct was carried out and cDNA constructs were subcloned into expression vector pEE6 for subsequent transfection into the RBL γ-chain deficient cell line. The digested vector pEE6 and pUC18 with wild-type and mutant rFcεRγ constructs were resolved in parallel on 1% TAE agarose gel *(section 2.9.8.4)*. The desired bands i.e.260bp (wild-type and mutant rFcεRγ constructs) and *6200bp (pEE6)* were excised from the agarose gel and purified using the Quantum Prep™ Freeze N Squeeze DNA gel extraction spin columns kit (Bio-Rad) *(section 2.9.8.4)*. The same procedure as described previously in section 3.6 was employed. The ligation reactions were transformed into XLI-Blue supercompetent cells *(section 2.9.5)*. Qiagen DNA miniprep kit was employed for small scale DNA

extraction *(section 2.9.6.1)*. Test digestion of 10µl miniprep plasmid DNA was carried out using the EcoRI and Hind III restriction enzymes *(section 2.9.8.1)* followed by resolving on TAE gel to confirm the presence of the desired fragment. The miniprep plasmid DNA was transformed into XLI-Blue competent cells (*Section 2.9.5)*. QIAGEN Plasmid Midiprep Kit was employed for large scale DNA extraction *(section 2.9.6.2)*. Test digestion of 10µl midiprep plasmid DNA was carried out using the EcoRI and Hind III restriction enzymes *(section 2.9.8.1)* followed by resolving on TAE gel to confirm the presence of the desired fragment **(figure 5.7)**.

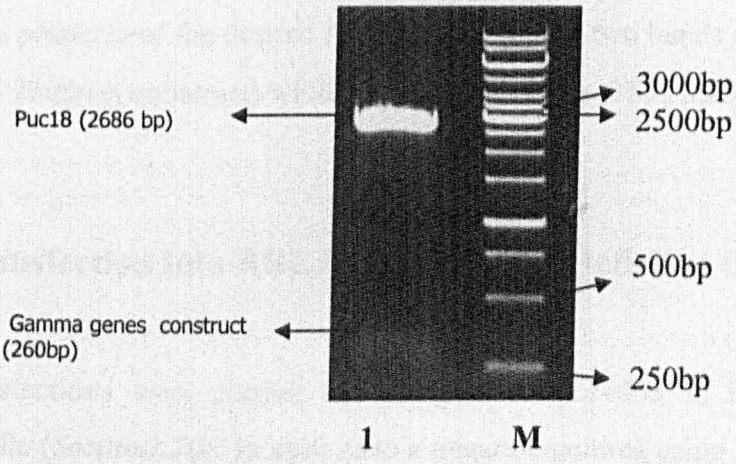


Figure 5.6 Test Digestion of Miniprep Plasmid DNA

Test digestion of 10µl miniprep plasmid DNA was carried out using the EcoRI and Hind III restriction enzymes (*Section 2.9.8.1*) followed by resolving on TAE gel to confirm the presence of the desired fragment. In Lane 1 two bands are visible 2686bp (pUC18) and 260bp (γ construct) while lane M is the 1Kbp DNA marker (*Section2.9.7*).

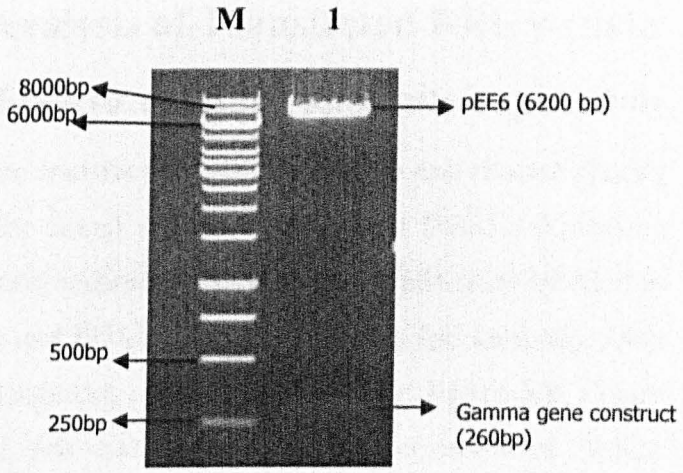


Figure 5.7 Test Digestion of Midiprep Plasmid DNA

Test digestion of 10μl midiprep plasmid DNA was carried out using the EcoRI and Hind III restriction enzymes (*Section 2.9.8.1*) followed by resolving on TAE gel to confirm the presence of the desired fragment. In Lane 1 two bands are visible 6200bp (pEE6) and 260bp (γ construct) while lane M is the 1Kbp DNA marker (*Section2.9.7*).

5.3.2: Transfection into RBL Gamma Chain Deficient Cell Line

Stable transfections were carried out using electroporation in the RBL γ-chain deficient cells (*Section2.10*). In each case a negative control using water in place of DNA was setup alongside. The same procedure as described previously in section 3.7.1 was employed.

The transfected cells were then used to carry out flow cytometric studies (*Section 2.6*) to ascertain the presence of the desired mouse FcεRIα receptors on the cell surface of RBL γ-chain deficient cells. Following flow cytometric studies stocks of the transfected cells were prepared by cryogenic preservation (*Section 2.3.2*) and employed for release assays and intracellular calcium mobilisation studies *(Section 2.8)*.

5.3.3: Flow Cytometric Analysis of Transfected RBL γ-chain Deficient cells for FcεRIα receptor subunits expression

The RBL γ-chain deficient cell line transfected with the rFcεRγ and mutant rFcεRγ cDNA constructs were assessed for clonal stability and highest FcεRIα expressing cells by employing the flowcytometry technique *(Section 2.6)*. Cells were labelled in series with a combination of mIgE and FITC labelled anti-mouse IgE antibody. Data from the FACS analysis of the transfected cell lines is shown in **figure 5.8**. Figure 5.8A shows the unlabelled control. The data demonstrate the expression of FcεRIα receptor subunit on the cell surface of parental RBL γ-chain deficient cells (**figure 5.8C**) at a level lower as compared to RBL-2H3 cell line (**figure 5.8B**). The RBL γ-chain deficient cells transfected with wild-type rFcεRγ cDNA construct (RBL-γ+) shows a reversion to expression of the FcεRIα receptor subunit to the same level (**Figure 5.8E**) as the parental RBL-2H3 cell line. The γ-chain deficient cells transfected with mutant rFcεRγ cDNA construct (T22A and T22S) also shows a reversion to expression of the FcεRIα receptor subunit to the same level (**figure 5.8F and G**) as the parental RBL-2H3 cell line. FACS analysis of the sham transfection carried out showed the same level of FcεRIα receptor subunit expression (**figure 5.8D**) as the parental γ-chain deficient cells.

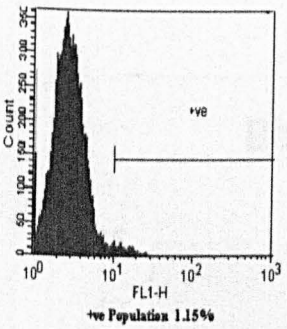
5.3.4: β-hexosaminidase Release Assays

The parental RBL-2H3, RBL γ-chain deficient cells and RBL γ-chain deficient cells transfected with the wild-type and mutant rFcεRγ cDNA constructs were monitored for β-hexosaminidase release by sensitising them with mIgE specific DNP-HSA followed by antigenic challenge (Wilson et al., 1993, Aketani et al., 2001, Dearman et al., 2005). The parental RBL γ-chain deficient cells and sham transfected cells demonstrated release in response to mIgE specific DNP-HSA which is much lower as compared to parental RBL-2H3 (**figure 5.9**). The transfected cell lines (RBL-γ+, T22A and T22S) in response to mIgE specific DNP-HSA released at a higher level as compared to the parental RBL γ-chain deficient cells (**figure 5.9**) but still much lower than the parental RBL-2H3 cells.

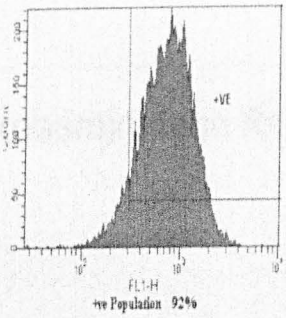
5.3.5: Intracellular Calcium Mobilisation

Intracellular Ca^{2+} mobilization in parental RBL-2H3, RBL γ-chain deficient cells and RBL γ-chain deficient cells transfected with the wild-type and mutant rFcεRγ cDNA constructs cell lines brought about by antigenic stimulus (DNP-HSA) was assessed using the Ca^{2+} indicator Fluo-3AM (*Section 2.8)* (Wilson et al., 1993, Aketani et al., 2001, Dearman et al., 2005). IgE sensitized cells loaded with Fluo-3AM were analysed using a FACSort flow cytometer preset for Fluo-3AM studies. Data were recorded in the form of a density plot of mean fluorescence against time. After an initial background reading of 30-50 seconds, cells were activated with the appropriate cross-linking agent (DNP 100ng/ml) and in the event of no calcium mobilization being witnessed the samples were challenged with a calcium ionophore (ionomycin) to ensure that the cells had been loaded with Fluo-3AM.

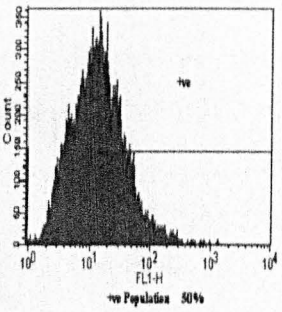
The RBL-2H3 cells sensitized with mIgE exhibited an increase in mean fluorescence peaking at 26 seconds after being activated by mIgE-specific DNP-HSA (**figure 5.10A**). In case of RBL γ-chain deficient cells and RBL γ-chain deficient transfected cells no calcium mobilization could be observed on being sensitized with mIgE and activated by mIgE-specific DNP-HSA (**figures 5.10B, C, D, E and F**). The RBL γ-chain deficient cells and RBL γ-chain deficient transfected cell lines on failure to elicit calcium mobilization with mIgE-specific DNP-HSA were activated by ionomycin and exhibited an immediate increase in mean fluorescence which was maintained until a slow decrease occurred relating to a depletion of intracellular Ca^{2+} (**figures 5.10B, C, D, E and F**). The RBL γ-chain deficient cells and RBL γ-chain deficient transfected cell lines support β-hexosaminidase release by challenging them with mIgE specific DNP-HSA but with the release being much lower as compared to parental RBL-2H3 cell line *(Section 5.2.1.6)*. The methodology of assessing the intracellular calcium mobilization using the Ca^{2+} indicator Fluo-3AM (*Section 2.8)* in the RBL γ-chain deficient cells and RBL γ-chain deficient transfected cell lines after sensitising with mIgE and activating by mIgE-specific DNP-HSA indicated that these cell lines were incapable of supporting any intracellular calcium mobilisation.



