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ABSTRACT

FcR, members of the immunoglobulin (Ig) superfamily, bind to specific Ig isotypes with varying affinities to trigger complex immune defense responses. The many functions of the three classes of FcR specific for IgG, FcγRI, FcγRII and FcγRIII, are thought to be a result of the rich structural diversity amongst these receptors. FcγRI, the focus of my studies, is comprised of a 72 kD ligand binding α chain noncovalently associated with γ chain, a 20 kD homodimerized signaling subunit that also associates with FcγRIIIa, FcεRI, and FcαR. FcγRI is distinguished from the other FcγR members by a unique third extracellular domain and high affinity for monomeric IgG. In this dissertation I propose that a novel role for γ chain is to enhance the affinity of FcγR for ligand. My findings demonstrate that FcγRI requires γ chain association to attain high affinity binding of monomeric IgG.

In addition, I analyzed the quaternary structure of FcγRI to test the hypothesis that γ chain augments receptor affinity by inducing homodimerization of the α chains. In this thesis I present evidence for homodimerization of the FcγRI α chains, a unique observation amongst FcR. Interestingly, the dimer seemed to form independently of γ chain association, suggesting that γ chain might stabilize the α chain dimer to enable
high affinity binding of IgG. The presence of a tetramer-sized complex, however, was
dependent upon γ chain association. Additionally, my findings indicated an
unidentified 30 kD molecule associated with the dimer and tetramer complexes,
suggesting that the complete quaternary structure of FcγRI remains to be defined.

The human FcγRI is encoded by three highly homologous genes (A, B, C)
resulting in six transcripts. In this dissertation I characterize the protein encoded by an
alternatively spliced transcript from gene B, FcγRI b2, in comparison to the bona fide α chain, FcγRI a1. I have shown that like FcγRI a1, the 45 kD b2 protein is present
in a human monocyte cell line and is capable of associating with the signal-generating γ subunit. An epitope-tagged FcγRI b2 fusion protein bound IgG despite being primarily localized intracellularly in transfected COS cells. The effects of the structural
differences between a1 and b2 on cell surface localization and ligand binding are
analyzed utilizing several a1/b2 chimeras. My findings give the first definitive
evidence that the FcγRI b2 transcript expresses a protein product which may have an alternate function from that of FcγRI a1.
This dissertation is dedicated with love and appreciation to my parents.
ACKNOWLEDGMENTS

While impossible to express my gratitude in its entirety, I wish to thank my adviser, Dr. Clark Anderson, for the opportunity, support and encouragement to develop as a scientist in his laboratory. With Dr. Anderson's patient guidance I have acquired an appreciation for the highest standards of scientific investigation that will serve me well in a career in science.

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LIST OF ABBREVIATIONS

cDNA complementary DNA
CYT cytoplasmic
EA antibody-coated erythrocytes
EC extracellular
Fab fragment antigen binding
Fc fragment crystallizable; constant domain of immunoglobulin
FcεR IgA FcR
FcγR IgG FcR
FcεR IgE FcR
FcR Fc receptor
GPI glycosyl phosphatidylinositol
hu human
IFN interferon
Ig immunoglobulin
ITAM immunoreceptor tyrosine-based activation motif
kb nucleotide kilobase pairs
kD kilodalton
mAb monoclonal antibody
MFI mean fluorescence intensity
MHC major histocompatibility complex
mu murine
PCR polymerase chain reaction
Ra rabbit
TM transmembrane
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CHAPTER 1

INTRODUCTION

The immune system functions as the body's defense mechanism by conferring resistance to foreign substances called antigens. Target antigens are bound by antibodies, which are highly specific components of the immune response found in the humoral, or fluid branch, of the immune system (1). The primary component of antibody molecules, also called immunoglobulin (Ig), is a tetrameric complex consisting of two identical polypeptide "heavy chains" and two identical polypeptide "light chains" (2, 3). The Ig classes and subclasses, or isotypes, are defined by their heavy chains. There are five Ig classes, IgM, IgD, IgG, IgA and IgE with the human IgG class further divided into four isotypes, IgG1, IgG2, IgG3 and IgG4 (4). The amino terminal domain of the heavy and light chains varies on each Ig, and thus is called the variable domain. Ig bind antigens through the variable region, also called the Ig Fab portion (2). The carboxyl terminal region of the heavy chains comprises the constant domain, or Fc portion of Ig. Specific cell surface proteins, called Fc receptors (FcR), bind to the Fc portion of Ig. Since the Ig molecule appears to function only in
binding to antigens, it is dependent upon interactions with FcR to stimulate effector systems (5). FcR thus provide the link between the cellular and humoral branches of the immune system.

There are FcR specific for each Ig class, including a family of receptors for IgG called FcγR (7) (Table 1.1). Like all FcR, FcγR belong to the immunoglobulin superfamily, of which Ig is the prototypic molecule (3). Members of the Ig superfamily have a common genetic organization, sequence homology, and similar three-dimensional structures (2, 8). There are three classes of FcγR: FcγRI, FcγRII and FcγRIII (1, 7, 9-11). The FcγR classes are further defined by their cell type distribution, structure, ligand binding affinity, and class specific binding to IgG subtypes. The ligand-binding α chains of most FcγR are integral membrane proteins that have glycosylated extracellular domains, a short transmembrane domain and a variable cytoplasmic domain. Like all FcR, the extracellular domains of FcγRII contain Ig-like folds consisting of two antiparallel β-sheets linked by a disulfide bond (10). After binding the antigen-antibody complexes, also referred to as immune complexes, FcγR cluster and trigger a variety of protective responses including phagocytosis, endocytosis, antibody-dependent cytotoxicity, release of inflammatory mediators, enhancement of antigen presentation, regulation of B cell activation, and participation in T cell development (12-29).

The diversity of functions triggered by the single ligand IgG has been attributed to the complexity of structure among FcγR (30, 31). Each receptor in this class is represented by multiple genes and transcripts as well as different isoforms and
associated subunits. To gain a better understanding of the specific roles FcγR have in
the immune system characterizing the structure-function relationships of these receptors
is necessary. While scientists have known about the actions of antibodies and FcR for
more than thirty years (32, 33), it has only been within the last ten years that the FcγR
genes have been isolated and their protein products characterized. Recently there have
been increased efforts to define the molecular interactions of FcγR ranging from the
binding of Ig to associations with signal transducing molecules. The primary focus of
my studies has been structural analysis of the high affinity receptor for IgG, FcγRI.

While FcγR are one of the most extensively studied FcR classes, the
characterization of FcγRI is still ongoing. When I began my graduate studies, the
FcγRI genes had just been isolated by our laboratory (34). Subsequently, several
transcripts have been identified (35) and FcγRI has been shown to associate with a
subunit necessary for receptor function (36). In this chapter I will present the progress
in the characterization of FcγRI leading up to my current studies, focusing primarily on
the relationship of receptor structure to function.

FcγRI

FcγRI Distribution and Affinity

The ligand-binding α chain of the human FcγRI is a 72 kD integral membrane
glycoprotein consisting of a 55 kD core protein and six N-linked carbohydrates (1, 37,
38). This receptor is unique from the other members of this class in having three Ig-
like extracellular domains while FcγRII and FcγRIII each have only two such domains.
FcγRI is expressed constitutively on monocytes and macrophages, is inducible on neutrophils and eosinophils (10, 39, 40), and is significantly upregulated by the cytokine IFN-γ (34, 41-45).

FcγRI shows isotype specificity for IgG (46), binding human IgG3 and IgG1 with high affinity, IgG4 with reduced affinity, and binding virtually no human IgG2. This receptor also shows isotype specificity for murine IgG, which is often used in studies with FcγR because it is readily available. FcγRI binds murine IgG2a and IgG3 with high affinity, but binds poorly to murine IgG1 and IgG2b. A defining characteristic of FcγRI is its high affinity for monomeric IgG (47, 1, 5). FcγRII and FcγRIII primarily bind immune complexes, having low affinity for monomeric IgG (1, 5). The sites on IgG required for high affinity binding by FcγRI have been studied at the molecular level (48-51). Two regions on IgG within the C\(_{H2}\) domain have been identified as sites crucial for high affinity binding by FcγRI (52, 53). The hinge-link region of both human IgG1 and IgG3 contains two residues, Leu(234) and Leu(235), that appear to be essential for FcγR binding. Substitutions at these sites result in decreased binding by 10-100-fold. Similarly, the hinge-proximal region of the C\(_{H2}\) domain in IgG1 and IgG3 contains a crucial Pro(331) residue whose substitution reduces receptor binding by 10-fold. Although these two sites are separated by almost 100 residues, a β sheet formation in the C\(_{H2}\) domain brings the Proline residue to within 11 angstroms of the hinge-link region (52) suggesting that these two regions could form a single binding site. Interestingly, substitution of the nearby Ala(339) to
Thr in IgG1 increases the affinity binding of FcγRI (54). Since each heavy chain of the Fc portion contains this FcγRI binding site, IgG is a potentially bivalent molecule (2).

Since a major structural difference between the α chain of FcγRI and the other members of the FcγR class is the third extracellular domain, the high affinity binding of FcγRI for monomeric IgG \((K_a=10^{9-9} \text{M}^{-1})\) (46, 55) has been attributed to the presence of this third domain. However, human and murine FcγRI α chains transfected into COS cells bind ligand with an affinity five-fold lower \((K_a=5.0\times10^7 \text{M}^{-1})\) than that measured on cells that naturally express the receptors (56, 57). These results suggested that some factor(s), present in the cells that naturally express FcγRI and absent in COS cells, confers the high affinity binding. One potential candidate is a protein shown to associate with FcγRI, the γ chain subunit. Chapter 2 investigates the effect of γ chain association on the affinity of FcγRI for IgG.

**FcγRI Associates with γ Chain Subunit**

The FcγRI α chain does not contain any known signaling motifs in its cytoplasmic domain, and thus relies upon an associated subunit to aid in triggering cell responses. FcγRI noncovalently associates with γ chain (36, 58), a signal generating subunit of 10 kD that forms disulfide-linked homodimers. Located on chromosome 1 (59, 60), the gene for γ chain spans 4 kb and has five exons encoding a protein that has a short five amino acid extracellular domain, a single transmembrane domain, and a cytoplasmic domain containing the immunoreceptor tyrosine-based activation motif (ITAM) sequence required for signal transduction (61-63). This subunit also associates
with the α chain of the FcγRIIIa isoform, the high affinity IgE FcR (FceRI) and the IgA FcR (FcαR) and is required by these receptors for their functions (64-70). While γ chain association is essential for cell surface expression of FcγRIIIa (71) and FceRI (72, 73), the α chains of both FcγRI and FcαR can be surface expressed in COS cell transfections without γ chain association (56, 69). Interestingly, FcγRI in mice containing a disrupted γ chain gene do not bind ligand, suggesting the murine receptor is dependent upon γ chain association for cell surface expression in native cells (74). Clustering of the receptors by binding immune complexes triggers the initiation of transmembrane signaling by inducing tyrosine phosphorylation of several cellular proteins, including the γ chain subunit and several tyrosine kinases (75-81). Since FcγRI has no intrinsic protein kinase activity, it requires association with non-receptor tyrosine kinases. After FcγRI binds ligand and is clustered, γ chain is phosphorylated and interacts with the non-receptor protein tyrosine kinase Syk, which is also phosphorylated after receptor clustering (82). The Src kinase Lyn associates both with FcγRI and γ chain in the receptor complex, and shows increased phosphorylation after FcγRI clustering (83).

**FcγRI Structure**

Before it was known that some receptors contained multiple subunits, references made to "FcγR" were primarily alluding to the ligand-binding α chains of the receptors. In many contexts "FcγR" is still synonymous to "the α chain". More frequently, however, the term FcγR encompasses all polypeptide chains comprising the functional receptor including the α chain and any associated subunits. The complete
quaternary structure of FcγRI and the manner in which its components interact continues to be defined. Due to high affinity binding of ligand as well as association of the α chain with γ chain, FcγRI is often structurally compared to the high affinity receptor for IgE, FcεRI. It is theorized that FcγR and FcεR have evolved through duplication of a common gene (9), and indeed these receptors share homology in both their genes, predicted α chain structures, and associated subunits. FcεRI, which is expressed upon mast cells, basophils and Langerhans cells, functions to initiate the allergic response (84, 67, 72, 73). FcεRI is a tetrameric structure composed of a ligand-binding α chain, a β chain, and the same γ chain homodimer that also associates with FcγRI. The α chain, a 40-50 kD integral membrane glycoprotein with seven sites for N-linked glycosylation (85), is the ligand binding subunit of FcεRI. The α chain has two Ig-like extracellular domains that share homology with two of the three extracellular FcγRI domains. FcεRI, like FcγRI, relies upon associated protein subunits to transmit cell signaling. Besides functioning in signal transduction, the γ and β chain subunits are also required for cell surface expression of the FcεRI α chain. While it was determined that the murine FcεRI α chain requires association with both the γ and β chains for cell surface expression, the human FcεRI requires only γ chain to permit expression at the cell surface (67, 72, 73). Like FcγRI, the FcεRI binds its ligand IgE with high affinity (K_a=10^{10} M^{-1}) (84). Although solubilized extracellular domains of the FcεRI α chain have been shown to form dimers (86), other studies suggested that the stoichiometry of the FcεRI to be one α, one β and two disulfide-linked γ chain subunits (87).
The stoichiometry of the FcγRI components has yet to be determined. While the α chains of many cell surface receptors are known to dimerize (88, 89), there has been no report for receptor dimerization among FcR belonging to the Ig-superfamily. There is, however, some evidence for dimerization of an IgG receptor. The human homologue of the neonatal rat Fc receptor, FcRn, is distinct from other FcγR because it is structurally related to MHC Class I molecules (90). This receptor is involved in IgG transport and has been postulated to have a role in regulating the catabolism of IgG (91, 92). FcRn binds IgG in a pH dependent manner, binding with high affinity at pH 6, and with little affinity at physiological pH 7.5 (93, 94). FcRn associates with β2-microglobulin, a subunit that also associates with MHC Class I molecules (90), forming a complex in which two receptor heterodimers bind a single IgG ligand (95-97). In chapter 3 the quaternary structure of FcγRI is studied to determine if the α chains of this receptor dimerize to confer high affinity binding of IgG.

**FcγRI Genes**

The human FcγRI α chain is encoded for by three genes, A, B, and C (34, 35). These genes were isolated from a human genomic library and defined in restriction enzyme site analysis. Compared with the A gene, the C gene is missing an NcoI site while the B gene lacks the same NcoI site as well as a HindIII site. The three FcγRI genes localize to chromosome 1 (35, 98-100) on bands 1p12 and 1q21 flanking the centromere (101). These genes have greater than 98% homology at the nucleotide level and are believed to be products of gene duplication. The 5′-flanking region of the genes contains two major transcription initiation sites. The first site has upstream
modified CAAT and TATA boxes, while the second, more upstream site is utilized only after cells have been incubated with IFN-γ (34). The FcγRI genes span 9.4 kb and contain six exons (34, 35). As with all FcγR, the splice junctions of the exons occur between the first and second nucleotide of an amino acid codon (34, 39). The leader peptide of the three genes is encoded in two exons, S1 and S2. The S1 domain contains the 5'-untranslated region and sequence for a portion of the leader peptide responsible for directing the peptide to the endoplasmic reticulum (ER) during translation. The S2 domain, only 21 bp in length, encodes the signal peptidase cleavage site, predicted to be between glycine-15 and glutamine-16. This exon is highly conserved and a similar miniexon has been described for all FcγR classes (102, 103). Each Ig-like domain (EC) is encoded on a separate exon. While the EC1 and EC2 domains share homology with the EC domains of FcγRII and FcγRIII, the EC3 domain is unique to FcγRI. The transmembrane and cytoplasmic domains of the FcγRI genes are encoded by a single exon (TM/C). The nucleotide sequence predicts a short 21 amino acid transmembrane domain and a highly charged cytoplasmic domain of 61 amino acids.

The FcγRI genes B and C have several nucleotide changes from gene A occurring in the exons (35). In the EC2 domain of gene B, a nucleotide change mutates a Thr to a Met, while in gene C nucleotide mutation changes a Leu to a Pro, and an Arg to a His. Genes B and C also share several differences from gene A. In the EC2 domain just upstream of the EC3 domain border, three nucleotides are replaced with six different residues mutating a Val to a Gln and Tyr. The most distinct
structural difference between the FcγRI genes is that while the gene A predicts a three
EC integral membrane protein, genes B and C contain nucleotide changes in their EC3
domain that result in premature stop codons predicting soluble receptors. Gene B has a
nucleotide difference that mutates a Gln to a termination codon, while the gene C has a
nucleotide deletion that produces an in-frame termination codon shortly downstream of
the mutation.

**FcγRI Transcripts**

Named for their genes, six FcγRI α chain transcripts, a1, a2, b1, b2, b3, and c,
have been characterized (35, 104) (Fig.1.2). Containing all six exons, the 1.1 kb a1
transcript encodes a three extracellular (EC) domain membrane-spanning receptor
believed to represent the putative bona fide FcγRI. The a2 transcript is 0.87 kb
splicing out both the S2 and EC1 exons. The 0.6 kb b3 transcript, a product of the B
gene, also splices out the S2 and EC1 domains in addition to the unique third EC
domain. Since the S2 domain contains a portion of the leader sequence containing the
signal peptidase sequence, these receptors would likely be anchored in the cell
membrane at both the amino terminus and the transmembrane domain. This could
suggest that the a2 and b3 isoforms are not functional and instead represent
pseudogenes. Both b1 and c are full length 1.1 kb transcripts that contain gene-specific
premature stop codons in EC3. The predicted receptors would contain no
transmembrane or cytoplasmic domains, and therefore could not be membrane bound.
While these isoforms do not represent classical cell surface FcγR, there is precedence
for such receptors. There is a soluble FcγRII splice variant, FcγRIIa2, that splices out
its transmembrane domain (105, 106), and soluble FcγR have also been described in human serum (107, 108). A final transcript from gene B, the 0.87 kb b2, splices out the third extracellular domain of FcγRI, thus eliminating the premature stop codon in EC3. The sequence of this isoform predicts a two extracellular transmembrane bound FcγR structurally similar to the other FcγR classes, FcγRII and FcγRIII.

The FcγRI isoforms currently under much investigation are a1 and b2. RNAse protection analyses have indicated that a1 and b2 are the most abundant FcγRI transcripts in U937 cells, a human monocyte cell line that naturally expresses FcγRI, suggesting the potential for functional receptors (35). Since the sequence of the a1 isoform is very similar to the previously characterized FcγRI cDNA p135 (56), it seems likely that a1 is the prototypic high affinity FcγRI. The two extracellular domain b2 isoform is intriguing because of its predicted structural similarity to the low affinity FcγR classes. Since the EC1 and EC2 domains of the FcγRI α chain share high homology with the corresponding domains of both the FcγRII and FcγRIII α chains (6, 9), this predicts an isoform of FcγRI that might bind IgG with the same affinity and isotype specificity as these receptors. In support of this speculation, a previous study reported that a murine FcγRI/FcγRII chimera containing the EC1 and EC2 domains of FcγRI and the transmembrane and cytoplasmic domains of FcγRII was expressed and had similar binding specificity for FcγRII when transfected in COS cells (109). Of the six FcγRI transcripts, the a1 and b2 transcripts appear to hold the most promise for representing functional FcγR and have become the focus of our studies.
The size of the protein product of α1 as characterized by *in vitro* translation assays was approximately 70 kD (104), the expected size for FcγRI. Anti-FcγRI monoclonal antibodies (mAbs) also immunoabsorbed a 70 kD protein from radioiodinated COS-7 cells transfected with FcγRI α1 cDNA. Additionally, FcγRI α1 transfected into COS cells bound the appropriate IgG isotypes in rosetting assays. Together these results indicated that the FcγRI α1 isoform has the expected size and same basic functions of FcγRI.

The size of the protein product of β2 as characterized by *in vitro* translation assays was approximately 45 kD, similar in size to FcγRII. Interestingly, although RNAse protection analyses of COS cells transfected with β2 cDNA indicated an abundance of β2 transcript, no protein was detected after immunoabsorption of radioiodinated transfected cells with anti-FcγRI mAbs. This preliminary data suggested that the β2 protein was not recognized by FcγRI mAbs. Since FcγRII b2 represents such an intriguing isoform, chapter 4 describes the continued characterization of this receptor including analyses of the structural differences between the α1 and β2 isoforms.

*Other FcγR Members*

Binding to IgG is a major function of FcγR that has been analyzed at the molecular level with both FcγRII and FcγRIII (7, 10). These studies have identified specific amino acid stretches crucial for the binding of ligand. Additionally, there is an FcγRII polymorphism identified in which a single amino acid residue difference dramatically affects ligand binding. Although detailed analyses of the structural
requirements for FcyRI ligand binding have yet to be reported, chapter 4 addresses this aspect of the receptor. Since the members of the FcyR class share both structural and functional homology, findings related to FcyRII and FcyRIII may be relevant to FcyRI studies. Therefore, brief descriptions of these FcyR have been included in this chapter.

**FcyRII**

FcyRII is a 40 kD integral membrane glycoprotein expressed on a wide variety of immune cells including monocytes, macrophage, neutrophils, eosinophils, basophils, Langerhans cells, B cells, platelets, and a subpopulation of T cells (110). FcyRII, which has two extracellular Ig-like domains, has low affinity for monomeric IgG (Ka < 10^7M^-1) but binds immune complexes efficiently, showing isotype preference for human IgG3, and equivalent binding specificity for murine IgG2a and IgG2b. Located on chromosome 1q23-24, three genes, A, B, and C, encode FcyRII. Alternative splicing results in transcripts encoding six isoforms with highly homologous extracellular domains and divergent cytoplasmic domains (1, 111). There are two products of the A gene, FcyRIIa1 and FcyRIIa2. FcyRIIa1 has an ITAM sequence within its cytoplasmic domain, making this receptor unique since most other FcR have separate signaling subunits. FcyRIIa2, a product of an alternatively spliced transcript in which the transmembrane domain has been removed, is a soluble receptor. Three highly homologous isoforms are produced by the B gene. Except for an additional 19 residues in its cytoplasmic domain, FcyRIIb1 is identical to FcyRIIb2. Similarly, FcyRIIb3 is nearly identical to FcyRIIb2 except for a deletion of 21 residues in its
cytoplasmic domain. The FcγRII C gene appears to be a result of a crossover event between the A and B genes, and encodes a receptor containing the extracellular domains of FcγRIIb and the cytoplasmic domain of FcγRIIa.

Due in part to its wide cellular distribution and self-contained signaling sequences, the structure of the FcγRIIa1 isoform has been extensively studied. FcγRIIa mutants and chimeras were utilized to determine that the receptor binding site for IgG resides in the EC2 domain close to the interface of EC1 and EC2 (112-114). The major binding site has been localized to a stretch of eight amino acids spanning Asn 154-Ser 161. Two additional regions important for IgG binding are Ser 109-Val 116, and Phe 129-Thr 135. Some individual amino acid substitutions in these regions, especially within the Asn 154-Ser 161 stretch, result in loss of ligand binding, while others actually increased ligand binding.

The FcγRIIa1 isoform also has a genetic polymorphism characterized by a single amino acid difference that dramatically affects ligand binding affinity (115-117). The FcγRIIa-R131 form has an Arginine at position 131, and is characterized by strong binding to murine IgG1, and poor binding to human IgG2. The FcγRIIa-H131 form has a His at position 131 resulting in poor binding of murine IgG1 and strong binding to human IgG2. Many amino acid substitutions at this site result in loss of IgG binding, indicating the importance of this residue in ligand binding. It has been speculated that this FcγRII polymorphism might influence susceptibility to diseases in which the human IgG2 is the predominant antibody isotype, and in vitro the phenotype of FcγRII on platelets affects the response to heparin-induced thrombocytopenia (118).
These studies indicate that the EC2 domain of FcγRII participates in binding IgG, and regions in this domain shown to be important for ligand binding are sensitive to even single amino acid substitutions.

**FcγRIII**

FcγRIII is a 50-80 kD glycoprotein expressed in monocytes, macrophages, NK cells, and a subpopulation of T cells (119). The size differences observed with this receptor have been attributed to differences in N-linked glycosylation (10). FcγRIII is encoded by two genes, A and B, located on chromosome 1q23-24. FcγRIIIa, a product of the A gene, requires association with either γ chain or the closely related ζ chain to enable cell surface expression (62, 64, 120). In mast cells, FcγRIIIa is found associated with both the γ and β chains of the FceRI complex (121). The transmembrane domains of both FcγRIIIa and FceRI have an identical stretch of ten consecutive amino acids (122). It has been proposed that this stretch of residues results in retention in the endoplasmic reticulum, and association with γ chain masks this site to allow surface expression of the receptors. FcγRIIIa binds ligand with a "medium" affinity ($K_a = 2 \times 10^7 M^{-1}$) (1) that is higher than FcγRIIIb or FcγRII, but lower than FcγRI. FcγRIIIa shows isotype specificity and binds human IgG1 and IgG2 efficiently while binding IgG3 and IgG4 inefficiently. In order of decreasing affinity, FcγRIIIa has specificity for the murine isotypes IgG3 > IgG2a > IgG2b > IgG1. Although not as well defined as in FcγRII, the EC2 domain is also proposed to contain the IgG binding site for FcγRIII. A stretch of eight highly hydrophilic amino acids predicted to lie in a loop between two β strands appear to be crucial for FcγRIII binding of IgG (123).
The product of the B gene, FcγRIIIb, is the only cell surface localized FcγR that is not an integral membrane protein (124, 125). This receptor is attached to the outer leaflet of the cell membrane via a glycosyl-phosphatidylinositol (GPI)-linkage. This receptor binds monomeric IgG with low affinity ($K_a < 10^7 M^{-1}$). FcγRIIIb has equivalent isotype specificity for human IgG1 and IgG2, but has low affinity for IgG3 or IgG4, and binds murine IgG with the same specificity as FcγRIIIa. Although the gene for FcγRIIIb contains sequence for both a transmembrane and cytoplasmic domain, a single amino acid (Ser 203) in the EC2 domain of FcγRIIIb results in the receptor being GPI-linked (71, 126, 127). Similar to FcγRII, the IgG binding site(s) in FcγRIII appears to reside in the EC2 domain of the receptor (128, 129).

**Aims of the Dissertation**

In summary, FcγR trigger a variety of protective immune responses after binding IgG. Although as members of the Ig-superfamily FcγR share a certain homology, it is thought that the structural heterogeneity among these receptors accounts for the diversity of functions. Therefore, to better understand the roles of these receptors in the immune system it is crucial to dissect the effects of FcγR structure on function. FcγRI is perhaps the least characterized member of the FcγR class. At the initiation of my studies, the three FcγRI genes had been identified (34, 35), and the six transcripts were in the midst of being analyzed (104). Also during this time a signaling subunit, γ chain, was shown to associate with FcγRI (36) and become phosphorylated upon receptor activation (83). The objectives of my studies were to further characterize the structure-function relationships of FcγRI. Chapter 2 analyzes the
relationship of the FcγRI association with γ chain as it relates to receptor affinity for IgG. After results from this study led to postulations that γ chain association altered receptor structure, the quaternary structure of FcγRI was analyzed as reported in chapter 3. The characterization of the FcγRI isoforms is continued in chapter 4 focusing on analysis of the FcγRI b2 isoform. Additionally, this chapter examines the structural requirements for ligand binding and cell surface expression of FcγRI.
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1.1 Summary of FcγR characteristics

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Table 1.1: Summary of FcγR characteristics. T, transmembrane; S, soluble, G, glycosyl phosphatidylinositol-linked; Ka, equilibrium association constant; kD, kilodalton; hu, human; mu, murine.
## 1.2 LIST OF FIGURES

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Figure 1.1: Structure of an immunoglobulin G (IgG) molecule. H, heavy chain; L, light chain; V, variable domain; C, constant domain; S-S, disulphide bond.
Figure 1.2: Diagram of six FcγRI transcripts. The nucleotide sizes of the coding regions derived from the gene exons are depicted above diagrams representing the transcripts. S, signal sequences; EC, extracellular domains; TM/C, the transmembrane/cytoplasmic region. Dashed v-shaped lines indicate alternative splicing of the coding regions. Dotted straight lines indicate 3' untranslated regions. a1, a2, FcγRI gene A transcripts; b1, b2, b3, FcγRI gene B transcripts; c, FcγRI gene C transcript; *, nucleotide differences that result in amino acid changes; *, deletion resulting in a premature termination codon; ATG, translation initiation codon; TAG, TGA, translation termination codons.
1.3 LIST OF REFERENCES


58. Scholl, P.R., and R.S. Geha. 1993. Physical association between the high-affinity IgG receptor (FcγRI) and the γ subunit of the high affinity IgE receptor (FceRIγ). *Proc. Natl. Acad. Sci.* 90:8847-8850.


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A NOVEL ROLE FOR THE Fc RECEPTOR γ SUBUNIT: ENHANCEMENT OF FcγR LIGAND AFFINITY

Kathy Miller, Anne-Marie Duchemin, and Clark Anderson
2.1 INTRODUCTION

The Fc receptors (FcR), binding the Fc region of Ig, provide an important link between cellular and humoral branches of the immune system by triggering several immune responses including phagocytosis, endocytosis, antibody-dependent cytotoxicity, release of inflammatory mediators, and enhancement of antigen presentation upon immune-complex mediated clustering (1,2). Three classes of receptors for IgG (FcyR) have been identified - FcyRI, FcyRII and FcyRIII - and all but one are integral membrane proteins constituted of glycosylated extracellular Ig-like domains, a short transmembrane domain and a cytoplasmic domain. FcyRIIIb is not a transmembrane receptor, being anchored to the membrane by a glycosyl phosphatidylinositol (GPI) link. FcyRI is distinguished from the other FcyR members in having a unique third extracellular domain while FcyRII and FcyRIII are two extracellular domain receptors. FcyRI and FcyRIIIa both associate with γ chain, a 20 kDa disulfide-linked homodimeric signal-generating subunit that associates also with the high affinity IgE FcR (FceRI) and the IgA FcR (FcαR) (3,4,5). In addition to functioning as a signal transducing subunit, γ chain also has a role in the cell surface expression of some of these receptors; for example, FceRI and the FcyRIIIa isoform require association with γ chain for expression in COS cells (6,7). Expression of FcyRI and FcαR in eukaryotic cells, however, is independent of γ chain cotransfection (8,9).
FcγR bind IgG with different affinities and show class specific binding to IgG subtypes. A defining characteristic of FcγRI is its high affinity for monomeric IgG, while FcγRII and FcγRIIIb bind primarily immune complexes, having low affinity for monomeric ligand. The FcγRIIIa isoform binds monomeric IgG with an intermediate affinity, lower than FcγRI, but higher than FcγRIIIb and FcγRII (10). Although the high affinity binding of FcγRI for monomeric IgG (Ka = 10^{8.9} \text{ M}^{-1}) (11,12) has been attributed to the presence of the third extracellular domain (8,13,14), FcγRI transfected into COS cells binds ligand with an affinity five-fold lower (Ka of 5.0 \times 10^{7} \text{ M}^{-1}) than that measured on cells that naturally express the receptor (8,14). These results suggest that some factor(s), present in the cells that naturally express FcγRI and absent in COS cells, participates in the high affinity binding. Among these factors one potential candidate is the γ chain subunit. In the present study, we demonstrate that the affinity of both the human and the murine FcγRI increases when γ chain is cotransfected with these receptors, and that the affinity increase correlates with the association with γ-chain and is independent of the cytoplasmic domain of FcγRI. We also observe that the intermediate affinity of FcγRIIIa appears to be dependent upon γ chain association.
2.2 MATERIALS AND METHODS

Cells and Cell Culture. U937, a human monocyte cell line from ATCC, and N10F7, a mouse 3T3 fibroblast cell line stably transfected with human FcγRI cDNA, were maintained in RPMI 1640 (Life Technologies, Gaithesburg, MD, U.S.A.) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, U.S.A.), 2mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. COS-7 cells were cultured in Dulbecco's Modified Eagle Medium with the supplements listed above. The N10F7 medium also contained 0.5mg/ml G418 (Life Technologies). U937 cells were cultured with 100 U/ml human recombinant IFNγ (Genentech, San Francisco, CA, U.S.A.) 48 hours before each experiment to increase FcγRI expression.