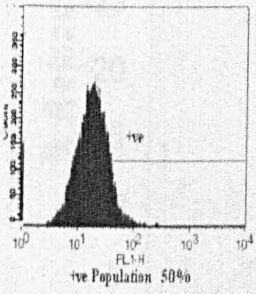
A. Control



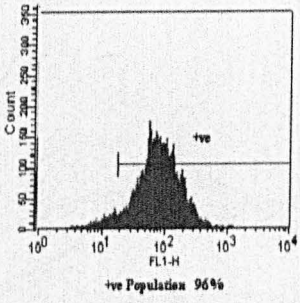
B. RBL-2H3



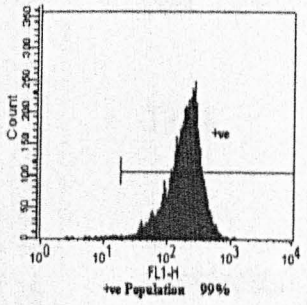
C. RBL γ-chain deficient



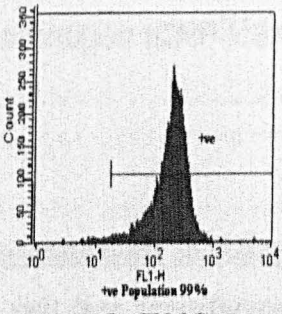
D. Sham transfection



E. RBL-γ+



F. T22A



G. T22S

Figure 5.8: Assessment of cell surface expression of mouse FcεRIα receptors in RBL-2H3, RBL γ-chain deficient cell line, RBL γ-chain deficient cell line transfected with wild type (RBL-γ+) and mutant rFcεRγ constructs (T22A and T22S) and sham transfection by flowcytometry

RBL-2H3 (5.8B), RBL γ-chain deficient cells (5.8C), RBL γ-chain deficient cells transfected with the rFcεRγ construct (RBL-γ+) (5.8E), RBL γ-chain deficient cell line transfected with mutant rFcεRγ constructs (T22A and T22S) (5.8F and G respectively), and sham transfection (5.8D) were harvested labelled in series with a combination of mouse IgE and anti-mouse FITC labelled IgE and assessed for mouse FcεRIα receptor expression.

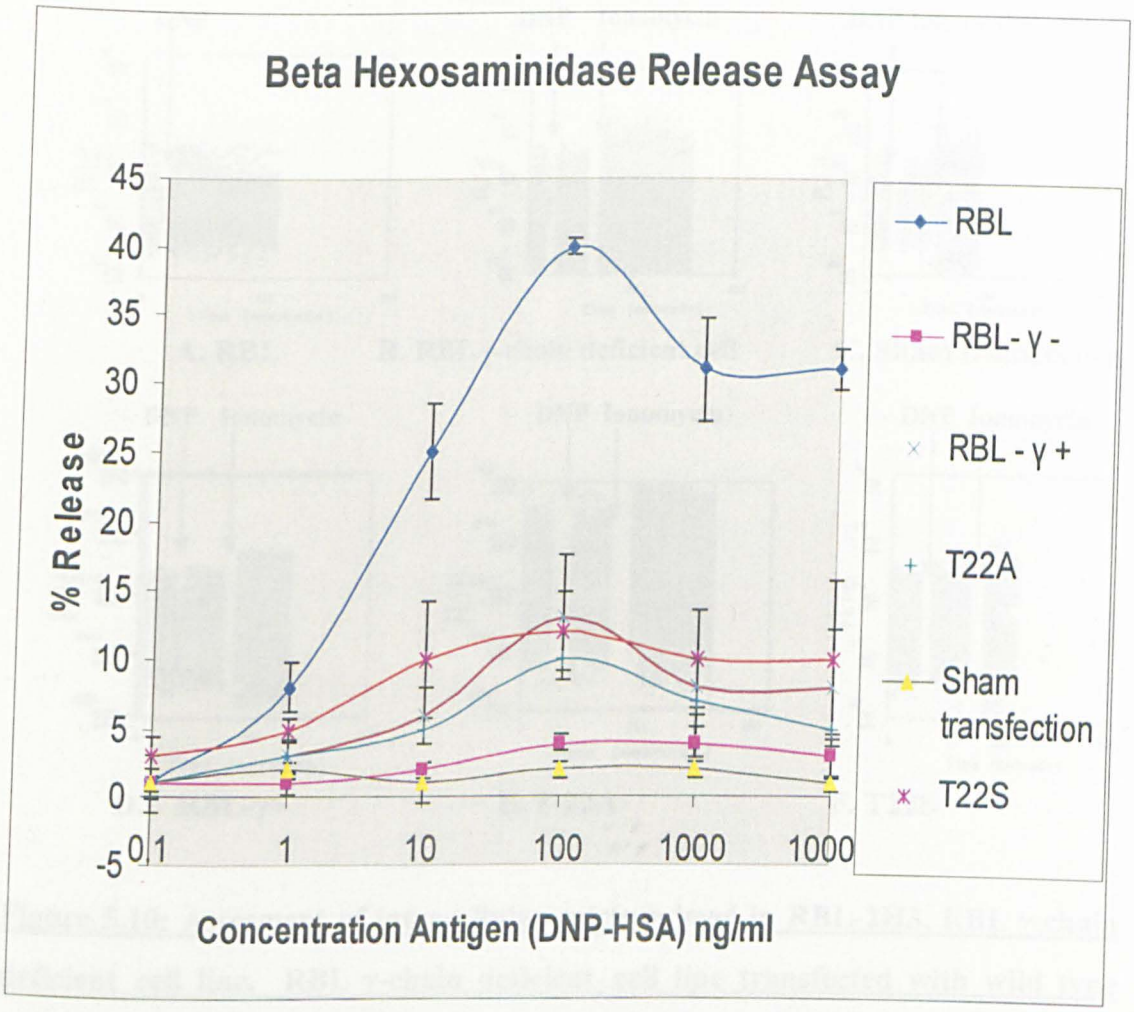


Figure 5.9: Release of β-hexosaminidase in RBL-2H3, RBL γ-chain deficient cell line, RBL γ-chain deficient cell line transfected with wild type (RBL-γ+) and mutant rFcεRγ construct (T22A and T22S) and sham transfection in response to an IgE mediated antigenic stimulus

Cells were cultured, harvested, re-suspended at $0.5x10^6$/ml in appropriate media with DNP-specific mIgE (SPE, 1/500) and plated into 96 well plates for 16h. Next day, cells were washed and activated with DNP-HSA cross-linking agent (0.1-10000ng/ml) for 20 minutes prior to incubation with β-hexosaminidase substrate for 2 hours. β-hexosaminidase release was assessed as described in *section 2.4*. Data are presented as mean ± S.D. from three separate experiments performed in triplicate.

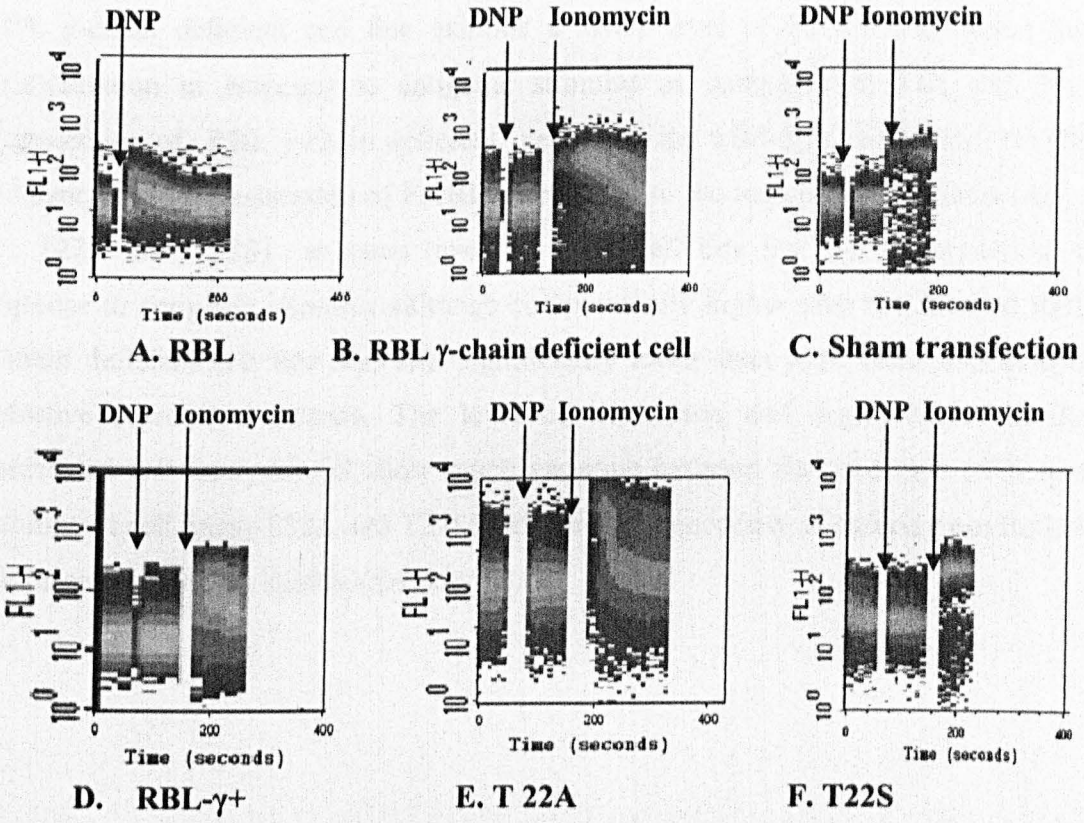


Figure 5.10: Assesment of intracellular calcium level in RBL-2H3, RBL γ-chain deficient cell line, RBL γ-chain deficient cell line transfected with wild type (RBL-γ+) and mutant rFcεRγ constructs (T22A and T22S) and sham transfection following activation in the absence of extracellular calcium.

RBL-2H3 (5.10A), RBL γ-chain deficient cell line (5.10B), RBL γ-chain deficient cell linel transfected with the rFcεRγ construct (RBL-γ+) (5.10D), RBL γ-chain deficient cell line transfected with mutant rFcεRγ constructs (T22A and T22S) (5.10E and F respectively), and sham transfection (5.10C) were sensitised with DNP-specific mIgE (SPE-7, 1/500) for 16 hours. IgE sensitised cells were washed in BSS and harvested with CDS. Cells were loaded with Fluo 3-AM (5μM) for 30 min at room temperature in the dark. Samples were washed and re-suspended at $2x10^6$ /ml in DPBS (without $CaCl_2$ or $MgCl_2$). After an initial background reading the cells were activated with DNP-HSA at a concentration of 100ng/ml. Following excitation at 488nm emitted fluorescence was recorded at 525nm using a FACSort flow cytometer (BD biosciences). The cells were then activated with ionomycin (10μM, Figure5.10B, C, D, E and F). Results are representative of three separate experiments.