Plasmid Constructs and Transfections. The human FcγRI cDNA cloned into the pCDM vector, was described by Ernst et al (3). The human γ-chain cDNA in vector pSVL was a kind gift from Dr. Jean-Pierre Kinet (National Institute of Allergy and Infectious Diseases, Bethesda, MD, U.S.A.). FcγRIIib, FcγRIIa, FcγRIIIa GPI, and murine γ chain cDNAs were all generous gifts from Dr Jeffrey V. Ravetch (Memorial Sloan-Kettering Cancer Center, New York, N.Y., U.S.A.). FcγRIIIa and FcγRIIib cDNA were further subcloned in the vector pcDNA1 (Invitrogen, San Diego, CA, U.S.A.). The murine FcγRI cDNA was a kind gift from Dr. Duane W. Sears (University California-Santa Barbara, CA,
U.S.A.), the human macrophage mannose receptor cDNA in the vector pHMR was from Dr. Benjamin A. Kruskal (Children's Hospital, Boston, Ma, U.S.A.) and the pRSVCAT vector was from Dr. Lai-Chu Wu (The Ohio State University, Columbus, OH, U.S.A.). A truncated human FcγRI, designated FF, was constructed by PCR amplification using FcγRI cDNA as a template, and truncation confirmed by sequencing. The FcγRI sense primer, containing a stop codon at the predicted amino acid residue 319, was 5'-GAC AAT ACG TAA AGA ACT GAA ATG AAA GAA-3', and the antisense primer was 5'-ACT GAG CCG CTG CTA CGT GG-3'. The cDNAs were transiently transfected into COS-7 cells by the diethylaminoethyl-dextran method as described previously (3) using 1-2 μg/ml of DNA, and the cells were analyzed 48 hours after transfection.

**Ligand Binding Assays and Scatchard Analysis.** Approximately 5 x 10⁵ COS-7 cell transfectants or N10F7 cells per well were cultured in 24 well plates overnight to confluence. Cells were incubated in triplicate for 2 to 4 hours on ice with 0.15 ml of increasing concentrations (0.5 to 10 or 30 μg/ml) of monomeric ¹²⁵I-IgG2a labelled with an efficiency of 95% by the chloroglycouril method. After the cells were washed to remove unbound ¹²⁵I-IgG, they were solubilized in 0.5 ml 3N NaOH and radioactivity measured with a gamma scintillometer. For cells in suspension, unbound ¹²⁵I-IgG was removed by centrifuging the cell suspension through oil (bis(2-ethylhexyl)phthalate: dibutylphthalate::1:1.1) as previously described (11). The nonspecific binding was determined by the amount of radioactivity binding to wells containing mock transfected COS-7 and untransfected 3T3, or by incubating the COS-7, N10F7 transfectants, and U937 with radioligand in the presence of 100 fold excess unlabeled IgG2a. Both methods
of determining nonspecific binding resulted in background counts that represented 0.1-
0.3% of the input of radioactivity. Binding data were analyzed using the Collection of
Radioligand Binding Analysis Programs (Elsevier-BIOSOFT, Cambridge, U.K.). Results
were expressed as the mean ±SEM of 3 to 13 experiments, and statistical analysis was
performed using the Student’s t test.

Immunoadsorption and Western Analysis. Anti-FcγRI mAb 197 (mIgG2a) was obtained
from Medarex (Annandale, NJ, U.S.A.). The rabbit anti-γ chain serum was a generous
gift from Dr. Jean-Pierre Kinet (National Institute of Allergy and Infectious Diseases), and
mAb anti-γ chain 4D8 from Dr. Jarema Kochan (Hoffman-La Roche, Nutley, NJ, U.S.A.).
COS-7 transfectants were lysed for 1 to 2 hours on ice in 1% Digitonin (Calbiochem, San
Diego, CA, U.S.A.), 20 mM triethanolamine pH 7.8, 150 mM NaCl, 0.12% TritonX-100,
and 2 mM phenylmethylsulfonyl fluoride (Sigma), and clarified supernatants were
incubated with mAbs bound to Sepharose 4B (Pharmacia, Piscataway, NJ, U.S.A.)
coupled to goat F(ab')2 anti-mouse IgG (Pierce, Rockford, IL, U.S.A.) as previously
described (3). Immunoadsorbed proteins, eluted by boiling in Laemmli sample buffer
containing 5% 2-mercaptoethanol, were separated by SDS-PAGE in parallel with
Rainbow protein molecular weight markers (Amersham, Arlington Heights, IL, U.S.A.),
and were transferred to nitrocellulose membranes. After blocking, the blots were
incubated sequentially with 1/1000 dilution of anti-γ chain rabbit serum and horseradish
peroxidase-linked protein G (Biorad, Hercules, CA, U.S.A.), followed by Enhanced
Chemiluminescence detection (ECL, Amersham).
FACS Immunofluorescence. 2 x 10^5 COS-7 cells that had been transfected 48 hours earlier with cDNAs for FcγRIIIa and γ chain, FcγRIIIb, or the FcγRIIIa GPI mutant were sequentially incubated with anti-FcγRIII mAb 3G8 and with FITC-labeled F(ab')2 fragments of goat anti-mouse IgG and were analyzed on an Elite EPICS FACS (Coulter, Hialeah, Fl, U.S.A.). Fluorescence data from 10,000 cells were expressed as the number of positive cells (percentage of the cells brighter than the mock transfected COS-7 cells) and as the mean fluorescence intensity (MFI) of the positive cells.
2.3 RESULTS

*The affinity for ligand of both the human and the murine FcγRI increased when the receptors were cotransfected with the γ chain subunit in COS cells.* Both the human and the murine FcγRI cDNAs were transfected into COS cells either alone or with γ chain, and the affinity of FcγRI for IgG was measured in ligand binding assays using $^{125}$I-labeled IgG2a, a murine IgG isotype that both species of FcγRI bind with high affinity (11). The saturation binding data, corrected for nonspecific binding and analyzed by the Scatchard method, produced a straight line for both the human FcγRI and murine FcγRI COS cells transfected with and without γ chain, indicative of a single population of receptors with a single affinity for IgG (Fig. 2.1). The equilibrium association constants (Ka) of the FcγRI transfectants were determined from the slope of the line obtained in the Scatchard plots (Table 2.1). The human and the murine FcγRI transfected into COS cells bound monomeric IgG2a with a Ka of $3.2 \pm 0.3 \times 10^7 \text{M}^{-1}$ and Ka of $4.9 \pm 0.4 \times 10^7 \text{M}^{-1}$ respectively (Table 2.1), similar to affinities previously reported for these transfectants (8,14). The affinity for ligand of FcγRI expressed in another fibroblast cell line, a murine 3T3 cell line stably transfected with a human FcγRI cDNA, was found to be nearly identical, with a Ka of $3.5 \times 10^7 \text{M}^{-1}$. 

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When human γ chain was cotransfected with human FcyRI in COS cells, the binding affinity of the receptor for monomeric IgG2a was increased five-fold to a Ka of 15.6x10⁷ M⁻¹ (Fig. 2.1 and Table 2.1). The binding affinity of FcyRI expressed on IFNγ treated U937 cells, a human monocytic cell line that naturally expresses both FcyRI and γ chain, was also measured and an average Ka of 19.0x10⁷ M⁻¹ was found, in agreement with previous reports (11) and very similar to the binding affinity measured on the COS cells cotransfected with the human FcyRI and γ chain.

Similar results were obtained with the murine FcyRI cotransfected with γ chain in COS cells. Cotransfection of murine γ chain induced a two-fold increase in the affinity of the receptor for mIgG2a with a Ka of 9.1±0.3x10⁷ M⁻¹, an affinity comparable to the one reported on the murine macrophage cell line P388D, Ka of 11.0x10⁷ M⁻¹ (12). The difference in binding affinities for both the human and the murine FcyRI measured when the receptor was transfected alone or with γ chain in COS cells was demonstrated to be statistically significant when analyzed by the Student’s two-tailed t test.

The human and the murine γ chain, which share 86% amino acid identity (15) substituted equally for each other in increasing the binding affinity of FcyRI. The human FcyRI cotransfected with the murine γ chain showed a Ka of 14.5x10⁷ M⁻¹, almost identical to the affinity measured when cotransfected with human γ chain. The binding affinity of the murine FcyRI when cotransfected with the human γ chain was also nearly identical to that measured when the receptor was cotransfected with the murine γ chain, with a Ka of 9.2x10⁷ M⁻¹.
To exclude the possibility that the increase in binding affinity of FcγRI for ligand was a non-specific effect of cotransfection, both the human and the murine FcγRI were cotransfected in COS cells with several control cDNAs including the human macrophage mannose receptor cDNA and several expression vectors, pHMR, pRSVCAT, and pcDNAI. The binding of these FcγRI transfectants was analyzed as before and the resulting Ka determined by the Scatchard method were $3.2\pm0.3\times10^7\text{ M}^{-1}$ and $4.1\pm0.7\times10^7\text{ M}^{-1}$ for human and murine FcγRI respectively when cotransfected with nonspecific cDNA (Table 2.1). Thus, cotransfection of non-specific cDNA did not change the affinities of the human and the murine FcγRI.

Cotransfection of γ chain with human FcγRI also appeared to affect the number of expressed receptors. Binding of monomeric IgG2a to COS cells cotransfected with human FcγRI and γ chain showed a consistent decrease in the total concentration of receptor sites, as shown by the Bmax values: $5.0\pm1.0\times10^{-10}\text{ M}$ for FcγRI cotransfected with γ chain, as compared to $16.0\pm4.0\times10^{-10}\text{ M}$ when FcγRI was transfected alone, or $13.0\pm1.0\times10^{-10}\text{ M}$ when it was cotransfected with control cDNAs. Although expression levels in transient transfectants can be variable, the consistency with which γ chain decreased the Bmax of the human FcγRI suggested that the change in the concentration of receptor sites may be specific to γ chain cotransfection. Unlike the human receptor, however, the Bmax measured for the murine FcγRI cotransfected with γ chain was not different from the Bmax obtained with FcγRI alone.

*The changes in binding affinity for IgG correlate with the association of γ chain with FcγRI.* We have previously shown that the association of the human FcγRI with
human γ chain can be reconstituted in COS cells (3). To confirm that this association was present in our experimental conditions and to determine if the murine FcγRI association with murine γ chain was also reconstituted in COS cell transfections, we analyzed transfectants by Western blot after immunoadsorption with antibodies against human FcγRI (197) or γ chain (4D8) (Fig. 2.2). Since antibody 197 is an IgG2a isotype, it was also used as a ligand to adsorb the murine FcγRI because of the lack of specific antibodies for this receptor. The anti-γ chain immunoblot antibody recognized a 10 kDa protein copurifying with FcγRI in immunoadsorbates from cells transfected with human FcγRI and human γ chain (lane 2, A) and from cells transfected with murine FcγRI and human γ chain (lane 4, A). A band the appropriate size for γ chain was also copurified from cells in which the human FcγRI was cotransfected with the murine γ chain (lane 4, B), as well as from cells cotransfected with the murine FcγRI and the murine γ chain (lane 5, B). Direct immunoadsorption of γ chain with anti-γ chain mAb 4D8 confirmed the presence of γ chain in the appropriate cells (lanes 2 and 4, A; lanes 4 and 5, B).

The association of FcγRI with γ chain and the subsequent increase in binding affinity are independent of the cytoplasmic domain of the receptor. To investigate if the transmembrane domain of FcγRI was sufficient for association with γ chain as well as for the increase in binding affinity mediated by γ chain, a truncated form of the human FcγRI containing the extracellular and transmembrane domains of the receptor without the cytoplasmic domain (FF) was constructed and transfected into COS cells with or without γ chain. Western blot analysis with an anti-γ chain antibody showed a 10 kDa sized band (Fig. 2.2, lane 7 A) in cells that had been cotransfected with the truncated construct and γ
chain, and immunoadsorbed with either anti-FcγRI or anti-γ chain antibodies, indicating that γ chain was expressed in these cells and was coimmunoadsorbed with the truncated FcγRII mutant.

The increase in binding affinity of FcγRI induced by γ chain was not affected by the absence of the cytoplasmic domain of the receptor. Cos cells transfected with the truncated FcγRI alone bound monomeric 125I-IgG2a with a Ka of 6.9±0.8x10^7 M⁻¹, whereas after cotransfection with γ chain, cells bound 125I-IgG with a Ka of 20.3±4.3x10^7 M⁻¹, similar to the Ka of 19.0 x 10⁷ M⁻¹ measured for FcγRI on U937 cells (Table 2.1), indicating that the cytoplasmic domain of FcγRI is not required for enhanced binding affinity. The binding affinity of the truncated FcγRI transfected without γ chain was two-fold higher than that of the wild type receptor (p=0.025); however, no significant difference was seen in the affinity of the two receptors cotransfected with γ chain (p=0.400).

**Association with γ chain confers medium affinity binding to FcγRII.** To determine if the medium affinity of FcγRIIIa for monomeric IgG is due to its association with γ chain, COS cells were transfected with either the FcγRIIIa isoform, which requires cotransfection and association with γ chain for cell surface expression, or the FcγRIIIb isoform, a GPI-linked form of the receptor which does not associate with γ chain. We also transfected an FcγRIIIa mutant resulting in a GPI-linked form of the receptor whose expression is independent of γ chain association. The three FcγRIII constructs transfected in COS cells were analyzed simultaneously for ligand affinity by IgG binding assay and for expression by flow cytometry. Table 2.2 shows the results of representative
Scatchard and flow cytometric analyses of the same set of transfected COS cells comparing ligand binding affinity and surface expression of the three FcγRIII. The affinity of FcγRIIIα cotransfected with γ chain (1.2x10^7 M⁻¹) demonstrated an approximate 10-fold increase when compared to the affinity measured for the FcγRIIIα GPI mutant (0.1x10^7 M⁻¹), while the affinity of FcγRIIIb could not be calculated due to the low level of ligand binding. The expression of the three FcγRIII was analyzed with an anti-FcγRIII antibody by FACS to insure that the lower binding and affinities measured for FcγRIIIb and FcγRIIIα GPI were not due to low levels of expression (Table 2.2). The three FcγR were expressed in COS cells, with FcγRIIIα-γ chain expressing less than FcγRIIIb or FcγRIIIα-GPI mutant confirming an absence of correlation between binding affinity and levels of receptor expression. Similar results were obtained in 2 additional ligand binding assays and flow cytometric analyses.
2.4 DISCUSSION

The functions that γ chain provides in its association with FcγR continue to be defined. We have now shown that γ chain, in addition to its roles in receptor expression and signal transduction, modifies the affinity of FcγR for ligand. In this study we demonstrated that the high affinity for monomeric IgG that characterizes both the human and murine FcγRI was dependent upon γ chain cotransfection with the receptor, thus explaining the discrepancy between the lower binding affinity reported for FcγRI transfected into COS cells and the higher affinity measured on cells that naturally express the receptor. Association of γ chain increased 5-fold the binding affinity of human FcγRI, and 2-fold the affinity of the murine receptor. While the level of affinity increase was lower in the mouse, γ chain brought the affinity of both species of FcγRI to the levels reported on cells that naturally express the receptors. The differences observed between the human and the murine FcγRI when cotransfected with γ chain in COS cells did not appear to be due to the species of γ chain cotransfected with the receptors, as similar results were obtained when each receptor was cotransfected with γ chain from the other species, suggesting differences within the structure of the receptors themselves. In fact, the reported range of binding affinity for the human FcγRI, Ka of $10^{5-9} M^{-1}$ (10), is slightly higher than the affinity reported on murine macrophages, Ka of $1.1 \times 10^{9} M^{-1}$ (12), an
observation our data supports. Our findings also suggested that the FcγRIIIa isoform, the "medium affinity" FcγR, has a higher affinity for monomeric IgG than the FcγRIIIb isoform due to its association with γ chain. The affinity of an FcγRIIIa GPI mutant, expressed without the requirement of γ chain, was ten-fold less than the affinity measured for FcγRIIIa cotransfected with γ chain.