5.3.6 Conclusions

RBL γ-chain deficient cell line exhibits a lower level of FcεRIα expression and degranulation in response to antigenic stimulus as compared to RBL cell line. Transfection of RBL γ-chain deficient cells with the wild-type and mutant rFcεRγ constructs lead to restoration of FcεRIα expression in the transfected cell lines (RBL-γ+, T22A and T22S) to same levels as RBL cell line but the degranulation in response to antigenic stimulus although comparatively higher than the parental RBL γ-chain deficient cell line was still significantly lower than RBL cells indicating a defective secretory response. The level of expression and degranulation in the transfected cell lines did not show much variation between the wild-type (RBL-γ+) and mutant cell lines (T22A and T22S) indicating the necessity of carrying out further site-directed mutation studies (Section 6.4.1).

CHAPTER 6

DISCUSSION

CHAPTER 6

DISCUSSION

6.1: Introduction

The aim of the study was to understand the interaction between the subunits of high affinity immunoglobulin E receptor (FcεRI) and its ability to mediate transmembrane signalling. Immunoglobulin E is key player in producing the allergic phenomena (Ishizaka and Ishizaka, 1967, Yazdanbakhsh et al., 2001, Maizels and Yazdanbakhsh, 2003) that is responsible for causing multiple debilitating allergic diseases which are being labelled as the epidemic of the 21st century (Isolauri et al., 2004). FcεRI is composed of ligand binding α-chain (Hakimi et al., 1990, Blank et al., 1991, Ra et al., 1993), signal-amplifying β-chain (Scharenberg and Kinet, 1997, Kinet, 1999) and signal-transducing γ subunits (Kinet, 1999). A model for the interaction was first described by Faber and Sears but rejected by our group on the basis of data obtained *(Section 1.11)* (Iodice, 2006) and the aim of the present study was to design experiments capable of assessing predictions made by the new molecular model structure.

Firstly: Site-directed mutations (*Chapter3 and 4*) were carried out in the D194 residue within the TM domain of the huFcεRIα subunit which were then transfected into Rat Basophilic Cells (RBL-2H3.1) to produce rat/human chimeric receptors and assess the effect of the mutations on subsequent receptor expression and signalling.

Secondly: Transfection into the RBL cell line (*Chapter 5*) of chimeric constructs created using the extracellular (EC) domain of the human high affinity IgE receptor alpha subunit (huFcεRIα) spliced onto the rodent gamma TM and cytoplasmic domain (CD) to achieve the surface expression of the huFcεRIα and activate them independent of the endogenous rodent FcεRIα.

Thirdly : Assessment of a model system for studying the effects of mutations carried out in γ subunits by using a gamma chain deficient RBL cell line (Bocek et al., 1995) and transfecting it with wild type (rFcεRγ) and mutant gamma constructs (T22A and T22S).

6.2: Detection of huFcεRI Expression and Mediator Release in Transfected RBL.2H3.1 Cells

The RBL-2H3.1 cells were transfected with the mutant huFcεRIα cDNA constructs (D194T, D194I, D194V, D194S and D194R) *(Section3.7.1)* and assessed for the cell surface expression of the huFcεRIα subunit and the response to the antigenic stimulus employing measurements of mediator release, intracellular Ca^{2+} mobilisation, and phosphorylation of the γ-subunit and Syk kinase as functional read-outs for signal propagation via the activator receptor complex. The results obtained showed that the mutation of the polar aspartic acid, at position 194 of the transmembrane domain of the huFcεRIα, to a slightly smaller polar threonine residue and subsequent transfection into parental RBL-2H3.1 cell line (D194T) resulted in a functional rat/human chimeric receptor, the huFcεRIα in association with the endogenous rodent FcR-β and FCR-γ subunits, which was not only able to express the human receptor as shown by flow cytometric results (*Section 4.2.1*) but also degranulate in response to antigenic stimulus (huIgE/NIP-HSA) as exhibited by β-hexosaminidase assay (Section 4.2.2) and intracellular calcium mobilsation studies (*Section 4.2.3*).

In contrast the mutations to isoleucine (D194I), valine (D194V), serine (D194S) and arginine (D194R) and transfection into RBL-2H3.1 cells failed to create the assembly of a functional chimeric receptor complex as seen by flow cytometric studies *(Section 4.2.1)*, β-hexosaminidase assays *(Section 4.2.2)* and intracellular calcium mobilsation studies (*Section 4.2.3*). This data harmonizes with the previous study by our group suggesting the importance of a polar residue at the 194 position (Iodice, 2006).

Previous studies by our group showed that mutations to asparagine (D194N) and leucine (D194L) lead to surface expression of the huFcεRIα subunit but it was only D194N which had a functionally viable receptor which was able to respond to antigenic stimulus, thus pointing to the importance of a polar residue at the 194 position as it is expected that the polar Asp serves to stabilise the receptor complex by forming side-chain/side-chain inter-helical hydrogen bonding with the other subunits (Iodice, 2006). It had already been shown that mutations to alanine (D194A), glutamic acid (D194E) and lysine (D194K) carried out previously failed to induce

huFcεRIα expression in transfected cells. According to the model proposed *(Section 1.9)* the side-chain of residue 194 is placed in a closely packed central position at the interface between the transmembrane helices where there will be severe restraints as to which side chain can be accommodated. This is consistent with the results which demonstrates that the residue at 194 be a polar residue of medium size (Asp, Asn, Thr) computed of making two separate hydrogen bonds with the Thr22 residue in the two FcR-γ subunits. The mutation to almost similar sized non-polar Ile (D194I) failed to exhibit surface expression as it is essentially different in polarity despite occupying similar space as Asp. The polar Thr mutation (D194T) is able to maintain receptor expression and function while the almost similar sized non-polar valine (D194V) is disfavoured as it is incapable of hydrogen bonding. It is evident that only slightly larger polar residues Glu (D194E) and polar Lys (D194K) and much larger sized polar Arg (D194R) were also not found to be suitable replacement although like Asp, Asn and Thr, the residues Glu, Lys, and Arg should also be capable of making the predicted hydrogen bonds thus reflecting the critical nature of the packing restraints at this position. Similarly the smaller sized residues non-polar Ala (D194A) and polar Ser (D194S) also failed to act as a suitable substitution at the 194 position as no surface expression was witnessed in the D194A and D194S transfected cells. It is possible that in all the above-mentioned mutations the correct assembly of the three transmembrane helices is compromised and thus correct assembly at the cell surface does not take place. In studies on COS-7 cells by Varin-Blank and Metzger (1990) a polar to non-polar mutation (D195A, homologous residue to D194) TM mutation in the rat FcεRIα subunit resulted in marked reduction in cell surface expression. Similarly in another study by Gosse et al. (2005) a chimeria consisting of the EC domain of huFcεRIα, TCRζ TM having a D15A mutation and CT domain exhibited a reduction in cell surface expression.

These data point to the importance of the D194 residue in receptor expression and downstream signalling as previously many studies have highlighted the importance of TM conserved residues of the immune receptors (TCR, BCR, IgA receptor, FcεRI) on receptor subunit assembly, expression and function (Varin-Blank and Metzger, 1990, Audoly and Breyer, 1997, Cain et al., 2001, Kunjibettu et al., 2001, Gosse et al., 2005).

6.3: Transfection of RBL Gamma Chain Deficient Cell with Wild-Type and Mutant rFcεRγ Constructs

A mutant of the RBL-2H3 cell line with FcεRI deficient in the γ subunit, which is essential for the expression FcεRI, was employed in the study (Draberova and Draber, 1995) with the aim of assessing the FcεRIα and γ -chain interaction in transmembrane signalling. The RBL γ-chain deficient cells were stably transfected with the wild-type and mutant rFcεRγ cDNA constructs using electroporation technique *(Section 5.3.2)*. The RBL γ-chain deficient cells and RBL γ-chain deficient cells transfected with the wild-type and mutant rFcεRγ cDNA constructs were assessed for the cell surface expression of the FcεRIα subunit, the response to the IgE-mediated antigenic stimulus (DNP-HSA) by measuring the release of mediators during degranulation and intracellular Ca^{2+} mobilisation.

Initial assessment of parental RBL γ-chain deficient cell line for FcεRIα expression by flow cytometry exhibited presence of FcεRIα subunit at a level much lower than the parental RBL-2H3 cell line, but not a complete deficiency (Section 5.3.3), and in response to antigenic stimulus degranulation was observed as seen by β-hexosaminidase release assay but at a level lower than in parental RBL-2H3 cells *(Section 5.3.4)*. The RBL γ-chain deficient cells transfected with the wild-type (rFcεRγ) construct (RBL-γ+) expressed the FcεRIα at a level similar to RBL-2H3 cell line as shown by flow cytometric studies and in response to antigenic stimulus degranulation could be demonstrated as seen by β-hexosaminidase release assay but at a level lower than in the parental RBL-2H3 cells. The results are similar to data obtained in a previous study carried out by Bocek et al (1995).

After successfully demonstrating that by transfecting RBL γ-chain deficient cells with rFcεRγ construct reversion of full FcεRIα can be obtained, the RBL γ-chain deficient cells were transfected with mutant rFcεRγ cDNA constructs in which the threonine residue at position 22 in the TM region of the rodent gamma subunit was replaced with polar serine (T22S) and non-polar alanine (T22A). RBL γ-chain deficient cells transfected with mutant rFcεRγ cDNA constructs expressed the FcεRIα at a level similar to RBL-2H3 cell line as shown by flow cytometric studies (*Section 5.3.3*) and in response to antigenic stimulus β-hexosaminidase release was

obtained although at a level significantly lower than RBL-2H3 cells but higher than the parental RBL γ-chain deficient cells (*Section 5.3.4)*. The molecular basis for the lower level β-hexosaminidase release in the transfected cell lines (RBL-γ+, T22A and T22S) as compared to the parental RBL-2H3 observed in the present study awaits further investigation but might be attributable to an incomplete assembly of the receptor complex resulting in inefficient signal propagation, a phenomenon commonly observed in RBL cells variants (Cohen-Dayag et al., 1992). A sham transfection carried out did not support any increase in the FcεRIα expression (*Section 5.3.3)* in the parental RBL γ-chain deficient or increase in degranulation in response to antigenic stimulus (*Section 5.3.4*). The level of FcεRIα expression restoration is similar in all the three transfections (RBL-γ+, T22A and T22S) carried out and the β-hexosaminidase release was also of same level with no difference apparent between the wild-type and mutant rFcεRγ cDNA constructs.