We had previously postulated that γ chain associated with FcγRI through the transmembrane domain of the receptor due to homology of this region to the transmembrane domains of FceRI and FcγRIIIa (3), both shown to associate with γ chain through their transmembrane domains (16). With only five putative extracellular amino acids (15), it is unlikely that γ chain associated with FcγRI or induced its change in affinity through an interaction at the extracellular level. Analysis of a truncated FcγRI lacking its cytoplasmic domain not only supported the hypothesis that γ chain associated with the transmembrane domain of the receptor, but also demonstrated that the cytoplasmic domain of the receptor was not required for the increased binding affinity conferred by association with γ chain. In fact, we observed a slight but significant increase in the affinity of the truncated FcγRI when compared to the wild-type FcγRI that suggested the cytoplasmic domain may actually have an inhibitory effect upon binding affinity. However, when the receptors were cotransfected with γ chain there was no longer a significant difference in the binding affinity of the truncated FcγRI compared to the wild-type receptor, suggesting γ chain association may overcome any inhibitory effect of the FcγRI cytoplasmic domain to confer the appropriate receptor structure for high affinity binding.

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It has previously been inferred that the unique third extracellular domain of FcγRI is responsible for its high affinity binding of monomeric IgG (8,14). In one study, a murine FcγR chimera lacking the third extracellular domain and containing only the first two extracellular domains of FcγRII spliced to the transmembrane and cytoplasmic domain of FcγRII bound IgG with the same low affinity and binding specificity as FcγRII (13). While we have shown that γ chain association is required for maximum binding affinity, FcγRI expressed without γ chain still bound IgG with an affinity higher than the other FcγR, indicating that the high affinity binding of this receptor may be a result of contributions from the extracellular domain along with the association to the γ chain subunit. The role of the extracellular domain was also apparent in our FcγRIII experiments. Both of the two GPI-linked forms of FcγRIII, a and b, bound ligand with low affinity; however, the affinity of FcγRIIIa could be measured while the binding of FcγRIIIb was too low for an accurate affinity to be determined, even though the two receptors were expressed at similar levels. These findings suggested that the six amino acid differences between the extracellular domain of FcγRIIIa and FcγRIIIb (17), in addition to association with γ chain, contributed to the higher binding affinity of this receptor. For both FcγRI and FcγRIII, it appeared that the structure of the extracellular domains affects binding affinity, and that association with γ chain further increases the affinity for ligand, probably through a change of the quaternary structure of the receptors.

While increased binding affinity due to an associated subunit is a novel observation amongst FcγR, a similar phenomenon has been described for members of the cytokine receptor family. Like FcγR, hematopoietic cytokine receptors are characterized by
pleiotropy and redundancy, and lacking a tyrosine signaling motif in their cytoplasmic
domain they associate with common subunits including gp130, KH97, and the IL-receptor
γ chain (18, 19, 20, 21, 22) that are responsible for mediating the receptor signal. These
cytokine receptors, like FcγRI, when expressed alone bind ligand with relatively low
affinity but are transformed to high affinity receptor complexes upon association with their
subunits. An explanation has been proposed for the affinity increase of these receptors
upon association with the signaling subunit: receptors that do not have built-in signaling
motifs and rely instead upon associated subunits might require a higher binding affinity for
ligand to give the receptor the needed time to transmit signal through the associated
subunit (18). This hypothesis may also be relevant to FcγR since FcγRII, which contains
a signaling motif within its cytoplasmic domain, binds monomeric IgG with lower affinity
than FcγRI and FcγRIIIa, which both rely on association with γ chain to transmit signals
to the cell. It would appear that the ability of γ chain to modify the affinity of FcγR for
ligand is not only a novel observation amongst FcR, it may also have a wider significance
suggesting that in addition to their roles in signal transduction, associated subunits may
regulate receptor-ligand interaction.

This work was supported by U.S. Public Health Service Award R01-CA44983.
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### Table 2.1. Equilibrium association constants for binding of monomeric IgG to FcγRI with and without γ chain.

The results are tabulated from ligand-binding assays and Scatchard analyses of the binding of monomeric $^{125}$I-IgG2a to various cell types and COS-7 cells transfected with human or murine FcγRI cDNAs or a truncated form of FcγRI lacking the cytoplasmic domain (FF), with or without human or murine γ chain (hγ, mγ) cotransfection. Control cDNAs (control) cotransfected with FcγRI were the human mannose receptor cDNA and several expression vectors. $Ka$ represent the mean of $n$ ligand-binding assays ±SEM. *The differences in the affinities of FcγRI for IgG measured when the receptor was transfected with or without γ chain was statistically significant as determined by the Student's two-tailed $t$ test, with $P$ values of $<0.001$ for hFcγRI and $<0.001$ for mFcγRI. †The affinity of the mFcγRI expressed on the murine macrophage cell line P388D as reported by Unkeless, et al. (12).

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Transfected cDNA</th>
<th>$Ka$ (M⁻¹)</th>
<th>$n$</th>
<th>$Ka$ (M⁻¹)</th>
<th>$n$</th>
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<td></td>
<td>19.0</td>
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<td></td>
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<tr>
<td>P388D</td>
<td></td>
<td></td>
<td></td>
<td>11.0†</td>
<td></td>
</tr>
<tr>
<td>3T3 (N10F7)</td>
<td>FcγRI</td>
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<td>2</td>
<td></td>
<td></td>
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<tr>
<td>COS-7</td>
<td>FcγRI</td>
<td>3.2 ±0.3</td>
<td>13</td>
<td>4.9 ±0.4</td>
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<tr>
<td></td>
<td>FcγRI + hγ</td>
<td>15.6 ±1.4*</td>
<td>10</td>
<td>9.2</td>
<td>2</td>
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<tr>
<td></td>
<td>FcγRI + mγ</td>
<td>14.5 ±0.5</td>
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<td>9.1 ±0.3*</td>
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<tr>
<td></td>
<td>FcγRI + control</td>
<td>3.2 ±0.3</td>
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<td>4.1 ±0.7</td>
<td>3</td>
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<td>FF</td>
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<td>6.9 ±0.8</td>
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<tr>
<td>FF + hγ</td>
<td></td>
<td>20.3 ±4.3</td>
<td>3</td>
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Table 2.1. Equilibrium association constants for binding of monomeric IgG to FcγRI with and without γ chain. The results are tabulated from ligand-binding assays and Scatchard analyses of the binding of monomeric $^{125}$I-IgG2a to various cell types and COS-7 cells transfected with human or murine FcγRI cDNAs or a truncated form of FcγRI lacking the cytoplasmic domain (FF), with or without human or murine γ chain (hγ, mγ) cotransfection. Control cDNAs (control) cotransfected with FcγRI were the human mannose receptor cDNA and several expression vectors. $Ka$ represent the mean of $n$ ligand-binding assays ±SEM. *The differences in the affinities of FcγRI for IgG measured when the receptor was transfected with or without γ chain was statistically significant as determined by the Student's two-tailed $t$ test, with $P$ values of $<0.001$ for hFcγRI and $<0.001$ for mFcγRI. †The affinity of the mFcγRI expressed on the murine macrophage cell line P388D as reported by Unkeless, et al. (12).
<table>
<thead>
<tr>
<th>Transfectant</th>
<th>Binding Affinity $K_a (10^9 \text{M}^{-1})$</th>
<th>FACS analysis</th>
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<td>$\text{Fc} \gamma \text{RIIIa} + \gamma$</td>
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<td>$\text{Fc} \gamma \text{RIIIb}$</td>
<td>&lt;0.1</td>
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<tr>
<td>$\text{Fc} \gamma \text{RIIIa GPI mutant}$</td>
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<td>22.1</td>
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Table 2.2: Analysis of ligand binding and expression of $\text{Fc} \gamma \text{RIII}$ transfected in COS-7 cells. The table contains the equilibrium association constants of one representative ligand binding assay of monomeric $^{125}\text{I-}\text{IgG2a}$ to COS-7 cells transfected with $\text{Fc} \gamma \text{RIIIa}$ and $\gamma$ chain, $\text{Fc} \gamma \text{RIIIb}$, and an $\text{Fc} \gamma \text{RIIIa}$ GPI mutant, each point done in triplicate as described in Figure 1. The surface expression of the three $\text{Fc} \gamma \text{RIII}$ constructs from the same set of transfected COS-7 cells was analyzed in FACS with the anti-$\text{Fc} \gamma \text{RIII}$ mAb 3G8. The results are expressed as the mean of the % of cells brighter than negative controls (%+cells) and the mean fluorescence intensity of the positive cells (MFI). Similar results were obtained in 2 additional ligand binding assays and flow cytometric analyses.
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Figure 2.1: Scatchard plots of monomeric IgG binding to human and murine FcγRI transfected with and without γ chain in COS-7 cells. The Scatchard plot of one representative $^{125}$I-IgG2a ligand binding assay is shown in which all points were done in triplicate and corrected for nonspecific binding, demonstrating the binding of ligand by FcγRI transfected alone (○) or with γ chain (●). Panel A, human FcγRI (hFcγRI) with and without human γ chain (hγ); Panel B, murine FcγRI (mFcγRI) with and without murine γ chain (mγ); Bound (B), Molar concentrations of $^{125}$I-IgG2a bound specifically to transfectants; Free (F), Molar concentration of unbound $^{125}$I-IgG2a.
Figure 2.2: FcγRI association with γ chain in COS-7 transfectants. Digitonin lysates of COS-7 cells transfected (COS Tf) with human FcγRI (hRI) or murine FcγRI (mRI) with or without human γ chain (hy) (Panel A, lanes 1-5), and with or without murine γ chain (my) (Panel B, lanes 1-5) were immunoadsorbed (IA) with anti-FcγRI (> FcγRI, mAb 197; Panel A, one half the volume of the lysates; Panel B, two-thirds the volume of the lysates) and anti-γ chain mAb 4D8 (> γ). Lanes 6 and 7 of Panel A are from a separate experiment showing the association of γ chain with a truncated human FcγRI mutant lacking its cytoplasmic domain (FF) transfected with or without human γ chain. The eluates were analyzed by Western blot with rabbit anti-γ chain antibody and ECL detection. Lanes 3, 4 and 5 of Panel A > FcγRI are from a longer exposure time. kD, kilodalton.
2.7 LIST OF REFERENCES


CHAPTER 3

EVIDENCE FOR HOMODIMERIZATION OF THE LIGAND-BINDING α CHAINS OF THE HIGH AFFINITY Fc RECEPTOR FOR IgG (FcyRI)

Kathy L. Miller, and Clark L. Anderson
3.1 INTRODUCTION

Receptors that bind the Fc portion of immunoglobulin (Ig), FcR, function at the surface of immune cells to trigger a variety of protective mechanisms in response to antigenic substances (1). The FcR specific for IgG (FcγR) are FcγRI, FcγRII and FcγRIII (2). After binding immune complexes, these receptors cluster and trigger an array of biological functions including phagocytosis, endocytosis, antibody-dependent cytotoxicity, release of inflammatory mediators, enhancement of antigen presentation, regulation of B cell activation, and participation in T cell development (reviewed in 3, 4). To ascertain how FcγR convey their effects upon a cell, it is important to first understand the manner in which the structural components of the receptor interact. The high affinity receptor for IgG, FcγRI, consists of noncovalently associated α and γ polypeptide chains (5, 6). A member of the Ig superfamily, the α chain of FcγRI is a 72 kD three extracellular domain integral membrane glycoprotein (7, 8). Since there are no signaling sequences intrinsic to the cytoplasmic region of the α chain (9), FcγRI relies upon the γ chain to initiate cell signaling. The γ chain subunit, a 20 kD disulfide-linked homodimer, is an integral membrane protein with a short five amino acid extracellular region and a cytoplasmic domain containing the immune tyrosine activation motif (ITAM) sequence required for signal transduction (10-13).
Like FcγRI, many FcR form oligomeric complexes (14, 15). The α chains of several FcR also associate with the FcγRI γ chain subunit, including another member of the FcγR class, FcγRIIIa, the high affinity receptor for IgE, FceRI, and the receptor for IgA, FcαR (16-19). The FceRI complex has an additional associated subunit, the β chain (20). To add to the diversity, FcγRIIIa also associates with the FcεRI β chain subunit in some cell types, and in others substitutes γ chain with the homologous T cell receptor ζ chain subunit (21, 22). While these subunits appear to function primarily in signal transduction, we recently demonstrated that the high affinity of the FcγRI α chain for monomeric IgG is dependent upon the γ chain association (23). This led us to hypothesize that the γ chain interaction induces dimerization resulting in two α chains binding to a single IgG molecule, thereby conferring high affinity binding of IgG.

Although dimerization of ligand binding subunits is a common theme amongst many cell surface receptors (24-26), it has not been demonstrated for FcR, which instead are thought to bind Ig through a monomeric α chain (1, 27). Since the Fc portion of Ig is itself a dimer (28), it is plausible that two FcγRI α chains could bind to a single IgG ligand. In this report we present evidence for dimerization of the ligand binding α chains of FcγRI utilizing iodination and Western blot analyses of both the human monocyte cell line U937 and FcγRI transfected COS cells. N-terminal FLAG and C-terminal histidine epitope-tagged FcγRI α chain cDNAs were engineered and cotransfected into COS cells to demonstrate homodimerization of the receptor α chains.
3.2 MATERIALS AND METHODS

**Cells and Cell Culture.** U937, a human monocyte cell line from ATCC was maintained in RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 2mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. COS-7 cells were cultured in Dulbecco's Modified Eagle Medium with the supplements listed above. U937 cells were cultured with 100 U/ml human recombinant IFNγ (Genentech, San Francisco, CA) 48 hours before each experiment to increase FcγRI expression.

**Plasmid Constructs and Transfections.** The human FcγRI a1 cDNA cloned into the pCDM vector, was described by Ernst et al (5). The human γ-chain cDNA in vector pSVL was a kind gift from Dr. Jean-Pierre Kinet (National Institute of Allergy and Infectious Diseases, Bethesda, MD). Utilizing PCR amplification with FcγRI a1 cDNA as a template, two cDNAs were constructed to express FcγRI a1 fusion proteins containing either an amino-terminal (a1F) or a carboxy-terminal (a1H) epitope-tag. Oligonucleotides for PCR were synthesized by Gibco BRL Life Technologies (Grand Island, New York). PCR was performed by adding 25 ng of a1 cDNA into a 100-µl volume reaction containing 10 mM Tris-HCL (ph 8.85), 2.0 mM MgSO₄, 25 mM KCl, 5 mM (NH₄)₂SO₄, 200 µM each of four dNTPs, 0.5 µM of each oligonucleotide.
primer, and 5 U of Pwo DNA polymerase (Boehringer Mannheim, Indianapolis, IN). The a1F sense primer (p91), which corresponded to nucleotide sequences 52-69 in EC1, was 5'-AAG CTT GAC ACC ACA AAG GCA GTG-3' and contained a HindIII restriction site. The antisense primer (p84), 5'-TCT AGA GAG CCG CTG CTA CGT GG-3', was the reverse compliment of nucleotides 1120-1137 in the TM/C region, including the translation termination codon, and contained an XbaI restriction site. The a1H sense primer (p87), 5'-AAG CTT TGC TCC CAC CAG C-3', coincided with nucleotides −12-25 in the SI domain (upstream of the translation start codon) and contained an XhoI site. The antisense primer (p76), 5'-CCG CTC GAG CGT GGC CCC C-3', was the reverse compliment of nucleotides 1115-1125 in the TM/C region immediately preceding sequence encoding the translation termination codon, and contained a HindIII restriction site. After addition of a 3' adenosine-overhang using Taq polymerase (Boehringer Mannheim), both PCR products were subcloned into the pCR2.1 vector (Invitrogen, San Diego, CA). The a1F cDNA was excised via the HindIII and XbaI restriction sites, and re-subcloned into the pFLAG-CMV-1 mammalian expression vector (Eastman Kodak Company, New Haven, CT) to match the reading frame with the N-terminal FLAG vector sequence encoding the octapeptide N-AspTyrLysAspAspAspAspLys-C. The a1H construct was inserted into the pETb20+ vector (Novagen, Madison, WI) utilizing the XhoI and HindIII sites. The entire a1 cDNA including sequence encoding the six C-terminal histidine residues was amplified by PCR using the sense primer p87 along with an antisense primer (p78), 5'-TCT AGA ATC TCA GTG GTG G-3', the reverse compliment of sequences encoding
the four terminal histidine residues, and subcloned into the pCDM mammalian expression vector. The 5' and 3' ends of both the aIF and aIH were sequenced by the chain termination method (29) using Sequenase 2.0 (Amersham Life Science, Cleveland, OH) to confirm in-frame insertion into the epitope-tag sequences within the vectors. The cDNAs were transiently transfected into COS-7 cells by the diethylaminoethyl-dextran method as described previously (23) using 1-2μg/ml of DNA, and the cells were analyzed 48 hours after transfection.

**Antibodies.** Anti-FcγRI mAbs 22 (mIgG1) and 32.2 (mIgG1) were acquired from Medarex (Annandale, NJ). The anti-FcγRI cytoplasmic tail mAb CT6 (mIgG1) and rabbit anti-FcγRI cytoplasmic domain peptide antiserum were prepared in collaboration with Dr. Pravin Kaumaya (The Ohio State University) against an 18-residue peptide (H3N^+VTI RLE LKR KKK WNL EIS-COO, residues 312-329) as previously described (30). Anti-FLAG mAb M2 (mIgG1) was obtained from Eastman Kodak Company.

**Iodinations and Chemical Crosslinking.** Cells were radioiodinated by the chloroglycouril (CGU) method (5) by incubating 1-3x10^7 cells in 0.7 ml PBS with 50 μl borate buffered saline, and 1 mCi Na^{125}I (500 mCi/ml) (ICN Pharmaceuticals, Inc, Irvine, CA) in a vial coated with 50 μg of CGU for 30 minutes at 4°C, and then washed with 5 mM KI in a buffer of 20 mM sodium phosphate buffer, 0.15 M NaCl, pH 7.5 (PBS). In some experiments cells were incubated in PBS either alone or with increasing concentrations of the homobifunctional thiol-cleavable chemical crosslinker.
Dithiobis(succinimidylpropionate) (DSP; Pierce, Rockford, IL) for 15-30 minutes at 4°C. The reaction was quenched by the addition of 50 mM Tris buffer, pH 7.5 for 7.5 minutes at 4°C.

**Immunoadsorptions.** Cells were lysed for 1 to 2 hours at 4°C with either TritonX-100 buffer (1% TritonX-100 (Sigma, St. Louis, MO), 2mM phenylmethylsulfonyl fluoride (PMSF; Sigma) in PBS) or digitonin lysis buffer (1% digitonin (Calbiochem, San Diego, CA) 20 mM triethanolamine pH 7.8, 150 mM NaCl, 0.12% TritonX-100, and 2 mM PMSF. Supernatants were clarified by centrifugation at 15,000 x G for 20 minutes and incubated with mAbs bound to Sepharose 4B (Pharmacia, Piscataway, NJ) coupled to goat F(ab')2 anti-mouse IgG (Pierce). The anti-FcγRI mAbs 22, 32.2 and the anti-FLAG mAb M2 were bound to the Sepharose-anti-mouse beads by incubating 50 μl of packed beads with 100 μl of 10μg/ml mAbs for 4 hours 4°C. Anti-FcγRI CT6 was coupled by incubating the beads with 100 μl of a 1:1 dilution of the CT6 antiserum. Immunoadsorbed proteins were eluted by boiling in Laemmli sample buffer containing SDS with or without 5% 2-mercaptoethanol. The immunoadsorbed proteins were separated by SDS-PAGE in parallel with Rainbow protein molecular weight markers (Amersham).

**Affinity Purifications.** The a1H protein was purified from cell lysates through binding of the six histidine residue-tag to divalent nickel cations immobilized on His-Bind metal chelation resin (Novagen). After washing twice with sterile deionized water, 200 μl of His-Bind resin was charged by incubating in a buffer of 50 mM NiSO₄ for 15 minutes at room temperature. The Ni²⁺ charged beads were washed once with TritonX-100
buffer with 2 mM PMSF and incubated with clarified a1H COS cell transfectant lysates (prepared as described above) for 4 hours at 4°C. The adsorbed protein was washed once with TritonX-100 buffer followed by four washes in a buffer consisting of 60 mM imidazole, 5 mM NaCl and 20 mM Tris-HCl, pH 7.9. In the experiment analyzing a1H and a1F COS cell cotransfectants, these four washes were more stringent (150 mM imidazole, 1.25 M NaCl and 50 mM Tris-HCl, pH 7.9) to prevent a1F from nonspecifically binding to the resin. The affinity purified protein was eluted from the His-Bind resin with a buffer containing excess imidazole (1M imidazole, 25 mM NaCl, 10 mM Tris-HCl, pH 7.9), which competes with the histidine for binding to the beads. Eluted proteins were precipitated with ice-cold trichloroacetic acid, redissolved in Laemmli sample buffer with or without 5% 2-mercaptoethanol, boiled and separated by SDS-PAGE as described above.

**Western Blot Analysis.** After separating by SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose membranes in a buffer of 25 mM Tris base, 190 mM glycine, 0.01% SDS and 20% methanol. Membranes were incubated overnight at 4°C in TBS-Tween (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 5% dry milk as a blocking agent. After blocking, the blots were incubated in TBS-Tween either for 1 hour at room temperature with 10 μg/ml anti-FLAG mAb M2 or with a 1:250 dilution of rabbit anti-FcγRI cytoplasmic domain peptide antiserum overnight at 4°C. After four washes the membranes were incubated with a 1/1000 dilution of horseradish peroxidase-linked protein G (Biorad, Hercules, CA, U.S.A.), and detected by Enhanced Chemiluminescence (ECL, Amersham).
3.3 RESULTS

**FcγRI Multimer-sized Complexes Detected on Surface Labeled U937 Cells.** In the first approach to investigate the quaternary structure of FcγRI, we analyzed U937 cells, a human monocytic cell line that expresses both the FcγRI α chain and its associated subunit γ chain, utilizing the cleavable crosslinker DSP to maintain FcγRI subunit associations. Radioiodinated IFN-γ treated U937 cells were chemically crosslinked, lysed and incubated with anti-FcγRI antibody. The immunoabsorbates were analyzed by SDS-PAGE in either nonreducing conditions to maintain the crosslinker (Fig.3.1 A) or reducing conditions to break the disulfide bond in the crosslinker (Fig.3.1 B). Iodinated bands of the appropriate size for an FcγRI α chain monomer (1x), dimer (2x), and tetramer (4x) were revealed in nonreducing conditions. The dimer-sized band running between the 97 kD and 200 kD protein molecular mass markers was distinct even in the absence of crosslinker (Fig.3.1 A, lane 1). The tetramer-sized band running above the 200 kD marker, although present without crosslinker, appeared to intensify with the addition of DSP (Fig.3.1 A, lanes 2-5). As the concentration of crosslinker increased, the amount of monomer- and dimer-sized bands decreased while the intensity of the tetramer-sized band intensified. In reducing conditions the multimer-sized bands were resolved to an FcγRI α chain monomer-sized...
band of 70 kD (Fig.3.1 B). Also evident was a 10 kD iodinated band the appropriate size for the associated subunit γ chain that intensified with the addition of crosslinker (Fig.3.1 B, lower panel), suggesting the presence of γ chain in the multimer-sized bands.

**FcyRI Dimer and Tetramer-sized Complexes Contain 70 kD, 30 kD and 10 kD Iodinated Proteins.** To confirm that the dimer and tetramer-sized complexes detected in U937 contained an FcyRI α chain monomer-sized protein as well as a γ chain-sized protein, two-dimensional gel electrophoresis was utilized. Radioiodinated IFN-γ treated U937 cells either without (Fig.3.2 A) or with (Fig.3.2 B) DSP crosslinking were lysed in Triton-X100 detergent and adsorbed with anti-FcyRI mAb. The eluates were analyzed by two-dimensional SDS-PAGE with the first dimension in nonreducing conditions to maintain the multimer complexes, followed by reducing conditions for the second dimension to resolve the multimers into their components. Even in the absence of crosslinker, distinct FcyRI α chain-sized 70 kD protein comprised the monomer, dimer and tetramer bands (Fig.3.2 A, top panel). The dimer-sized band actually appeared to be a doublet that was not detectable in one dimensional analysis (Fig.3.1 A). With longer exposure it became evident that in addition to the 70 kD protein, there was also a 30 kD iodinated protein included in the dimer and tetramer-sized complexes (Fig.3.2 A, bottom panel). The upper band of the dimer doublet, in fact, appeared to be a result of association with this 30 kD protein. In the absence of crosslinker no γ chain-sized band appeared, as expected since γ chain association with FcyRI α chain requires chemical crosslinking to be maintained in Triton-X100 detergent lysis (5).
With the addition of chemical crosslinker the resolution of the dimer and tetramer bands was diminished (Fig.3.2 B, top panel), and the 30 kD molecule was also difficult to distinguish. The DSP crosslinked U937 cells, however, did reveal a 10 kD γ chain-sized iodinated protein associated with both the dimer and tetramer-sized complexes (Fig.3.2 B, bottom panel). The 30 kD and the γ chain-sized proteins did not, however, appear to associate with the FcγRI α chain monomer in these assays.

**Presence of the FcγRI Dimer-sized Complex in COS Transfectants is Independent of γ Chain, while the Tetramer-sized Complex is Dependent upon γ Chain Cotransfection.** The suggestion of FcγRI dimers in the previous results prompted further analyses of these complexes and how they related to our hypothesis. To determine if the multimer-sized iodinated complexes observed in U937 cells could be reconstituted and to investigate further the role of γ chain in dimerization, cDNAs for either the FcγRI α chain alone or both the α and γ chains were transfected in COS cells. The transfectants were iodinated, lysed and immunoadsorbed with anti-FcγRI mAb followed by analysis on SDS-PAGE in nonreducing conditions (Fig.3.3).

Iodinated bands the appropriate sizes for an FcγRI α chain monomer and dimer were evident when the α chain was transfected alone (Fig.3.3, lane 2), indicating that γ chain association is not required for formation of the dimer-sized complex. The tetramer-sized structure was not evident in these transfectants. Upon cotransfection with γ chain, however, an iodinated band the appropriate size for an FcγRI α chain tetramer appears (Fig.3.3, lane 3). The presence of a 200 kD iodinated band was inconsistently observed with both iodinated COS cell transfectants and U937 cells. Two-dimensional SDS PAGE of the iodinated COS transfectants confirmed that the
dimer and tetramer bands contained a 70 kD protein (data not shown). No 30 kD iodinated band was observed in either FcγRI α chain transfectant, with or without γ chain.

**FcγRI α Chain Monomer and Dimer Complexes in COS Cell Transfectants are Recognized by Anti-FcγRI Antibody in Western Analyses.** Since both the U937 cells and the FcγRI COS cell transfectants were adsorbed with anti-FcγRI antibody, it was probable that the 70 kD iodinated protein within the multimer-sized complexes represented the FcγRI ligand binding α chain. While unlikely, however, it was also possible that the 70 kD protein was not the α chain but rather an unknown protein purified through an association with the receptor. To confirm that the FcγRI α chain was a component of the multimer-sized structures, COS cell transfectants were analyzed by Western blot with anti-FcγRI antibody. COS cells were transfected with an α chain cDNA containing sequence for a C-terminal histidine epitope-tag (Fig.3.5 A, RI-H) to allow for isolation of the protein without using antibody, since the heavy and light chains comprising the adsorbing antibody are detected by the HRP-conjugated secondary reagent in Western analysis and can interfere with interpretation of results. Lysates of the FcγRI α chain histidine tagged transfectants were affinity purified on a nickel charged column and the eluates analyzed by Western blot. In nonreducing conditions α chain monomer and dimer-sized bands were recognized by the anti-FcγRI antibody (Fig.3.4 A, RI-H). Transfectants analyzed by Western blot after two-
dimensional SDS-PAGE as described above indicated that in addition to the α chain monomer band, the 70 kD protein comprising the receptor multimer-sized bands was also recognized by the anti-FcγRI antibody (Fig. 3.4 B).

**FcγRI Homodimers are Detectable in COS Transfectants Utilizing Different Epitope Tags.** The results thus far suggested that FcγRI was forming complexes of the appropriate size for a receptor dimer and perhaps even tetramer, but they did not address whether the α chain was homodimerizing or forming heterodimers with another protein(s). To determine if homodimerization was occurring, two α chain proteins each with a different epitope tag were engineered: an N-terminal FLAG tag (Fig. 3.5 B, RI-F) and a C-terminal histidine tag (Fig. 3.5 A, RI-H). The epitope tags were placed at opposite ends of the α chain to prevent potential steric hindrance that might disrupt or prevent dimer formation. The strategy was to cotransfect the two epitope-tagged α chains, adsorb RI-H and Western blot for the presence of RI-F coadsorbing in a receptor homodimer (Fig. 3.5 B). The RI-H construct was adsorbed using nickel beads in metal-chelate affinity chromatography instead of by antibodies, which would purify FcγRI as ligand and lead to uninterpretable results.

The cDNAs of both α chain constructs were transfected in COS cells either individually or together and the lysates were adsorbed on a charged nickel column. The eluates were examined by SDS-PAGE in reducing conditions to resolve the dimers to α chain monomers, followed by Western blot for the presence of the FLAG-tag with an anti-FLAG antibody. Controlling for the specificity of the assay, analysis of RI-F transfectants indicated that the FLAG tag did not adsorb to the nickel column (Fig. 3.6,
lane 4) and the anti-FLAG antibody did not detect the histidine tag of RI-H in the Western blot (Fig. 3.6, lane 3). Only when the α chain constructs were cotransfected was a FLAG-specific 70 kD α chain band detected, indicating that RI-F was coadsorbing with RI-H as a homodimer (Fig. 3.6, lane 2).
3.4 DISCUSSION

Recently, we revealed that the high affinity of FcγRI for monomeric IgG was dependent upon association of the ligand binding α chain with the γ chain subunit (23). This observation led to the hypothesis that through association, γ chain was forming homodimers of the α chains enabling them to bind a single bivalent IgG molecule with high affinity. Two receptors binding to a single, usually bivalent ligand with high affinity is a common occurrence among many cell surface receptors, however the dimerization of ligand binding subunits has not been reported for Ig-superfamily FcR.

In this study we present evidence that such a structure is formed by FcγRI.

Anti-FcγRI antibodies detected α chain dimer-sized complexes on iodinated U937 cells, a human monocyte cell line that expresses FcγRI (Fig.3.1). The presence of proteins the size of the α and γ chains in these complexes (Fig.3.2) was consistent with our hypothesis of γ chain induced α chain dimers. Transfection of cDNAs representing these FcγRI subunits into COS cells, which do not endogenously express either polypeptide chain, allowed us to analyze the specificity of the proteins within the dimer complex as well as the role of γ chain in dimerization. The α chain dimer-sized complex was detected even in the absence of γ chain in these transfectants (Fig.3.3; Fig.3.4), suggesting that dimerization was occurring independently of γ chain
association. Western blot analyses with anti-FcγRI antibody confirmed that the FcγRI α chain was a component of the dimer-sized structure (Fig. 3.4), however they did not indicate whether the α chains were homodimerizing or heterodimerizing with an unknown protein of the same size. Two differentially epitope-tagged FcγRI α chain constructs (Fig. 3.5) cotransfected in COS cells and analyzed by Western blot confirmed that the α chains were homodimerizing (Fig. 3.6).

Although γ chain does not appear to be essential for inducing dimerization as we had hypothesized, our findings provide evidence for homodimerization of the ligand binding subunits of FcγRI, a novel observation amongst Ig-superfamily FcR (1, 32). Unlike many cell surface receptors that exist as monomers, forming dimers only after binding ligand (25, 26), the α chains of FcγRI appear to homodimerize even in the absence of IgG, similar to the type II transforming growth factor-β (TGF-β) receptor and the insulin receptor which have been shown to express as ligand-independent dimers (25). We speculate that the Ig-like extracellular domains of the FcγRI α chains might self associate similarly to the heavy and light chains in Ig, which itself is a homodimerized molecule (28). Such direct association of ligand binding subunits has been reported for other receptors, including the stem-cell factor receptor (SCF-R), which relies upon specific interactions between the extracellular Ig-like domains for homodimerization (31).