The technique of Ca^{2+} imaging *(Section 2.8)* for assessing intracellular calcium mobilisation in response to antigenic stimulus did not prove sensitive enough in case of RBL γ-chain deficient cells and RBL γ-chain deficient cells transfected with the wild-type and mutant rFcεRγ cDNA constructs to detect any intracellular calcium mobilization, although it is known that the FcεRI-mediated pathway culminating in exocytosis requires Ca mobilization.

6.4: Future Work

The current study has provided assessment of a model system (RBL γ- chain deficient cell line) for studying the affect of mutagenesis in the gamma subunit along the same line as was carried out in the α-chain. Since the γ-chain deficient cell line still supported some FcεRIα expression, a meaningful interpretation of the changes in the interactions between α and γ-chain interactions in the FcεRI at the molecular level requires further experimental analysis.

6.4.1: Mutational Analysis of FcR- γ Subunit

Mutations were carried out in the Thr22 position of the gamma construct which were subsequently transfected into the RBL γ-chain deficient cell line. The result obtained revealed no significant effect of the mutations (T22A and T22S) on the surface expression and degranulation levels as compared to the transfected wild-type cell line (RBL-γ+). There is need to carry out

further mutations in the Thr22 (T22V) and other residues in the γ construct. A possible residue which can be targeted is the K29 (rat FcεRI-γ) as this has been suggested by Farber and Sears (1991) to be involved in interaction with the D203 residue of the rat FcγRIIIα *(Section 1.9)*. The K29 residue is homologous to the K30 residue of huFcR-γ and D203 corresponds to D194 in huFcεRIα.

6.4.2 Alternative Model System

On the basis of data obtained in the current study the use of RBL γ-chain deficient cell line as potential model system has been found to be associated with certain limitations including the continued expression of FcεRI, even before transfection of the gamma constructs, and the defective secretory response to antigenic stimulus in the parental RBL γ-chain deficient and the transfected RBL γ-chain deficient cells. An alternative cell line which may be used is the COS-7. This cell line has been used for transfecting mutant FcεRI subunits in previous studies (Varin-Blank and Metzger, 1990, Kuster et al., 1990, Mao et al., 1993). If COS-7 cell line is co-transfected with the gamma and huFcεRIα constructs, used in the present study, together with co-transfection of gene constructs encoding the FcεRIβ subunits it may provide an alternative means of assessing the effects of mutations in the α and γ subunits although the use of this line only allows the assessment of cell surface expression as they do not support mediator release (Varin-Blank and Metzger, 1990, Kuster et al., 1990, Mao et al., 1993).

6.5: Conclusions

The present study provided further support for the importance of residue D194 in the transmembrane region on surface expression and downstream signaling of FcεRI. Various mutations carried out at the D194 position showed that a polar residue of medium size (D194T) was required for cell surface expression of FcεRI and successful downstream signalling leading to degranulation in response to antigenic stimulus. RBL-2H3 transfected with mutant huFcεRIα subunit cDNA constructs showed that mutations to almost similar sized non-polar Ile (D194I) and V (D194V), larger sized polar Arg (D194R), and smaller sized polar Ser (D194S) and non-polar Ala (D194A) lead to loss of surface expression of huFcεRIα subunit and abrogation of downstream signalling as evidenced by loss of degranulation in response to an IgE-mediated antigenic stimulus. These data point towards D194 residue as being a potential target for

developing future anti-allergy therapeutic strategies by inhibiting FcεRI-mediated signalling along the same lines as T- cell receptor signalling inhibition and "molecular wedge" anti-cancer drugs *(Section 1.9)*. Further research needs to be carried out with the aim of developing TM-derived peptides sequence targeting D194 residue.

The current study also investigated the validity of RBL γ-chain deficient cell line (mutant of RBL-2H3 cell line) as a potential model system for assessing the effect of mutation in the γ-chain to gain an improved understanding of α- and γ-chains interactions. A variant RBL-line proclaimed to be γ-chain deficient exhibited lower levels of FcεRI expression and degranulation than the parental RBL-2H3 cells. Transfections of these cells with wild-type and mutant γ-chain cDNA constructs lead to restoration of FcεRI expression to a similar level as seen in RBL-2H3 cell line. Measurement of degranulation in response to antigenic stimulus in the transfected RBL γ-chain deficient cells (RBL-γ+, T22A and T22S) also showed an increased level as compared to the parental cells (RBL γ-chain deficient) but was still at a much lower level than RBL-2H3 cell line pointing to defective receptor assembly and downstream signal signaling resulting in a reduced secretory response commonly observed in variant RBL cells (Cohen-Dayag et al., 1992, Bocek et al., 1995).

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APPENDIX I

Assessing the Role of Amino Acid Residues in the Transmembrane Domains Of The α- and γ-Chains of the High-Affinity Receptor Complex for Immunoglobulin E in Signal Transduction

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Running Title: Requirement of transmembrane D194 in human FcεRIα function

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Key Words: Allergy, FcεRI, Transmembrane, Polar residue

Abstract

The high-affinity receptor complex for IgE plays a pivotal role in allergic responses since cross-linking of the high affinity IgE receptor (FcεRI) on target cells initiates a signaling cascade facilitating release of inflammatory mediators causing allergic responses. The transmembrane regions of the ligand binding domain of the high-affinity IgE and low-affinity IgG receptors share an invariant motif (LFAVDTGL) containing a polar aspartate within a predominantly non-polar setting. The functional importance of this aspartate residue (D194) in FcεRI-mediated receptor signaling was assessed by site-directed mutagenesis. Rat basophilic leukemia cells (RBL-2H3) transfected with the human IgE binding subunit (FcεRIα), incorporating either asparagine (D194N) or threonine (D194T) polar substitutions, a functional rat/human chimeric receptor complex similar to wild type (D194) was formed. When activated via huIgE and antigen, mediator release, intracellular calcium mobilisation and tyrosine phosphorylation of γ-chain and Syk kinase was obtained while a non-polar substitution (D194L) supported cell surface expression but failed to initiate downstream signaling.

No cell surface expression of mutant huFcεRIα gene constructs was observed when D194 was replaced with the non-polar Ile (D194I) residue of similar size, the larger positively charged Arg (D194R) or lysine (D194K) residues, the negatively charged glutamate (D194E) or the smaller polar Ser (D194S) non-polar Ala (D194A) and V (D194V). These observations highlight importance of both the size and charge of amino acid residue at position 194 in determining IgE receptor subunit interactions, cell surface localization, distribution and signaling.

Introduction

The high-affinity IgE receptor (FcεRI) is expressed as a tetrameric complex ($\alpha\beta\gamma_2$) on mast cells and basophils and consists of an extracellular FcεRIα subunit that engages its ligand, a membrane spanning FcR-β subunit that acts to amplify signal transduction and a disulphide-linked FcR-γ subunit homodimer that relays the activation signal intracellularly (Metzger, 1992). The FcR-γ subunit is promiscuous and functions in homo- or hetero-dimeric forms in association with FcεRI, FcγRIII, FcγRI, FcαRI and TCR (Kinet, 1999). Following cross-linking or aggregation of the receptor via an antigenic stimulus, FcR-β and FcR-γ subunits become tyrosine phosphorylated within conserved immunoreceptor tyrosine-based activation motifs (ITAMs). Subsequent recruitment and activation of Syk kinase results in the initiation of numerous intracellular signaling pathways that culminate in the release of various preformed and *de novo* synthesized cellular mediators (Nadler et al., 2000).

Several studies have demonstrated the importance of amino acid chemistry within transmembrane (TM) regions in the assembly, function and stability of numerous cell surface receptors. In earlier studies, COS-7 cells transfected with mutant subunits of the high-affinity IgE receptor showed that even minor changes within the TM regions could invoke major effects on receptor function and loss of cell surface expression (Varin-Blank and Metzger, 1990). More recently, chimeric receptors consisting of the extracellular human FcεRIα (huFcεRIα), varying TM regions (wild-type and mutant TCR-ζ, the IL-2 receptor α-chain Tac, transferrin and the phosphatases PTP-α and CD45) and TCR-ζ cytoplasmic domains expressed in the RBL-2H3 cell line, demonstrated disparate effects on cell surface expression or subsequent signaling events depending on the source of the TM, thus substantiating an essential role for TM segments in FcR signaling (Gosse et al., 2005). The TCR consists of two extracellularly oriented heterodimeric glycoproteins (TCR-α and TCR-β) that are non-covalently linked to the CD3 complex (CD3-γ, -δ, -ε and -ζ) (Samelson, 2002). Previous investigations have established the importance of conserved charged TM residues within the TCR as crucial for receptor assembly, cell surface expression and functional competence (Call et al., 2002). Similarly, a D to N substitution in the second TM domain of the C5a receptor abolished signaling while ligand binding was preserved (Monk et al., 1994). Furthermore, site-directed mutagenesis to investigate

the role of a conserved R in the TM domain of FcαRI demonstrated the requirement of a positively charged residue in mediating a functional association with the FcR-γ subunit and consequent signal transduction (Morton et al., 1995).

The identification of an invariant eight amino acid motif (LFAVDTGL) containing a polar aspartate residue within the TM domains of FcεRIα and FcγRIIIα from rat, mice and humans (Farber and Sears, 1991; Ravetch and Kinet, 1991) suggested a functional role in receptor mediated signalling. Subsequently, a model was proposed depicting possible TM domain helix-helix interactions between charged residues in rat FcγRIIIα and FcR-γ, mediated in part by the negatively charged D and a corresponding positively charged K in the FcR-γ, to allow optimal counterbalancing of electrostatic interactions. Furthermore, due to the conserved nature of the invariant TM motif, the authors proposed that their model was also applicable to FcεRIα and FcR-γ TM domain helix-helix interactions (Farber and Sears, 1991).

The aim of the present study was to examine the role of the D194 residue within the TM domain of the huFcεRIα subunit on cell surface expression and FcεRI-mediated signaling. In the current study, the parental rat basophilic leukemia (RBL-2H3.1) cell line (Bingham et al., 1994), referred to hereafter as RBL-2H3, was chosen as host for gene constructs encoding wild-type and mutant huFcεRIα subunits. Previous studies have shown that in RBL-2H3 cells the transfected huFcεRIα subunit forms a functional chimeric rat/human receptor complex with endogenous FcR-β and FcR-γ subunits, which can be specifically activated via a human IgE (huIgE) mediated cross-linking stimulus to mediate cell secretion (Gilfillan et al., 1992; Wilson et al., 1993).