The findings in this report led us to reassess our hypothesis of how γ chain association confers high affinity binding to the FcγRI α chains. Since FcγRI α chains require γ chain for high affinity (23) but not for homodimerization in COS cells,
association of γ chain could act to stabilize the dimers by altering the structure of the α chains. Several sites on IgG have been identified as being important for high affinity binding by FcγRI (33, 34), and perhaps a γ chain induced conformational change enables the α chains to bind these sites differently than dimers that do not associate with γ chain. Future studies will be required to determine the exact nature of the interactions between FcγRI and IgG.

We also present observations of another FcγRI-complex larger than both the α chain monomer and dimer that was immunoadsorbed by anti-FcγRI antibody. Similar to the α chain dimer, this complex contained proteins of the appropriate sizes for both the FcγRI α and γ chains in U937 cells (Fig.3.1; Fig.3.2). While this larger FcγRI complex was observed in COS cells transfected with both the α and γ chains (Fig.3.3, lane 3), it was not detected without γ chain cotransfection (Fig.3.3, lane 2). These results indicated that, unlike the FcγRI α chain dimer, the presence of this larger receptor complex appeared to be dependent upon γ chain association.

Due to its size, this complex could represent a tetramer consisting of dimers of α chain homodimers formed through γ chain association. The implications of how a receptor tetramer might affect ligand binding affinity is unclear. Interestingly, this complex was not detected in Western blot analysis with FcγRI α and γ chain COS cell cotransfectants (data not shown). While this result could be attributed both to inadequate amounts of recovered receptor as well as the decreased sensitivity of this
technique compared with iodination, it is also possible that this complex does not contain the FcγRI α chain. Additional analyses will be necessary to determine the components and role of this tetramer-sized complex.

Results from this study indicated that the structure of FcγRI was more complicated than anticipated. In U937 cells there was evidence for another potential component of the FcγRI complex of approximately 30 kD in size (Fig. 3.2 A), suggesting that the complete quaternary structure of FcγRI has yet to be defined. This 30 kD protein associated with both the α chain dimer and tetramer sized complexes in U937 cells, but was not detected in COS transfectants. Interestingly, the dimer complex resolved to a doublet when analyzed by two-dimensional SDS-PAGE, of which the 30 kD protein appeared to associate with the larger of the two bands in U937 cells. The dimer-doublet was not observed in COS cell transfectants, consistent with the absence of the 30 kD protein in these cells. While the identity of the associated 30 kD protein is unknown, it is tantalizing to speculate that it is a homologue of the 30 kD β chain, a signaling subunit associated with the FceRI (20).

The FceRI α chain associates with two signaling subunits, the γ and β chains. The γ chain interacts with the syk kinase, while the β chain subunit links the receptor to the src kinase Lyn (35). In a recent report we identified a phosphorylated protein of similar size (30 kD) that associated directly with FcγRI (36). In the same report (and in others, 37) the Src kinase Lyn was shown to associate with FcγRI, leading to the conclusion that possibly a β chain homologue was associating with the receptor, linking FcγRI to the Src kinases. The β chain is a member of a large family of molecules that
traverse the cell membrane four times (38-40), resulting in both the amino and carboxyl protein termini localized in the cytoplasm. While the FceRI β chain is reported not to be expressed in the same cells as FcγRI (20), it is possible that a homologue of β chain is associating with FcγRI. One such candidate is the tetraspan molecule HTm4, a protein expressed specifically in hematopoietic cell lines, including U937 cells (41).

We have utilized reverse transcription PCR amplification to obtain the cDNA for HTm4, and are currently analyzing it in COS cell transfections with FcγRI and γ chain.

While the FceRI γ and β subunits are thought to interact with and function through an α chain monomer, our findings indicate that γ and β-sized proteins associate with FcγRI α chain multimers. Neither the γ chain-sized protein nor the 30 kD protein were detected associating with the α chain monomer in our assays (Fig.3.2), suggesting that these subunits might be unable to interact with the monomer. Since γ chain is necessary to initiate cell signaling, this result could indicate that unlike FceRI, the FcγRI α chain monomer is inactive, and the receptor is functional only with homodimerized α chains.

An intriguing observation throughout these studies was the maintenance of the FcγRI complexes even in the absence of chemical crosslinker. While the abundance of α chain monomer indicated that most protein-protein associations were disrupted, some dimer and tetramer-sized complexes remained intact in Triton-X100, a non-ionic detergent that consistently disrupts the α and γ chain interactions. Since the native structure of integral membrane proteins is often maintained in non-ionic detergents, the
α chains may still be capable of association. It was more surprising, however, that these complexes survived SDS, an ionic detergent that usually disrupts the native conformation of proteins. There have been reports, however, suggesting that some integral membrane proteins maintain structural associations in ionic detergent conditions that completely denature soluble proteins (42, 43). In the presence of detergent and β-mercaptoethanol, however, the FcγRI α chain dimer associations were completely disrupted. The reduction of the intradisulfide bonds of each α chain probably destabilized the tertiary structure of the peptides, thus allowing better access to the SDS detergent molecules and denaturation of the proteins.

The structural complexity amongst FcR results in diverse cellular responses triggered after binding immune complexes. In this report we add to this structural heterogeneity with our evidence for homodimerization of the ligand binding α chains of FcγRI, a unique observation for FcR. Since our results indicated that γ chain association was not required for α chain homodimerization, the role of this subunit in conferring the high affinity of the FcγRI α chains for IgG remains unclear. In a human monocyte cell line we also detected a poorly characterized tetramer-sized FcγRI complex, as well as a 30 kD β chain-sized protein that, along with γ chain, associated with the FcγRI α chain dimer and tetramers but not with the monomers. The findings in this report suggest that the complete structure of FcγRI has yet to be defined, and therefore continued characterization of the quaternary structure of FcγRI is required to understand better the various functions of this receptor.
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Figure 3.1: Chemical crosslinking of iodinated surface proteins on a monocytic cell line, U937, reveals bands the appropriate size for an FcγRI α chain monomer (1x), dimer (2x), and tetramer (4x). U937 cells cultured in IFN-γ were radioiodinated, incubated with increasing concentrations of a cleavable bifunctional crosslinking reagent, Dithiobis-succinimidylpropionate (mM DSP) and lysed in Triton-X100 detergent. The adsorbate was eluted from anti-FcγRI mAb coated sepharose beads and analyzed by SDS-PAGE either in nonreducing (A) or reducing (B) conditions, and visualized with autoradiography. kD, kilodalton.
Figure 3.2: Two-dimensional analyses of iodinated U937 cells. IFN-γ cultured radioiodinated U937 cells were incubated either without (A) or with (B) the crosslinking reagent DSP, lysed in Triton-X100 and adsorbed with anti-FcγRI mAb coated sepharose beads. The eluates were analyzed by two-dimensional SDS-PAGE, nonreducing (NR) in the first dimension and reducing (R) in the second dimension, and visualized by autoradiography. kD, kilodalton.
Figure 3.2 continued
Figure 3.3: FcγRI α chain multimer-sized bands are present in iodinated COS cell transfectants. COS cells transfected (COS Tf) with no cDNA (mock; lane 1), FcγRI α chain cDNA (RI; lane 2) or FcγRI α and γ chain cDNAs (Rlγ; lane 3) were radioiodinated, lysed in Triton-X100 and adsorbed with anti-FcγRI mAb coated sepharose beads. The eluates were analyzed by SDS-PAGE analysis in nonreducing conditions. Bands the appropriate sizes for an α chain monomer (1x), dimer (2x) and tetramer (4x) were detected. Lanes 1 and 3 are from a longer exposure time. kD, kilodalton.
Figure 3.4: Western analyses of FcγRI α chain COS cell transfectants. (A) Triton lysates of COS cells transfected (COS Tf) with an FcγRI α chain cDNA engineered to express a C-terminal histidine epitope-tag (RI-H) or with no cDNA (mock) were affinity purified with nickel-charged beads. The eluates were analyzed by SDS-PAGE in nonreducing conditions followed by Western blot with rabbit anti-FcγRI antiserum and ECL detection. (B) The RI-H eluted adsorbate was analyzed by two-dimensional gel electrophoresis in nonreducing (NR) in the first and reducing (R) conditions in the second dimension, followed by Western blot analysis as in (A). kD, kilodaltons.
Figure 3.4 continued
Figure 3.5: FcγRI histidine and FLAG epitope-tagged α chain constructs. (A) Diagram of a carboxyl-terminal histidine (RI-H) and an amino-terminal FLAG (RI-F) epitope-tagged FcγRI α chain cDNAs. L, leader sequence encoded in the S1 and S2 domains; EC1, EC2, EC3, sequences encoding extracellular domains 1-3; TM/C, sequence encoding the transmembrane and cytoplasmic regions; Histidine, sequence encoding the six histidine residues of the epitope-tag; FLAG, sequence encoding the mouse preprotrypsin leader sequence and the eight residue FLAG epitope-tag. (B) Depiction of an experiment to show homodimerization of FcγRI α chains using RI-H and RI-F cDNAs in COS cell transfections. H, carboxyl-terminal histidine tagged FcγRI α chain; F, amino-terminal FLAG tagged FcγRI α chain; EC, extracellular region; Tm, transmembrane region; CT, cytoplasmic region; Ni²⁺, nickel charged beads.
Figure 3.5 continued
Figure 3.6: Association of Histidine-tagged and FLAG-tagged FcγRI α chains. Detergent lysates of COS cells transfected (COS Tf) with no cDNA (mock; lane 1), a histidine-tagged α chain cDNA (RI-H; lane 3), a FLAG-tagged α chain cDNA (RI-F; lane 4) or an equal ratio of both cDNAs (RI-H/RI-F; lane 2) were affinity purified with nickel charged beads. The eluates were analyzed by SDS-PAGE in reducing conditions followed by Western blot with anti-FLAG mAb and ECL detection. kD, kilodalton.
3.6 LIST OF REFERENCES


CHAPTER 4

CHARACTERIZATION OF A NOVEL TWO-EXTRACELLULAR DOMAIN FcγRI ISOFORM: A PRODUCT OF THE B GENE

Kathy L. Miller, Anne-Marie Duchemin, and Clark L. Anderson
4.1 INTRODUCTION

Antibodies, or immunoglobulin (Ig), are proteins that bind to antigenic substances in the body via the variable, or Fab portion, forming immune complexes (1). The Fc portion of Ig is bound by FcR, cell surface receptors that are members of the Ig-superfamily (2). After binding to Ig immune complexes, these receptors provide an important link between the cellular and humoral branches of the immune system by triggering a myriad of defense responses which ultimately remove antigens from the body. The class of FcR that bind IgG is comprised of three members, FcγRI, FcγRII and FcγRIII (3). Since the diversity of functions among these FcγR has been attributed to their complexity of structure, it is necessary to characterize the structure-function relationships of these receptors to gain an insight into the specific roles FcγR have in the immune system.

The high affinity FcR for IgG, FcγRI, consists of two integral membrane proteins. The 72 kD ligand binding α chain (4) noncovalently associates with the 20 kD signaling subunit γ chain (5, 6), which is required for receptor function (7, 8). The FcγRI α chain is encoded in humans by three highly homologous genes, A, B and C (9, 10). Although these genes share 98% homology at the nucleotide level, they produce a total of six distinct transcripts, FcγRI a1, a2, b1, b2, b3 and c, which predict structurally diverse
proteins due to differential splicing and amino acid substitutions and insertions (10, 11). The two transcripts currently under investigation are FcγRI a1, a product of gene A believed to represent the α chain of the bona fide high affinity FcγRI, and FcγRI b2, a spliced product of gene B that predicts a receptor structurally similar to the other members of the FcγR class, FcγRII and FcγRIII (10).

The a1 transcript encodes an α chain that appears to typify the genuine FcγRI both structurally and functionally. The a1 transcript encodes a three extracellular (EC) domain, transmembrane-spanning α chain similar in sequence to a previously characterized FcγRI cDNA (12). An RNAse protection analysis indicated that a1 is the most abundant FcγRI transcript expressed in U937 cells (10), a human monocyte cell line that naturally expresses the receptor (13). In agreement with prior studies of FcγRI with native cells (4, 13), cDNA representing the a1 transcript transfected in COS cells expresses a membrane-bound 72 kD α chain protein at the cell surface that is recognized by all FcγRI monoclonal antibodies (mAbs) (11). The a1 α chain binds human and rabbit IgG in addition to showing the expected isotype specificity (3) for murine IgG, binding to IgG2a but not to the remaining murine IgG isotypes. Association of the a1 α chain with the FcγRI γ chain subunit, which, in addition to cell signaling is required for high affinity binding of IgG (14), is reconstituted in COS cell cotransfections. Together, these results indicate that the isoform encoded by the a1 transcript has the expected characteristics of the FcγRI α chain.

The b2 transcript represents a potentially novel isoform of FcγRI. There are several gene specific differences that predict amino acid discrepancies between the b2
and α1 isoforms (9, 10). N-terminal in the second EC (EC2) domain, a Thr in α1 is altered to Met in b2, while C-terminal in the same domain a Val in α1 is replaced by a Gln and Tyr. In the cytoplasmic region there is a single predicted residue difference of an Asp in α1 to Asn in b2. The most distinct structural difference is that b2 splices the EC2 domain directly to the transmembrane region eliminating the third EC (EC3) domain of the receptor (10). While the predicted amino acid sequence of the EC3 domain of FcγRI is unique amongst FcγR, the first two EC domains share significant homology with the corresponding domains of FcγRII and FcγRIII (12). Therefore, b2 could represent an FcγRI isoform similar in structure and function to these receptors, suggesting the potential for both a high and low affinity FcγRI. The size of the b2 protein product as determined by in vitro translation analyses is approximately 45 kD (11), similar to the reported sizes of both FcγRII and FcγRIII (15).

Despite being the second most abundant FcγRI transcript in U937 cells (10), it is unclear whether b2 expresses a protein product in these cells. While a 45 kD b2-sized protein was copurified with the α1 α chain from iodinated U937 cells with anti-FcγRI antibody, a similar result was also observed with iodinated α1 COS cell transfectants. Since no b2 was expressed in the α1 COS cell transfectants, this result raised doubt as to the identity of the b2-sized protein detected in U937 cells.

In this study we analyzed the FcγRI b2 isoform in comparison to FcγRI α1 utilizing several new approaches with the aim of determining the structures responsible for the differences in expression and ligand binding observed between the two isoforms. We developed an anti-FcγRI cytoplasmic domain-directed antibody capable
of detecting both the a1 and b2 isoforms, which we used in both immunoadsorptions and Western blot analyses. FLAG epitope-tagged a1 and b2 constructs were engineered and transfected into COS cell transfectants to compare the ligand binding and cellular localization of the two FcγRI isoforms. Our findings give the first definitive evidence that the FcγRI b2 transcript expresses a protein product and also suggest the possibility that this isoform might have an alternate function from FcγRI a1.
4.2 MATERIALS AND METHODS

Cells and Cell Culture. U937, a human monocyte cell line from ATCC was maintained in RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 2mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. COS-7 cells were cultured in Dulbecco’s Modified Eagle Medium with the supplements listed above. U937 cells were cultured with 100 U/ml human recombinant IFNγ (Genentech, San Francisco, CA) 48 hours before each experiment to increase FcγRI expression.