Our study demonstrates that cells transfected with wild-type (D194) and mutated variants (D194N, D194T and D194L) of the huFcεRIα subunit are expressed at the cell surface in association with endogenous FcR-β and FcR-γ subunits. Cell transfection with other mutant huFcεRIα variants was attempted, but constructs encoding D194A, D194E, D194V, D194S, D194I, D194R and D194K failed to support cell surface expression. D194, D194T and D194N transfected cell lines demonstrated FcεRI aggregation, tyrosine phosphorylation of FcR-γ and Syk kinase, intracellular Ca^{2+} mobilization and mediator release in response to antigen-mediated

activation, analogous to that observed with the RBL-2H3 cell line. In contrast, RBL-2H3 cells transfected with the mutant D194L gene construct supported cell surface expression but showed no evidence of FcεRI-mediated signaling in response to the same antigenic stimulus. Based on these observations, we propose that interaction between D194 in FcεRIα and T22 in FcR-γ TM domains is essential for expression of functional FcεRI.

Materials and Methods

Reagents and immunoglobulins - Cell culture reagents were purchased from Sigma-Aldrich (Dorset, UK) and fetal calf serum from Autogen Bioclear (Wilts, UK). All enzymes used for DNA manipulation and 'Complete Protease Inhibitor Cocktail' tablets were purchased from Roche Applied Science (Sussex, UK). DNA purification kits were purchased from Promega (Madison, USA) and Qiagen (Crawley, UK). DNA primers for PCR were synthesized in-house by Dr. A.J.G Moir in the Krebs Institute, University of Sheffield and VH Bio (Gateshead, UK). Dinitrophenol-human serum albumin (DNP-HSA) was obtained from Sigma-Aldrich and 4-hydroxy-3-iodo-5-nitrophenylacetyl-human serum albumin (NIP-HSA) was prepared from NIP-OSu (Sigma-Genosys). NIP-specific human IgE (or huIgE, JW8, a chimeric mouse/human IgE with specificity for the hapten NIP) tissue culture supernatant was obtained in house by culturing transfected myeloma cells, as was DNP-specific mIgE (or mIgE, SPE-7 supernatant). Human IgE and biotinylated goat anti-human IgE for FACS was purchased from Serotec (Kidlington, Oxford, UK) and Vector Laboratories Ltd. (Peterborough, UK), respectively. Anti-phosphotyrosine, clone 4G10 and anti-FcR-γ subunit antibodies were purchased from Upstate Biotechnology (Lake Placid, USA). Monoclonal and polyclonal antibodies to Syk kinase were purchased from Abcam Ltd. (Cambridge, UK) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Goat anti-mouse and anti-human IgE were both purchased as FITC conjugates from Bethyl Laboratories (Montgomery, USA). All other reagents and antibodies were obtained from Sigma-Aldrich, unless stated otherwise.

Expression vector construction and cell transfection - *cDNA* for huFcεRIα, (a kind gift from Drs U. Blank and J-P. Kinet) was used as a template for PCR to generate both the wild-type construct and constructs containing the targeted mutations of D194N, D194L, D194T, D194V, D194R, D194S, D194A, D194E, D194K and

D194I. Site directed mutagenesis by Overlap Extension PCR (Ho et al., 1989) or Quickchange Site- Directed Mutagenesis Kit (Stratagene, USA) was used to generate constructs. The following primers were generated:

5' external 5'- ATCAAGCTTATGGCTCCTGCCATG-3' [1]; 3' external 5'-ATGCTTGAATTCTCAGTTGTTTTTGGGGTT-3' [2]; Mutagenic primers; D194L5' 5'-GCTGTGCTCACAGAA-3' [3]; D194L3' 5'-TTGTGTGAGCACAGC-3'[4]; D194N5' 5'-GCTGTGAACACAGGATTA-3'[5] ; D194N3' 5'-TAATCCTGTGTTCACAGC-3'[6]; D194T5′ 5′- GGTGATTCTGTTTGCTGTG ACCACAGGCCTATTTATCTCAACTCAGCAG-3′[7]; D194T3' 5′- CTGCTG AGTTGAGATAAATAGGCCTGTGGTCACAGCAAACAGAATCACC-3′[8]; D194V5′ 5′- GGTGATTCTGTTTGCTGTGGTCACAGGCCTATTTATCTCAACT CAGCAG-3′[9]; D194V3′ 5′- CTGCTGAGTTGAGATAAATAGGCCTGTGAC CACAGCAAACAGAATCACC-3′ [10]; D194I5′ 5′- GGTGATTCTGTTTGCT GTGATTACAGGCCTATTTATCTCAACTCAGCAG-3′[11]; D194I3′ 5′ - CTG CTGAGTTGAGATAAATAGGCCTGTAATCACAGCAAACAGAATCACC-3′ [12]. D194S5′ 5′- GGTGATTCTGTTTGCTGTGTCCACAGGATTATTTATCTCA ACTCAGCAG-3′[13]; D194S3′ 5′ - CTGCTGAGTTGAGATAAATAATCCTGT GGACACAGCAAACAGAATCACC -3′[14]; D194R5′ 5′- GGTGATTCTGTTT GCTGTGCGTACAGGATTATTTATCTCAACTCAGCAG-3′[15]; D194R3′ 5′ - CTGCTGAGTTGAGATAAATAATCCTGTACGCACAGCAAACAGAATCACC-3′[16]; D194A5′ 5′- GCTGTGGCCACAGGATTA - 3′ [17]; D194A3′ 5′-TAATCCTGTGGCCACAGC - 3′ [18]; D194E5′ 5′- GCTGTGGAAACAGGATTA - 3′ [19]; D194E3′ 5′- TAATCCTGTTTCCACAGC - 3′ [20]; D194K5′ 5′-GCTGTGAAAACAGGATTA - 3′ [21]; D194K3′ 5′- TAATCCTGTTTTCACAGC - 3′ [22];

The restriction sites *Hind* III and *Eco*RI were incorporated into primers to facilitate cloning into the pEE6 expression vector (Celltech, Slough, UK). An additional silent mutation in the form of Stu I was also incorporated in primers [7-12] to aid in assessing the presence of mutation with the help of restriction digestion with Stu I and resolution on TAE agarose gel. Reaction mix for PCR was as follows: 5µl 10X reaction buffer (supplied with enzyme), 50ng template, 1µl dNTPs, primers 125ng each, ddH_2O to a final volume of 50µl to which 1µl of *PfuUltra* HF DNA polymerase was added. The PCR cycling conditions were as follows: 95°C/30 s, 95°C/30 s,

55°C/60 s and 68°C for 60sec/kb of plasmid length, 12-18 cycles, according to manufacturer's instructions. The PCR products were resolved on 1% TAE gels at 100 mA/h, and the resulting bands purified using Wizard® PCR kits according to the manufacturer's instructions (Promega). The initial round of PCR was used to generate a template for the second round (Ho et al., 1989), which was again purified as described, and digested with the restriction endonucleases *Hind* III and *Eco*RI to facilitate cloning, initially into pUC18 and subsequently into the expression vector pEE6. Ligation was carried out at each step using T4 DNA ligase according to the manufacturer's instructions. Resulting DNA clones were sequenced for authenticity at each step using Cogenics (Takeley,UK) and results were then analysed using ALIGN, CHROMA and CLONE computer programmes to compare the sequence with genomic huFcεRIα subunit and check mutations were 'in-frame'. The primers used for the sequencing were M13-48REV and M13For-40 to read the DNA from both ends. Transfection quality DNA was prepared using a commercial kit purchased from Qiagen. RBL-2H3 cells (10^7 cells/0.8ml) were transfected by electroporation (Bio-Rad, Hercules, CA) using 20μg of DNA in a cuvette at 250V, 960 μFD according to the manufacturer's instructions. 24 h post-transfection, selection media was added (800μg/ml Geneticin, G-418 sulphate, Invitrogen, Carlsbad, USA) for 5 days, after which a concentration of 400μg/ml Geneticin was maintained. Cells expressing high levels of the transfected receptor gene were sorted using flow cytometry.

Flow Cytometry - Cells transfected with gene constructs encoding D194, D194N, D194T, D194V, D194R, D194S, D194I and D194L huFcεRIα subunits were monitored and selected for highest receptor number expressing cells by flow cytometry. Briefly, cells ($1x10^6$-10^7/ml) were washed in PBS, harvested with a non-enzymatic cell dissociation solution (Sigma-Aldrich) and resuspended in wash buffer (PBS containing 1% fetal calf serum). Cells were incubated with huIgE (1-10μg, proportional to cell numbers) and biotinylated goat anti-human IgE (1/400 dilution) followed by a final incubation with streptavidin R-phycoerythrin (1/25 dilution). Samples were analysed using a FACsAria/ Facscalibur (BD Biosciences) or FASort/ FACS™ CYAN ADP flowcytometer (Dakocytomation). Following FACS sorting cells were grown and expanded for several generations and taken into suspension with cell dissociation medium prior to analysis.

Cell culture and activation - RBL-2H3 cell lines were maintained in culture as described previously (Bingham et al., 1994). D194, D194N, D194T, D194V, D194R, D194S, D194I and D194L transfected cell lines were maintained in the same media supplemented with Geneticin (400 μg/ml). For receptor activation, RBL-2H3 and huFcεRIα transfected cell lines were sensitized for 16 h with either DNP-specific mIgE (1 ug/ml) or NIP-specific huIgE (1 ug/ml), respectively. Cells were harvested in cell dissociation solution and resuspended at $5x10^6$/ml in a buffered solution (120mM NaCl, 5mM KCl, 25mM PIPES, 0.04mM $MgCl_2$, 1mM $CaCl_2$, final pH 7.4). Cells were activated with the appropriate cross-linking agent (DNP or NIP coupled to HSA) at a final concentration of 100ng/ml and solubilized in RIPA lysis buffer (1% NP-40, 150mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 50 mM Tris, pH 7.4, supplemented with 1 mM Na_3VO_4 and a Complete Protease Inhibitor Cocktail tablet). Post-nuclear supernatants (PNS) were obtained by centrifugation at 12000g for 15 min.

β-hexosaminidase release assays - Cells were cultured, harvested, resuspended at $0.5x10^6$/ml in appropriate media with DNP specific mIgE/NIP specific huIgE (SPE, 1/500) and plated 100μl/well into 96 well plates. Next days, cells were washed with a buffered solution and activated with DNP-HSA/NIP-HSA cross-linking agent (0.1-10000ng/ml) for 20 minutes prior to incubation with 50μl β-hexosaminidase substrate (p-nitrophenyl N-acetyl β-D-glucosaminide diluted to a final concentration of 2mM in citrate buffer, pH 4.5) for 2-3 hours at 37°C. The reaction was stopped by addition of 150μl 1M TRIS-HCl, pH 9.0, and product absorbance visualized using an ELISA plate reader set to 405nm wavelength (Millenia Kinetic Analyser) against substrate/buffer blank. Total β-hexosaminadase cell content was obtained by cell disruption using Sirganian buffer supplemented with 1% Triton-X100. Experiments were carried out in triplicate and mean values ± SD presented.

Ca^{2+}mobilization - Cells were harvested in cell dissociation solution and washed in PBS. $1x10^7$ cells were resuspended in the appropriate media and incubated at 37°C for 1 h with 10μg/ml of either DNP-specific mIgE or NIP-specific huIgE. Following incubation, cells were sedimented and resuspended at a density of $5x10^6$/ml in a

buffered solution (120mM NaCl, 5mM KCl, 0.04mM $MgCl_2$, 1mM $CaCl_2$, 25mM PIPES, final pH 7.4). The Ca^{2+} probe Fluo-3 AM was added at a final concentration of 5μM. Cells were incubated for 15 min at 37°C, after which aliquots of $1x10^6$ cells were analysed on a Facscalibur flow cytometer (BD Biosciences). After a baseline reading was taken, DNP-HSA or NIP-HSA was added as appropriate (100ng/ml). In the unresponsive cell lines (D194L, D194V, D194R, D194S and D194I), ionomycin (10μM) was used as a positive control, demonstrating that the cells had been adequately loaded with Fluo-3 AM.

Immunoprecipitation and immunodetection - A standard Bradford assay (Bio-Rad, Hercules, USA) was performed with an aliquot of PNS to allow normalization between samples. Normalised PNS was incubated with respective antibodies (1μg/mg PNS) overnight at 4°C and precipitated with Protein A-agarose (Upstate Biotechnology, Lake Placid, USA) for 4 h. Immunoprecipitates were washed in ice-cold RIPA lysis buffer and eluted in SDS sample buffer (50mM Tris, pH 6.8, 2% SDS, 10% glycerol and 100 mM DTT) prior to separation by 12.5% SDS-PAGE and subsequent electrophoretic transfer of protein to PVDF membranes (Amersham Biosciences). Membranes blocked either in 3% BSA (for anti-phosphotyrosine, clone 4G10) or 5% non-fat milk (for all other primary antibodies) in PBS/Tween (0.1%) were incubated with primary antibodies and HRP-conjugated secondary antibodies, as indicated, for 1 h each. Proteins were visualized by using the Enhanced Chemiluminescence (Amersham Biosciences).

Results

(i) Cell surface expression of huFcεRIα in transfected cell lines: Gene constructs encoding wild-type and mutant variants, where D194 in the TM domain of the huIgE receptor ligand binding subunit has been replaced by codons specifying charged, polar and non-polar amino acids, were transfected into RBL-2H3 cells. Stable cell surface expression was observed following transfection of the wild-type gene and genes encoding replacement of D194 by N, T or L, while variants encoding a substitution with A, E, V, I, S, R or K at this position showed no evidence of cell surface expression of mutant receptor subunits. Fig. 1 shows analysis of transfected cell lines by flow cytometry from a typical experiment demonstrating positive expression where stably transfected cell lines were subjected to repeated cell sorting to select for the highest expressing cells. Furthermore, co-immunoprecipitation studies confirmed expression of transfected huFcεRIα subunits in association with endogenous FcR-β and FcR-γ subunits in the transfected cell lines (Iodice, 2006).

(ii) Downstream signaling β-hexosaminidase release: Antigen-mediated cross-linking of the high-affinity IgE receptor on mast cells results in the release of various mediators, including β-hexosaminidase, thus providing a reliable system for assessing mast cell degranulation (Landegren, 1984). All cell lines were assessed for their ability to undergo degranulation in response to an IgE-mediated antigenic stimulus through endogenous FcεRI and transfected huFcεRIα receptors (Fig. 2). The D194, D194T and D194N transfected cell lines exhibited β-hexosaminidase release in response to huIgE/NIP-HSA (selectively activating cells sensitized via the transfected huFcεRIα receptor subunit). As expected the D194V, D194R, D194S, and D194I transfected cell lines, which show no surface expression of huFcεRIα by flow cytometry, did not support mediator release in response to a huIgE/NIP-HSA cross-linking stimulus. In contrast, the D194L constructs, while supporting cell surface expressing of huFcεRI, showed no release, indicating that this mutation at D194 residue results in abrogation of receptor-mediated degranulation.

(iii) Downstream signaling Ca^{2+} mobilization: IgE/antigen-mediated activation of mast cells results in the production of the secondary mediator IP_3 which promotes release of Ca^{2+} from ER localized stores to facilitate an increase in the concentration

of intracellular Ca^{2+}, a process essential to the degranulation response (Scharenberg and Kinet, 1998). Changes in intracellular Ca^{2+} levels were examined in all cell lines passively sensitized with the IgE for the appropriate species and subsequently loaded with Fluo 3-AM prior to challenge with the corresponding antigen. Ca^{2+} mobilization was assessed using flow cytometry. As shown in Fig. 3, cell lines showed an almost immediate increase in cellular Ca^{2+} levels following the addition of DNP-HSA, peaking around 20 s after stimulation. Similarly, the D194, D194T and D194N transfected cell lines demonstrated a significant increase in cellular Ca^{2+} levels following the addition of NIP-HSA. In contrast, the D194L, D194V, D194R, D194S, and D194I transfected cell lines showed no increase in levels of intracellular Ca^{2+} following the addition of NIP-HSA. The Ca^{2+} ionophore ionomycin was used as a positive control in the unresponsive D194L, D194V, D194R, D194S, and D194I transfected cell lines demonstrating the capacity of the transfected cells to support Ca^{2+} mobilization (Fig. 3).

(iv) Tyrosine phosphorylation of FcR-γ and Syk kinase: Previously, we and others have established tyrosine phosphorylation of multiple proteins within 10-20 s of IgE-receptor-mediated activation including the FcR-γ subunit ITAMs and Syk kinase as a prerequisite to degranulation (Paolini et al., 1991). Immunoprecipitation studies demonstrated tyrosine phosphorylation of FcR-γ subunits at ~14.3 kDa (Fig. 4) and Syk kinase at ~72kDa (Fig. 4) in response to antigen-mediated cross-linking of corresponding receptor subunits in the RBL-2H3, D194, D194T and D194N cell lines. In contrast, receptor activation of the D194L transfected cell line with NIP-HSA showed no evidence of tyrosine phosphorylation of either FcR-γ or Syk kinase. To determine if the block in signal transduction and subsequent Ca^{2+} mobilization was due to defective receptor function caused by the D194L mutation within the huFcεRIα TM domain of this variant, the tyrosine phosphorylation status of FcR-γ and Syk kinase was assessed following receptor-mediated activation. Receptor activation of D194L transfected cell line by NIP-HSA showed no evidence of tyrosine phosphorylation of either FcR- γ or Syk kinase suggesting a medium sized polar residue at position 194 in FcεRI facilitates receptor phosphorylation and subsequent signaling events leading to degranulation (Iodice, 2006).

Discussion

- RBL-2H3 cells when transfected with wild-type (D194) and mutated variants (D194N, D194T and D194L) of the huFcεRIα subunit expressed at the cell surface in association with endogenous FcR-β and FcR-γ subunits. D194, D194T and D194N, but not D194L transfected cell lines, evidenced robust tyrosine phosphorylation of FcR-γ subunits and Syk kinase, intracellular Ca^{2+} mobilization and degranulation in response to NIP-HSA. In contrast exchanging Asp with isoleucine (D194I), valine, (D194V) serine (D194S), alanine (D194A), glutamic acid (D194E), lysine (D194K) and arginine (D194R) failed to support cell surface expression of the mutant ligand binding domain. Interestingly we find that there is no correlation between IgE-mediated, antigen induced levels of mediator release and levels of receptor expression confirming earlier observations (Sayers et al., 1998). This rules out that a reduction in the numbers of the ligand binding domain of the receptor complex can account for the failure of the mutant receptor subunit to induce downstream signalling and degranulation.

Based on results obtained in the current study, a new structural model was developed to account for interactions in the TM domain between the D194 residue and FcR-γ subunits (Fig. 5). The model envisages the interaction between two FcR-γ subunit TM helical segments (residues 6-26) which are arranged in a parallel conformation and restrained by forming the disulphide bond between cysteine residues (C7) in each subunit. A third helix corresponding to the FcεRIα TM sequence (residues 181-189) was orientated against these two helices in order to optimize interactions. D194 is predicted to face into the interior of the three-helix bundle to form hydrogen-bonding interactions with T22 in both FcR-γ subunits. Since the FcR-β subunit is not essential for cell surface expression of human FcεRIα and endogenous FcR-γ subunits (Varin-Blank and Metzger, 1990), this model addresses only the interaction between the minimal essential receptor subunits involved in FcεRI-mediated signaling. The predicted interaction would stabilize all three residues in the interior of the TM helical bundle. The molecular model developed as a result of the current mutagenesis study differs significantly from that proposed previously (Farber and Sears, 1991), which suggested the negatively charged D203 (rat FcγRIIIα) homologous to D194 in the FcεRIα subunit, would interact with the positively charged K29 (FcR-γ). Our model predicts that K29 occurs in the relatively hydrophilic sequence RLKIQV and places

its location into the cytoplasmic rather than in the TM region. In addition, the earlier study assumed a transmembrane 1:1 stoichiometry (Farber and Sears, 1991), rather than a 2:1 association of FcR-γ and FcεRIα subunits, as envisaged in our model. Indeed, a recent study has implicated T22, along with other residues, in the formation of a central interface that is presumably involved collectively in interacting with the FcεRIα TM domain (Wines et al., 2006).

According to the model proposed the side-chain of residue 194 is placed in a closely packed central position at the interface between the transmembrane helices where there will clearly be severe restraints as to which side chains can be accommodated. This is consistent with the results which demonstrates that the residue at 194 be a polar residue of medium size (Asp, Asn, Thr) capable of making two separate hydrogen bonds with the Thr22 residue in the two FcR-γ subunits. The mutation to almost similar sized non-polar Ile (D194I) failed to exhibit surface expression as it is essentially different in polarity despite occupying a similar space as Asp. The polar Thr mutation (D194T) is able to maintain receptor expression and function while the almost similar sized non-polar valine (D194V) is disfavoured as it is incapable of hydrogen bonding. It is evident that only slightly larger polar residues Glu (D194E) and polar Lys (D194K) and much larger sized polar Arg (D194R) did not support cell surface expression, although like Asp, Asn and Thr, the residues Glu, Lys, and Arg should also be capable of making the predicted hydrogen bonds thus reflecting the critical nature of the packing restraints at this position. Similarly the smaller sized residues non-polar Ala (D194A) and polar Ser (D194S) failed to act as a suitable substitution at the 194 position as no surface expression was witnessed in the D194A and D194S transfected cells. It is possible that in all the above-mentioned mutations the correct assembly of the three transmembrane helices is compromised and thus correct assembly at the cell surface does not take place.