Plasmid Constructs and Transfections. The human FcγRI a1 and b2 cDNAs were cloned into the pCDM vector, as described by Ernst et al (5). The human γ-chain cDNA in vector pSVL was a kind gift from Dr. Jean-Pierre Kinet (National Institute of Allergy and Infectious Diseases, Bethesda, MD). A truncated a1 cDNA, a1-CT, was constructed and subcloned into the pCDM vector as described previously (14, chapter 2). Two cDNAs were constructed by exchanging the cytoplasmic regions of a1 and b2 utilizing a shared SnaBI site located upstream of the sequence encoding the single residue difference within the TM/C domains and a common vector site located 3’ of the cDNA inserts (Fig.4.1 A), resulting in an a1 chimera encoding the b2-specific Asn residue (a1CTb2) and a b2 chimera encoding the a1-specific Asp residue (b2CTa1). An
a1 cDNA chimera containing sequences encoding the four b2 residues in EC2 (a1E2b2) was constructed by utilizing both a1 and b1 cDNAs (Fig.4.1 B). The EC2 sequences of b2 and b1, a cDNA representing the full-length transcript from FcγRI gene B, are identical, although the b1 cDNA encodes the gene B-specific premature stop codon in the EC3 domain (9, 10). An NsII endonuclease restriction site in EC3 of a1 and b1 (upstream of the premature termination codon) and a shared vector site 5′ of the cDNA inserts were digested and the b1 EC2 fragment was isolated and ligated into the a1 backbone. A b2 chimera containing the EC3 domain of a1 (b2+E3) was constructed using the shared EC3 NsII endonuclease restriction site and a SnaBI site within the TM/C domain of both the a1 and b1 cDNAs. The a1 EC3 domain was isolated and substituted into the b1 cDNA (Fig.4.1 B). An a1 cDNA lacking sequence encoding the EC3 domain was engineered using ligation PCR (Fig.4.2). Oligonucleotides for PCR were synthesized by Gibco BRL Life Technologies (Grand Island, New York). PCR was performed by adding 25 ng of a1 cDNA into a 100-μl volume reaction containing 10 mM Tris-HCL (pH 8.85), 2.0 mM MgSO₄, 25 mM KCl, 5 mM (NH₄)₂SO₄, 200 μM each of four dNTPs, 0.5 μM of each oligonucleotide primer, and 5 U of Pwo DNA polymerase (Boehringer Mannheim). The sense primer (p1A), 5′-ATG GAG CCG CTG CTA CGT-3′, coinciding with nucleotides 1-15 in S1, and the antisense primer (p82), 5′-CTG CTG ATG TGT AGC GAT GC-3′, the reverse compliment of nucleotides 518-538 in EC2, were used to PCR amplify the a1 cDNA. The sense primer of a second set of primers (p37A), 5′-GAA TAT CTG TCA CTG TGA-3′, corresponding to EC2 nucleotides 539-556 of the a1 cDNA in the region differing from the b2 nucleotide sequence, and
the antisense primer (p2A), 5'-ACT GAG CCG CTG CTA CGT-3', coinciding with
the reverse complement of TM/C nucleotides 1120-1137, were used to PCR amplify
the b2 cDNA. The two PCR products were ligated and PCR amplified with p1A and
p2A, and the resulting cDNA was cloned into the pCR2.1 vector (Invitrogen, San
Diego, CA) after addition of a 3' adenosine-overhang using Taq polymerase
(Boehringer Mannheim). The a1-E3 chimera was transferred to the pCDM expression
vector in the correct 5' to 3' orientation. As with the a1 cDNA, the b2, a1E2b2 and
a1-E3 cDNAs were constructed to express fusion proteins containing the amino-
terminus FLAG octapeptide epitope-tag (a1F, b2F, a1E2b2F, a1-E3F) (Eastman Kodak
Company, New Haven, CT) as described in chapter 3. The 5' and 3' ends as well as the
modified regions of the chimeras were sequenced by the chain termination method (16)
using Sequenase 2.0 (Amersham Life Science, Cleveland, OH) to verify that the cDNA
nucleotide sequences were correct. The 5' and 3' ends of all FLAG-cDNA constructs
were also sequenced to confirm in-frame insertion into the epitope-tag sequence within
the pFLAG-CMV-1 vector (Eastman Kodak Company). The cDNAs were transiently
transfected into COS-7 cells by the diethylaminoethyl-dextran method as described
previously (14) using 1-2µg/ml of DNA, and the cells were analyzed 48 hours after
transfection.

Antibodies. Anti-FcγRI mAbs 22 (mIgG1), 32 (mIgG1), 3G8 (mIgG1) and 197
(mIgG2a) were acquired from Medarex (Annandale, NJ). The anti-FcγRI mAb 10.1
(mIgG1) was the kind gift of Dr. Nancy Hogg. The anti-FcγRI cytoplasmic tail mAb
CT6 (mIgG1) and rabbit anti-FcγRI cytoplasmic domain peptide antiserum were
prepared in collaboration with Dr. Pravin Kaumaya (The Ohio State University) against an 18-residue peptide (H$_3$N$^+$-VTI RLE LKR KKK WNL EIS-COO$^-$, residues 312-329) as previously described (11). The rabbit anti-\(\gamma\) chain serum was a generous gift from Dr. Jean-Pierre Kinet (National Institute of Allergy and Infectious Diseases), and mAb anti-\(\gamma\) chain 4D8 (mIgG2b) from Dr. Jarema Kochan (Hoffman-La Roche, Nutley, NJ). Anti-FLAG mAb M2 (mIgG1) was obtained from Eastman Kodak Company.

Immunoadsorptions and Western Blot Analysis. Cells were lysed for 1 hour at 4°C with TritonX-100 buffer (1% TritonX-100 (Sigma, St. Louis, MO), 2mM phenylmethylsulfonyl fluoride (PMSF; Sigma) in PBS) and the supernatants clarified by centrifugation at 15,000 \(\times\) G for 20 minutes. Anti-Fc\(\gamma\)RI CT6 was bound to Sepharose 4B (Pharmacia, Piscataway, NJ) coupled to goat F(ab')2 anti-mouse IgG (Pierce, Rockford, IL) by incubating 50 \(\mu\)l of packed beads with 100 \(\mu\)l of a 1:1 dilution of the CT6 antiserum. The rest of the mAbs were coupled to the Sepharose-anti-mouse beads by incubating 100 \(\mu\)l of 10\(\mu\)g/ml mAbs for 4 hours at 4°C. For the preclearing experiment, the cell lysates were adsorbed for four hours at 4°C on the first set of antibody coated beads. The supernatant was transferred to the second set of antibody conjugated beads and incubated overnight at 4°C. Immunoadsorbed proteins were eluted by boiling in Laemmli sample buffer with or without 5% 2-mercaptoethanol, separated by SDS-PAGE in parallel with Rainbow protein molecular weight markers (Amersham) and electrophoretically transferred to nitrocellulose membranes in a buffer of 25 mM Tris base, 190 mM glycine, 0.01% SDS and 20% methanol. Membranes were incubated overnight at 4°C in TBS-Tween (10 mM Tris-
HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 5% dry milk as a blocking agent. After blocking, the blots were incubated in TBS-Tween with either 10 μg/ml anti-FLAG mAb M2, a 1:250 dilution rabbit anti-γ chain serum for 1 hour at room temperature or a 1:250 dilution of rabbit anti-FcγRI cytoplasmic domain peptide antiserum overnight at 4°C. After four washes the membranes were incubated with a 1/1000 dilution of horseradish peroxidase-linked protein G (Biorad, Hercules, CA), and detected by Enhanced Chemiluminescence (ECL, Amersham).

Immunofluorescence Microscopy. Approximately 1-2 x 10⁵ transfected COS cells were seeded into 24 well plates on glass coverslips and cultured overnight. After washing in PBS, some cells were fixed in cold methanol for 7 minutes at -20°C and permeabilized for intracellular staining by incubating with 0.1% tween 20 in PBS for 10 minutes at room temperature. The cells were washed twice in PBS and incubated at 4°C for 30 minutes with 150 μl of 10μg/ml mAbs diluted in PBS, 0.1% BSA, 0.1% NaN₃ (PBS+). Cells to be analyzed for surface staining were washed twice in PBS and incubated with mAbs as described above. The COS cell transfectants, both with and without permeabilization, were washed three times in PBS+ for 2 minutes and incubated with a 1:50 dilution of FITC conjugated goat F(ab')₂ anti-mouse IgG (Caltag Laboratories, San Francisco, CA) in PBS+ for 20 minutes at 4°C in the dark. The cells were washed three times in PBS+, fixed in 1% paraformaldehyde and observed under a fluorescent microscope in oil immersion with 1000x magnification.

Flow Cytometry Analysis. 2 x 10⁴ COS-7 COS cells that had been transfected 48 hours earlier with cDNAs were sequentially incubated with anti-FLAG or anti-FcγRI mAbs and
with FITC-labeled F(ab')\textsubscript{2} fragments of goat anti-mouse IgG (Caltag Laboratories) and were analyzed on an Elite EPICS FACS (Coulter, Hialeah, Fl). Fluorescence data from 10,000 cells were expressed as the number of positive cells (percentage of the cells brighter than the mock transfected COS-7 cells).

*sRosetting Assays.* Human and sheep erythrocytes were coated with IgG by washing 100 \(\mu\)l of packed erythrocytes with PBS followed by incubation with subagglutinating concentrations of rabbit IgG (anti-sheep erythrocytes), human IgG (anti-Rh, RhoGam), or mouse IgG switch variant mAb (mIgG\textsubscript{1}, 2a or 2b) anti-human glycophorin A (17, a considerate gift from Dr. Jan van de Winkel) at 37°C for 45 minutes and washed in PBS. The opsonized erythrocytes were added to 24 well plates in which 2x10\textsuperscript{4} COS cell transfectants had been cultured overnight on glass coverslips. The plates were centrifuged at 400 g for 3 minutes and incubated at 37°C for 60 minutes. After washing three times in PBS to remove unattached erythrocytes, the transfected COS cells were fixed with 1% paraformaldehyde and examined under an inverted microscope at 400x magnification. The percentage of rosettes formed represents the number of cells binding erythrocytes versus the total number of cells per well.
4.3 RESULTS

Anti-FcγRI mAb Directed Against the Cytoplasmic Domain Recognizes Both FcγRI a1 and FcγRI b2. Anti-FcγRI antibodies directed to the extracellular region of the three-EC domain FcγRI a1 might not recognize FcγRI b2 due to the absence of the third extracellular domain, a speculation supported by our preliminary studies with FcγRI b2 transfected COS cells. To enhance the probability of detecting the b2 isoform, we designed a peptide against the cytoplasmic region of FcγRI a1 from which a monoclonal antibody for immunoadsorptions (mAb CT6) was developed. There is, however, a single amino acid difference between a1 and b2 in the region the peptide antibodies recognize (a1:Asp(215); b2:Asn(215)) which could potentially prevent binding to the b2 isoform. To determine if mAb CT6 is capable of binding to the cytoplasmic region of b2, an FcγRI a1 cDNA chimera containing the cytoplasmic domain of b2 was engineered (a1CTb2). Radioiodinated COS cells transfected with either a1CTb2 or a1 cDNAs were lysed, immunoadsorbed and analyzed on SDS-PAGE. Although mAb CT6 purified both 70 kD α chain proteins, the band representing a1CTb2 (Fig.4.3 A, lane 5) was not as intense as the a1 band (Fig.4.3 A, lane 2), indicating less adsorption of the chimera. Since the a1CTb2 and a1 bands appeared similar when adsorbed with an anti-FcγRI extracellular domain mAb 32 (Fig.4.3 A, lanes 1, 4), this suggested the intensity
difference observed with mAb CT6 adsorption was not simply due to lower expression levels of a1CTb2. Additionally, while similar amounts of a1 were adsorbed with mAbs 32 (Fig. 4.3 A, lane 1) and CT6 (Fig. 4.3 A, lane 2), much less a1CTb2 was recovered with mAb CT6 (Fig. 4.3 A, lane 5) as compared to mAb 32 (Fig. 4.3 A, lane 4). These results suggested that mAb CT6 can bind the b2 cytoplasmic region, but with low affinity due to the single b2-specific amino acid difference in the cytoplasmic region. Adsorption with an IgG isotype control (mAb 3G8; Fig. 4.3 A, lanes 3, 6) did not adsorb any iodinated protein.

We tested rabbit polyclonal antiserum raised against our cytoplasmic peptide (RaCT) in Western blot analysis after finding that mAb CT6 did not detect the FcγRI α chain in this method. Western blot analysis of both a1 and a1CTb2 COS cell transfectants adsorbed with mAb CT6 indicated that RaCT detected both a1 (Fig. 4.3 B, lane 1) and a1CTb2 (Fig. 4.3 B, lane 2). Similar to the iodination results, the a1CTb2 band was less intense than the a1 band. The surface expression levels of these transfected receptors appeared roughly similar as determined by rosetting assays. These assays, which measured the ability of the receptors to bind IgG coated sheep erythrocytes (which mimic immune complexes), indicated that approximately 30% of a1CTb2 transfectants as compared to 17% of a1 transfectants formed rosettes. Since the extracellular domains of the receptors are identical (thus predicting identical ligand binding ability) these results indirectly suggested that a1CTb2 expressed similar to a1 in the COS cell transfectants. The decreased amount of the a1CTb2 protein detected in the Western blot, therefore, is
likely a result of a decreased affinity of both mAb CT6 and RaCT for the b2 cytoplasmic region. No protein was detected from mock transfected COS cells (Fig.4.3 B, lane 3).

Evidence for FcγRI b2 Protein Expressed in a Human Monocytic Cell Line, U937. We analyzed U937 cells, which express relatively abundant amounts of b2 transcript (10), for the presence of the b2 protein by adsorbing cell lysates with anti-FcγRI antibodies. Detection of the b2 isoform in these cells was not straightforward, however. Since there is more α1 than b2 transcript expressed in U937 cells, there is also likely to be more α1 than b2 protein in these cells. Additionally, while anti-FcγRI mAb CT6 recognizes b2, it appears to bind α1 with a higher affinity. To enhance the chances of adsorbing the b2 isoform, U937 lysates were first incubated with an anti-FcγRI antibody, mAb 22, directed against the extracellular region of the α chain to remove the majority of the α1 protein. The precleared lysates were then re-adsorbed with either mAb CT6 or a series of FcγRI extracellular-directed antibodies, 22, 32, 10.1, 197, and analyzed in Western blot using a rabbit anti-FcγRI polyclonal antiserum. While every anti-FcγRI antibody re-adsorbed some remaining 70 kD α1 protein, only mAb CT6 adsorbed a distinct 50 kD b2 protein (Fig.4.4, lane 2). The anti-FcγRI mAb 197, however, appears to have adsorbed a less distinct protein the approximate size of b2 (Fig.4.4, lane 6). The mAb 197, a murine IgG2a, is an IgG isotype that FcγRI α1 binds with high affinity (4), suggesting the possibility that this antibody might detect b2 either directly or as ligand. None of the other anti-FcγRI antibodies adsorbed a b2-sized protein (Fig.4.4, lanes 3-5). The IgG mAb 3G8 (Fig.4.4, lane 1), which has no specificity for FcγRI, does not adsorb any α1 or b2 protein.

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FcγRI b2 FLAG Epitope-tagged Construct is Expressed in COS Cells and Associates with γ Chain. Since all native cells that we tested expressed both a1 and b2 transcripts, it was necessary to transfec b2 cDNA into COS cells, which do not express FcγRI, to enable analyses of the b2 isoform in an environment independent from a1. Unfortunately our initial attempts to immunoadsorb protein from cells transfected with b2 cDNA were unsuccessful, both with iodination and Western blot analysis, possibly due to the low affinity of mAb CT6 for this isoform. To assist in the detection of this isoform, we engineered amino-terminal FLAG epitope-tagged FcγRI a1 and b2 constructs (Fig.4.5). The leader sequence of FcγRI was replaced by the murine preprotrypsin leader sequence preceding the FLAG sequence in the CMV-1 vector. This leader sequence is required to direct newly synthesized proteins to the endoplasmic reticulum for membrane insertion. When expressed in COS cell transfectants, the FLAG fusion proteins were detected by a mAb directed against the FLAG epitope.