The D194N mutation maintains receptor function as TM and downstream signaling are not affected, possibly due to the fact that N shares similar properties with D, even though there is a charge difference. The signaling properties of the variant receptor subunit are most likely to be maintained by virtue of similar polar properties of the residues as it is expected that the polar Asp serves to stabilise the receptor complex by forming side-chain/side-chain inter-helical hydrogen bonding with the other subunits. Although the amide side chain of N does not ionize, it is polar and can act as a

hydrogen bond donor and acceptor, suggesting the importance of polarity, rather than charge, of TM residues in the initiation of downstream signaling events. In contrast, a previous study showed that mutation of D→N in the putative second TM domain of the C5 complement receptor (C5a) resulted in abrogation of intracellular signaling following transfection of the mutant receptor without affecting ligand binding (Monk et al., 1994).

D and L essentially differ in polarity, and the latter non-polar substitution at residue 194 is associated with a failure to transmit a cross-linking stimulus into a secretory response. The observed signaling defect could be due to a loss of polarity resulting from this substitution, compounded by a change in the size of the TM helix, which could affect the rotational and/or translational shift of the ligand binding subunit relative to other TM components that need to be in proper register to function co-operatively.

The model proposed for FcεRIα/γ chain interaction in the current study should be substantiated by probing the interaction with T22 in FcεRIγ. Using a variant of the RBL cell line described as FcεRIγ chain deficient, (gift of Prof. Draber, Univ of Prague) (Bocek et al., 1995) we still observed basal expression of the FcεRI complex in these cells rendering them unsuitable for verifying the predictions made by the model developed in our study (Rashid, 2009).

A more detailed understanding of the interaction between FcεRIα and FcεRIγ at the molecular level, may form the basis for the development of therapeutic agents aimed specifically at the inhibition of FcεRI-mediated cell signaling in allergic disease linked to mast cell desensitization and should guide the development of 'molecular wedges', analogous to anti-cancer drugs, which inhibit *neu* oncogene receptor aggregation or T-cell receptor signaling (Lofts et al., 1993; Manolios et al., 1997) and may have wider applications for the development of selective inhibitors of immunoreceptor-mediated pathologies.These core peptides are not only anchor proteins but appear to function by inhibiting downstream signalling by blocking receptor subunit interactions in the membrane. The most interesting aspects of these peptides is their ability to internalize to many cell types without any apparent side effects. (Huynh et al., 2003) suggesting they may form the basis for a new class of anti-allergic drugs.

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Abbreviations

The abbreviations used are: DNP-HSA, dinitrophenol-human serum albumin; FcR-β, FcεRI beta subunit; FcR-γ, FcεRI gamma subunit; IP, immunoprecipitation; huFcεRIα, human FcεRI alpha subunit; huIgE, human IgE; NIP-HSA, 4-hydroxy-3-iodo-5-nitrophenylacetyl-human serum albumin; PNS, post-nuclear supernatant; PVDF, polyvinylidene difluoride; RBL, rat basophilic leukemia; RIPA, radioimmunoprecipitation assay; Tac, IL-2 receptor α-chain; TM, transmembrane; WB, western blot.

Figure Legends

Fig. 1. *Assessment of cell surface receptor expression in RBL-2H3 cells transfected with gene constructs encoding wild-type and mutant huFcεRIα subunits.* Cells were harvested and labeled with human IgE, biotinylated goat anti-human IgE followed by streptavidin R-phycoerthrin. Cell populations were assessed for huFcεRI transfected receptor expression by flow cytometry. Figure A shows control for B, C and D. Figure E shows control for F, G and H. Figure I shows control for J and K.

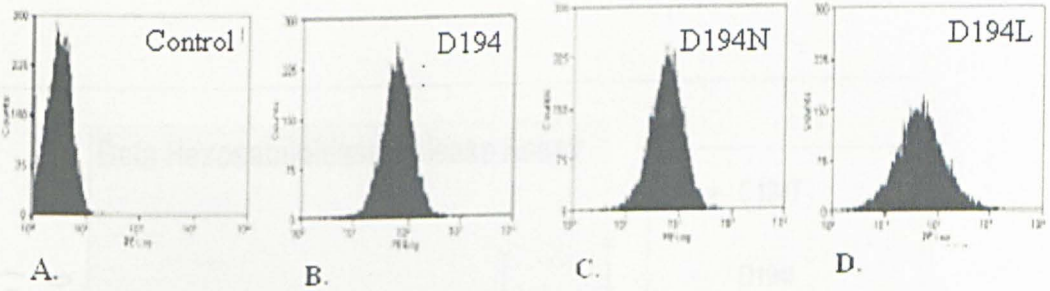
Fig. 2. *β-hexosaminidase secretion profiles of RBL-2H3 and transfected cell lines.* Cells were harvested, sensitized for 16 hours with DNP-specific mIgE or *NIP*-specific huIgE and activated with the appropriate cross-linking agent i.e. DNP-HSA or NIP-HSA (0.1-10000ng/ml) (16). Released β-hexosaminidase is expressed as a percentage of total β-hexosaminidase. Fig 2 shows cells activated through endogenous and transfected huFcεRIα receptors with DNP-specific mIgE/DNP-HSA and through transfected huFcεRIα receptors with NIP-specific huIgE/NIP-HSA. Data represent mean ± S.D. from three separate experiments performed in triplicate.

Fig. 3. *Ca^{2+} mobilization of RBL-2H3 and transfected cell lines in response to receptor activation.* Cells were sensitized with DNP-specific mIgE (1μg/ml) (A) and NIP-specific huIgE (1μg/ml) (B-J). IgE-primed cells were loaded with Fluo-3 AM (5μM) and analyzed on a Facscalibur flowcytometer (BD Biosciences) (see Materials and Methods). A base reading was obtained prior to the addition of the appropriate cross-linking agents (100ng/ml DNP-HSA, NIP-HSA or 10μM ionomycin). Data are representative of five separate experiments. (A) Cells sensitized with DNP-specific mIgE/ DNP-HSA as control (B) D194T (C) D194I (D) D194V (E) D194R (F) D194S (G) D194 (C) D194N (D) D194L (E) D194L sensitized with ionomycin (10μM).

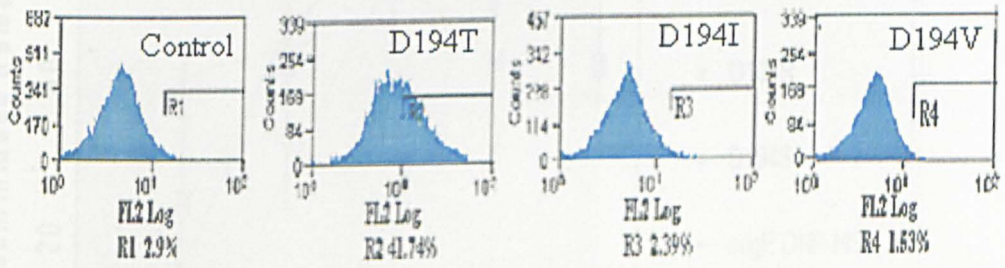
Fig. 4. *Tyrosine phosphorylation of FcR-γ and Syk kinase following aggregation of endogenous and transfected huFcεRIα receptors.* Cells sensitized overnight with DNP-specific mIgE (RBL-2H3) or NIP-specific huIgE (D194, D194N, D194T and D194L) were harvested and activated with the appropriate cross-linking agent i.e. DNP-HSA or NIP-HSA (100ng/ml) for 2 min. Cells were lysed in RIPA buffer and immunoprecipitation of the PNS (RBL-2H3 cells ~3mg/ml and huFcεRIα transfectants ~6mg/ml) was performed either with anti-phosphotyrosine (clone 4G10) or polyclonal anti-Syk antibodies. Immunoprecipitates were resolved by SDS-PAGE, transferred to PVDF membranes and probed with monoclonal anti-phosphotyrosine (clone 4G10), anti-Syk or polyclonal anti-FcR-γ antibodies as indicated. FcR-γ quantification was achieved by immunodetection of the PNS (taken prior to immunoprecipitation) with antibodies to the FcR- γ subunit. Data are representative of seven separate experiments.

Figure 5. *Modeling of transmembrane helices.* The transmembrane regions of both FcεRIα and FcR-γ subunits were identified from NCBI annotation and confirmed by hydropathy plots (Kyte and Doolittle, 1982) . A model was constructed from two FcR-γ subunit helices (linked by a disulphide bond between C7 in each FcR-γ subunit) and one helix of the Fc(RIα subunit (centre), all with inter-helical angles of approximately -25°. This aimed at exposing the majority of hydrophobic side chains on the outside of the three-helix bundle, so as to permit favorable interactions with lipid tail groups in the membrane. The model positioned D194 of the FcεRIα subunit in hydrogen bonding distance from T22 residues present in both FcR-γ subunits (indicated by arrows). This model was then subjected to energy minimization using the X-PLOR program (Brunger et al., 1987) in order to improve stereochemistry and to optimize contacts.

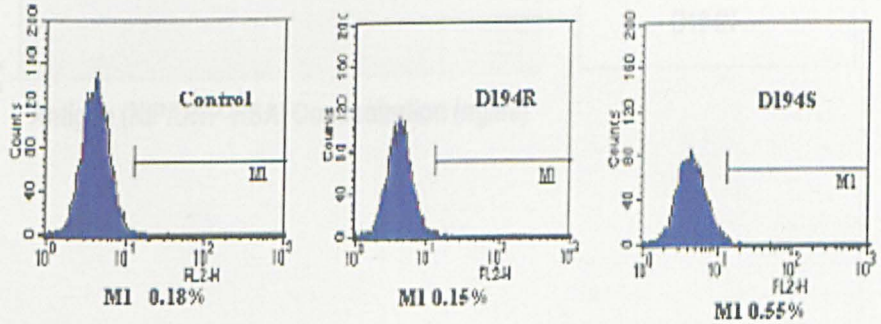
Figure1



A. B. C. D.



E. F. G. H.



I. J. K.

Figure 2

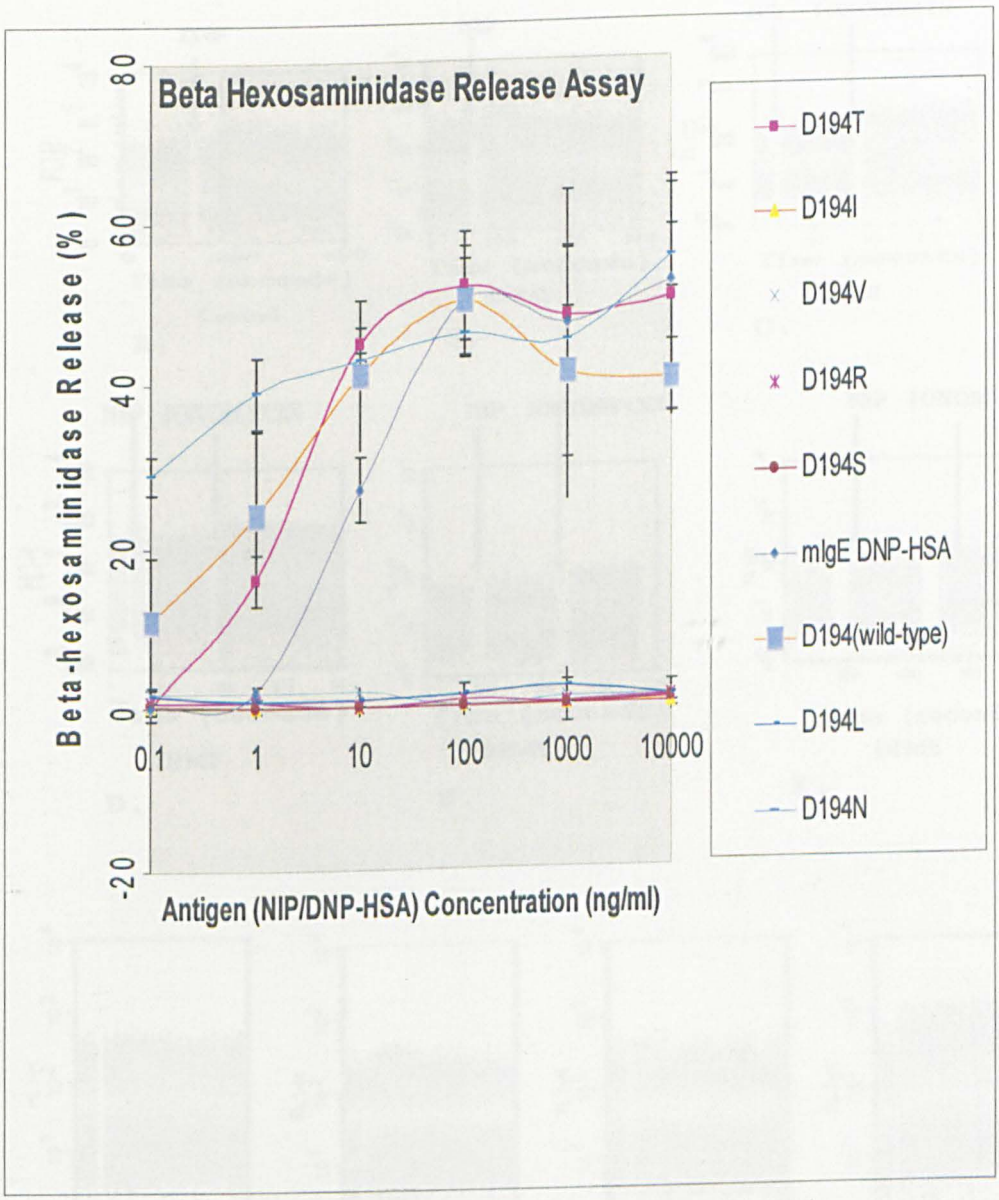
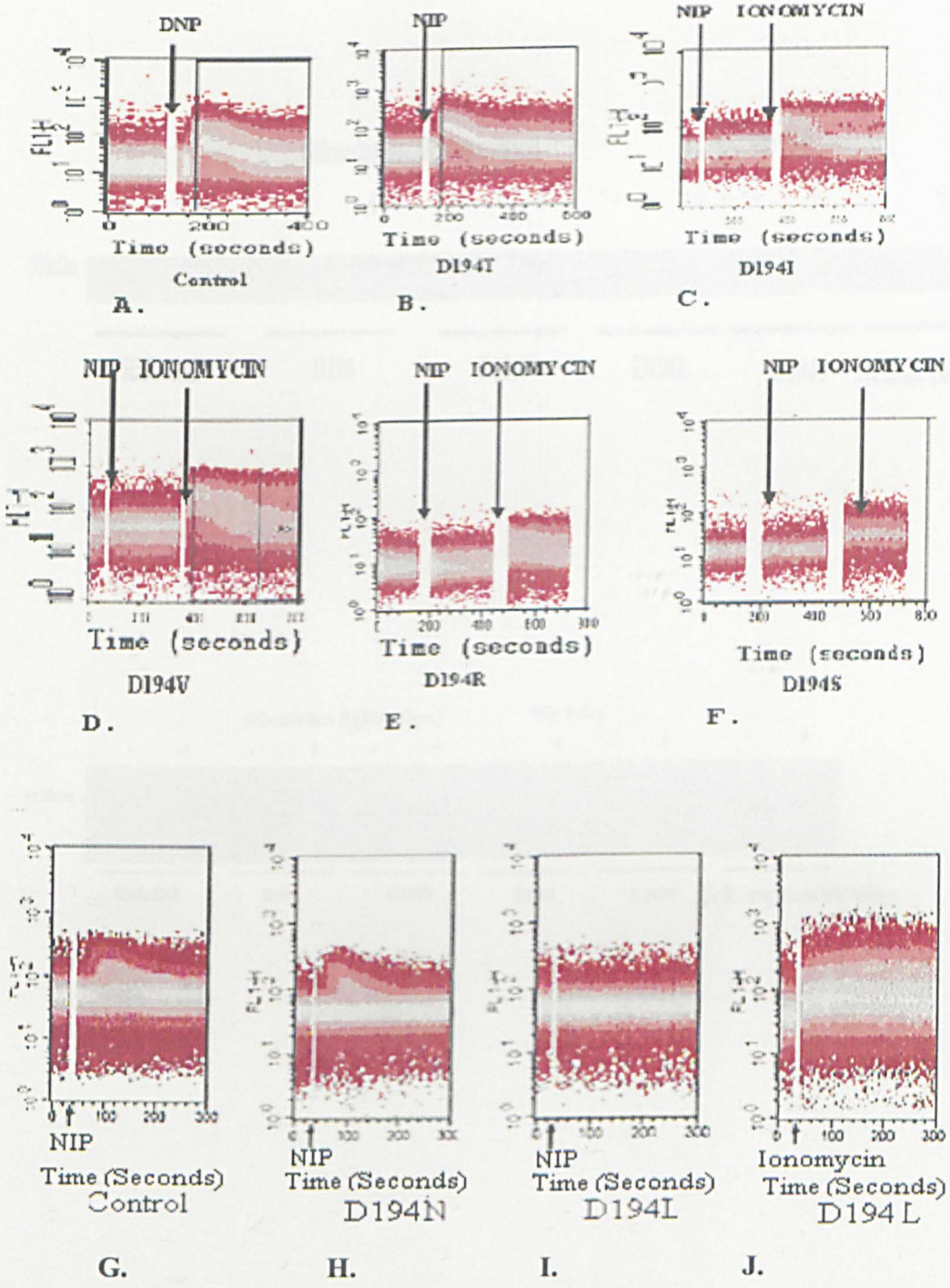

Beta Hexosaminidase Release Assay
Beta-hexosaminidase Release (%)
80
60
40
20
0
-20
0.1
1
10
100
1000
10000
Antigen (NIP/DNP-HSA) Concentration (ng/ml)
D194T
D194I
D194V
D194R
D194S
mIgE DNP-HSA
D194(wild-type)
D194L
D194N

Figure 3


DNP
Time (seconds)
Control
A.
NIP
Time (seconds)
D194T
B.
NIP IONOMYCIN
Time (seconds)
D194I
C.
NIP IONOMYCIN
Time (seconds)
D194V
D.
NIP IONOMYCIN
Time (seconds)
D194R
E.
NIP IONOMYCIN
Time (seconds)
D194S
F.
NIP
Time (Seconds)
Control
G.
NIP
Time (Seconds)
D194N
H.
NIP
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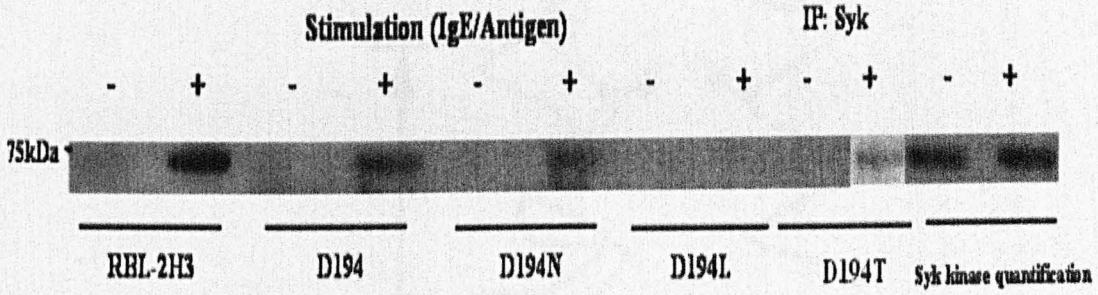
I.
Ionomycin
Time (Seconds)
D194L
J.

Figure 4


Stimulation (IgE/Antigen)
IP: Syk
- + - + - + - + - + - +
75kDa
RBL-2H3
D194
D194N
D194L
D194T
Syk kinase quantification

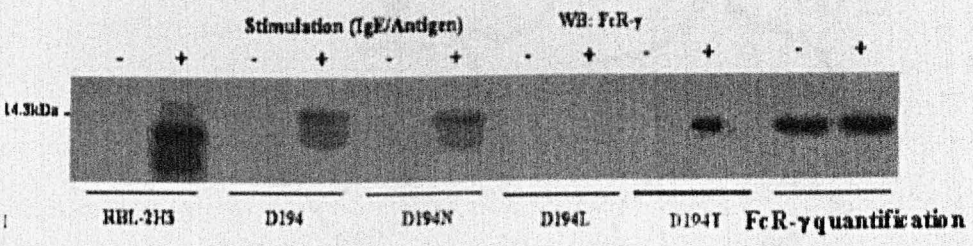

Stimulation (IgE/Antigen)
WB: FcR-γ
- + - + - + - + - + - +
14.3kDa
RBL-2H3
D194
D194N
D194L
D194T
FcR-γ quantification

Figure 5