FcγRI b2 FLAG (b2F) cDNA was transfected into COS cells and the fusion protein was adsorbed with anti-FLAG mAb M2 followed by analysis in Western blot with the same antibody. The FcγRI a1-FLAG (a1F) protein adsorbed from COS cell transfectants revealed the expected 70 kD protein (Fig.4.6 A, lane 1). A 50 kD protein was adsorbed from the b2F transfectant indicating that the protein was being translated in the COS cells (Fig.4.6 A, lane 2). The b2F band was not as intense as the a1F band, however, suggesting that less b2 was available for adsorption. No protein was adsorbed from COS cells transfected only with the FLAG vector (Fig.4.6 A, lane 3).
To determine if b2, despite structural differences from a1, was capable of associating with the FcγRI signaling subunit γ chain, COS cells were transfected with a1F or b2F, both with and without γ chain cDNA. The transfectants were lysed in digitonin, a detergent that allows maintenance of γ chain association (5), followed by adsorption with either anti-FLAG mAb M2 or anti-γ mAb 4D8. Both a1F and b2F cotransfected with γ chain and adsorbed with anti-FLAG antibody copurified a 14 kD protein recognized by rabbit anti-γ antiserum in Western blot analysis (Fig. 4.6 B, lanes 2, 5) that was the same size as the protein adsorbed with anti-γ antibody 4D8 (Fig. 4.6 B, lanes 3, 6). The 14 kD band was absent in conditions where a1F and b2F were transfected without γ chain (Fig. 4 B, lanes 1, 4).

Immunofluorescent Microscopy and Flow Cytometric Analysis of FcγRI-FLAG COS Transfectants Reveals the Majority of b2 Receptor is Located Intracellularly.

Having shown that the b2 protein was expressed in COS cell transfectants, we next determined the cellular localization of this isoform in comparison to a1. COS cells transfected with cDNAs for a1F, b2F or FLAG vector, pFV, (Fig. 4.7 A; Fluorescence), were incubated with anti-FLAG mAb M2 followed by a fluorescent secondary antibody. Phase microscopy depicted the cell shape and perimeters (Phase). To insure detection of cell surface proteins, COS cell transfectants were not fixed prior to incubation since fixation could permeabilize the cells. Fluorescence microscopy of the a1F transfectants revealed many cells with bright fluorescent staining defining both the outline and the surface of the cell (a1F, Fig. 4.7 A). As expected for the α chain of FcγRI, these results indicate an abundance of a1F protein at the cell surface of these transfectants. In
comparison, fewer COS cells transfected with b2F showed any positive surface fluorescence. With only an occasional bright cell, most positive b2F transfectants displayed faint, diffuse fluorescence that barely defined the perimeter of the cell (b2F, Fig.4.7 A), suggesting that less b2 protein was at the cell surface compared to the a1 transfectants. These results were confirmed by flow cytometric analysis of these cells which indicated only 10% of the b2 transfectants were brighter than the negative controls as compared to 40% measured for a1 transfected COS cells. Only background fluorescence was detected for pFLAG vector transfected COS cells (pFV, Fig.4.7 A).

Some transfected COS cells were fixed and permeablized to allow for detection of cytoplasmic expression of the two FcγRI isoforms using fluorescence microscopy (Fig.4.7 B, Fluorescence). Phase microscopy depicted the cell shape and perimeters (Phase). Some a1F transfectants showed predominantly surface staining, as depicted by the portion of the cell to the left in Fig.4.7 B, while other cells showed both surface staining along cytoplasmic staining, as shown in the edge of the cell to the right (a1F, Fig.4.7 B). The staining of the permeablized b2F transfectants, however, was restricted to the cytoplasm (b2F, Fig.4.7 B). Bright fluorescence around the nuclear envelope extended throughout the cell in a meshed, net-like pattern suggestive of the endoplasmic reticulum (18-20). Although the shape of the cells were delineated by this reticular pattern, there was no defined cell perimeter, indicating little b2 receptor was localized at the cell surface. COS cells transfected with the FLAG vector showed only background levels of fluorescence (pFV, Fig.4.7 B) Cotransfection of γ chain with both a1 and b2 did not alter the cellular localization patterns of the FcγRI isoforms (data not shown).
FcyRI b2 Binds IgG in Rosetting Assays. For the b2 isoform to function as an FcγR it must bind IgG. The ability of this isoform to bind immune complexes in comparison to a1 was tested by incubating transfected COS cells with sheep erythrocytes coated with IgG. By mimicking immune complexes, IgG coated erythrocytes in rosetting assays are capable of detecting receptors that bind monomeric ligand with both high and low affinity. Since FcγRI b2 is structurally similar to the low affinity two-EC domain FcγRII and FcγRIII α chains, we wanted to determine if it also bound IgG with the same isotype specificity as these receptors. Therefore, in addition to rabbit IgG, which FcγR readily bind, and a mixture of human IgG, we analyzed ligand binding using several murine IgG isotypes in the rosetting assays. FcγRI a1 and the lower affinity FcγRIII have binding specificity for IgG2a, while the low affinity FcγRII shows specificity for IgG1 and IgG2 (3).

Abundant rosettes (35-40%) were formed on the a1F transfected COS cells with erythrocytes coated with rabbit IgG (Fig.4.8 A), a mixture of human IgG, and the murine IgG2a isotype. Since typically less than half of the cells in transient assays are successfully transfected, these results indicated that the majority of a1 expressing cells formed rosettes. Despite immunofluorescent evidence indicating approximately 10% b2-F cell surface expression, only rare rosettes (1-3%) were detected on b2F transfected COS cells with rabbit IgG coated erythrocytes. Although some rosettes formed on the b2F transfectants were as dense as those formed on a1, most were less robust (Fig.4.8 B). No rosettes were formed on b2 transfectants by EA coated with either the mixture of human IgG or the murine IgG1, 2a or 2b isotypes. Cotransfection of γ chain with b2F did not measurably
improve the ligand-binding efficiency of this isoform. No rosettes were formed on COS cells transfected with the pFLAG vector alone (data not shown).

_FcγRI Chimeras are Utilized to Analyze the Structural Differences between the α1 and β2 Isoforms._ The differences in surface expression and ligand binding observed between the FcγRI α1 and β2 isoforms can be attributed to the structural heterogeneity between the two isoforms. In an attempt to further dissect this structure-function relationship, several FcγRI α1/β2 chimeras were constructed and analyzed in COS cell transfectants. Our strategy was to mutate α1 to be more like β2, and β2 more like α1, focusing on the individual differences between the isoforms. Several chimeras addressed the single residue difference between the isoforms in the cytoplasmic region. Similar to α1CTβ2 (Fig. 4.3; Table 4.1, column 2) which contains the β2-specific Asn, the cytoplasmic region of β2 was replaced with that of α1, resulting in a β2 chimera, β2CTα1, containing the α1-specific Asp residue (Table 4.1, column 6). An engineered premature stop codon in an α1 cDNA resulted in a construct, α1-CT, lacking the entire cytoplasmic region (Table 4.1, column 3). Several chimeras also addressed the extracellular differences between the α1 and β2 proteins. The EC2 domain of β2 was substituted into α1 producing a chimera, α1E2β2 (Table 4.1, column 4), that differs from the α1 isoform (Table 4.1, column 1) by only the three β2-specific amino acids in the EC2 domain. The chimera β2+E3 (Table 4.1, column 5) is identical to α1E2β2, except it also contains the β2-specific asparagine residue in the cytoplasmic region. In the chimera α1-E3 (Table 4.1,
column 7), the EC3 domain of the α1 isoform was deleted to create a two-extracellular structure similar to the β2 isoform (Table 4.1, column 8) except that the EC2 domain contained α1-specific residues.

The chimera COS cell transfectants were analyzed by immunofluorescence microscopy and/or flow cytometric analyses utilizing a series of anti-FcγRI mAbs including anti-FcγRI cytoplasmic mAb CT6 and a panel of extracellular domain-directed antibodies (mAbs 22,32,197). The chimeras were also examined in rosetting assays with human, rabbit and murine IgG, as summarized on Table 4.1. Results of the α1CTβ2 chimera were identical to those of α1, and neither ligand binding nor anti-FcγRI mAb recognition were rescued by the α1-specific cytoplasmic residue in the β2CTα1 chimera. The truncated α1-CT chimera results mirrored α1 (except for recognition by the CT6, which was negative since this construct did not contain the cytoplasmic region).

Cotransfection of γ chain with the chimeras did not alter the results. These findings indicated that the cytoplasmic domain did not alter protein structure in a manner that affected either ligand binding or recognition by anti-FcγRI mAbs.

Results from analyses of the extracellular domain chimeras α1E2β2, β2+E3 and α1-E3 were similar to those observed for the β2 isoform. While there was slight recognition by mAb CT6 after cell permeabilization, the results were inconclusive and inconsistent. None of the extracellular domain-directed antibodies detected these chimeras. Additionally, no detectable rosettes were formed on the chimera COS cell transfectants with the IgG isotypes tested. Since α1-E3 is structurally similar to β2, this isoform might be expected to perform similarly in these assays. Results with the α1E2β2 and β2+E3
chimeras were more surprising, however, since there were only a few b2-specific amino acid differences between these chimeras and the a1 isoform. The similar observations between the a1E2b2 and b2+E3 chimeras further indicated that the single cytoplasmic residue difference did not appear to affect receptor function. There were no differences observed in the results when γ chain was cotransfected with the chimeras. Since the lack of both anti-FcγRI extracellular domain antibody recognition and ligand binding could be explained by inadequate cell surface expression, we engineered the a1-E3 and a1E2b2 chimeras to be expressed as N-terminal FLAG fusion proteins, as described earlier. The a1-E3-FLAG (a1-E3F) and the a1E2b2-FLAG (a1E2b2F) chimera cDNAs were transfected into COS cells.

The cellular localization of the chimeras when transfected in COS cells was determined by immunofluorescent microscopy as described above. Both the a1E2b2F and the a1-E3F chimeras showed similar surface fluorescence patterns as a1F, with bright fluorescence indicating ample cell surface expression (Fig. 4.9, Surface). After permeabilizing the COS cell transfectants, a combination of reticular and cell surface fluorescence was observed for both the a1E2b2F and the a1-E3F (Fig. 4.9, Intracellular). Despite the b2-specific changes in the chimeras, the cellular localization of these proteins did not dramatically differ from that observed with the a1F transfectants, suggesting that the cytoplasmic retention of the b2 isoform must be due to the combination of amino acid differences in the EC2 domain along with the loss of the EC3 domain. Only background fluorescence was detected with COS cells transfected with the FLAG vector (V).
The chimeras were also transfected in COS cells and re-analyzed for recognition by several anti-FcγRI extracellular-directed antibodies (mAbs 22, 32 and 197) or by the anti-FcγRI cytoplasmic mAb CT6 in immunofluorescent microscopy. While both alCTb2F and al-E3F were recognized by mAb CT6 after permeablizing the cells, neither chimera was detected by any anti-FcγRI extracellular antibody, confirming the results on Table 4.1. These findings suggested the importance of both the EC2 and EC3 domains for antibody recognition.

Since the anti-FLAG mAb immunofluorescence microscopy results indicated abundant cell surface expression, we analyzed the FLAG-chimera transfectants for the ability to bind human, rabbit or murine IgG complexes in rosetting assays. Supporting the findings on Table 4.1, rosettes did not form on the a1E2b2F and a1-E3F COS cell transfectants. In the same assays rosettes were formed on a1-F transfectants, but none were formed on the b2-F transfectants, suggesting that the chimeras bound IgG in the same inefficient manner as the b2 isoform. These results indicated that both the EC2 and EC3 domains each have important roles in binding ligand.

The sizes of the FcγRI chimeras transfected in COS cells were analyzed on Western blot with anti-FLAG mAb M2 after adsorption with the same antibody. While differing from a1F by only three amino acids in the EC2 domain, the a1E2b2F chimera was slightly smaller, indicating a faster mobility on SDS-PAGE than the a1F protein (Fig.4.10 A, lanes 2,3). Protein doublets approximately 50 kD in size were adsorbed from both the a1-E3F (Fig.4.10 B, lane 1) and b2F (Fig.4.10 B, lane 2) COS cell transfectants. Although b2F was not previously detected as a doublet (Fig.4.6 A), the proteins in this
assay were separated by SDS-PAGE in conditions that allowed for better resolution of proteins of this size. The a1-E3F protein band detected in the Western blot analysis was more intense than the b2F band, suggesting more a1-E3F protein was adsorbed. Due to its cytoplasmic localization, it is possible that some b2 protein is trapped in the insoluble material and lost after clarification of the cell lysate. No protein was detected in COS cells transfected only with the FLAG vector (Fig. 4.10 A, lane 1; B, lane 3).
4.4 DISCUSSION

The multiple genes and transcripts of the FcγRI α chain are characteristic of the diversity found within the human class of FcγR. Along with a transcript representing the \textit{bona fide} three-EC domain α chain of the high affinity receptor for IgG, FcγRI a1, a novel transcript predicting a two-EC domain FcγRI α chain isoform, FcγRI b2, has also recently been characterized (10). In this report we utilized an anti-FcγRI mAb directed to the cytoplasmic region of the receptor α chain to obtain evidence that FcγRI b2 protein is present in the human monocyte cell line U937, which expresses both FcγRI a1 and b2 transcripts (10). Compared with a1 protein levels, less b2 protein was detected by Western blot analysis (Fig.4.4). While the lower abundance of b2 protein is consistent with the reported FcγRI transcript levels in U937 cells, this finding could also be explained by a lower affinity of the antibody for the b2 isoform (Fig.4.3). The presence of b2 protein in cells known to naturally express FcγRI suggested this isoform was potentially functional and deserving of further studies.

Since it would be difficult to characterize b2 on U937 cells due to the abundance of a1 protein, we utilized transiently transfected COS cells, which do not express FcγRI. To insure detection of the transfected proteins, we constructed the FcγRI isoforms to express N-terminal FLAG epitope-tags (a1F, b2F) which were then
detected with a specific antibody. The 50 kD FcγRI b2 protein expressed in U937 cells and transient COS cell transfections (as an N-terminal FLAG epitope-tagged fusion protein, Fig.4.5) (Fig.4.6 A) is similar in size to the two-EC domain members of the FcγR class, FcγRII and III (3, 15), supporting the b2 transcript sequence which predicts a two-EC domain protein. In COS cell transfections the b2 protein associated with γ chain (Fig.4), the signaling subunit shown to associate with the FcγRI a1 α chain (5, 6, 21). While this finding was not entirely unexpected since the transmembrane domain (the region of γ chain association) is identical between FcγRI a1 and b2, it indicated that the amino acid substitution of Asn to Asp in the cytoplasmic domain of b2 did not interfere with γ chain association. Since the FcγRI b2 isoform, like a1, does not have known signaling sequences in its cytoplasmic domain, γ chain association further implies that b2 is a potentially functional isoform.

The FcγRI b2-FLAG fusion protein displayed different cellular localization and ligand binding efficiency as compared to FcγRI a1 in COS cell transfections. The majority of the b2 protein was localized within the cytoplasm of the cell in what appeared to be the endoplasmic reticulum (ER), while most of the a1 isoform was expressed at the cell surface as expected for the α chain of FcγRI (Fig.5). Although the a1 isoform is expressed at the cell surface in COS cell transfections without γ chain association, it is possible that FcγRI b2, similar to FcγRIIIa and the human FcεRI (22-25), requires association with the subunit to rescue surface localization. Potentially the structural differences in the FcγRI b2 isoform result in improper folding and retention of the protein in the ER. Association with γ chain might insure correct processing of
b2 allowing for transport to the cell surface. FcγRI b2 cotransfected with γ chain, however, still localized to the ER, indicating that the association of this subunit did not rescue cell surface expression. If the b2 isoform functions at the cell surface like FcγRI a1, it might require another associated subunit similarly to the murine FcεRI. The FcεRI α chain associates with the γ and β chain signaling subunits, and while the human receptor requires only γ chain association for expression in COS cell transfections, the murine FcεRI α chain requires association of both γ and β chains for surface localization (26). Possibly all components of FcγRI have not been defined yet, and another associated subunit could be required with γ chain for proper b2 receptor expression and function. Future plans, therefore, include the development of methods to study FcγRI b2 in a cell line that naturally expresses this isoform in order to determine the cellular localization as well as investigate the possibility of an additional associated subunit.

A series of FcγRI a1/b2 chimeras revealed that the discrepancies in surface expression observed between the two isoforms were not due to the single cytoplasmic residue difference, but rather were a result of the collective differences in the extracellular domains. Two FLAG-tagged chimeras containing the EC differences between the a1 and b2 isoforms showed abundant cell surface localization similar to the a1F isoform when analyzed in COS cell transfections (Fig.4.9). These findings indicated that separately, neither the b2-specific residues within the EC2 domain nor the absence of the EC3 domain disrupted intracellular protein folding, processing and transport in a detectable manner. The cytoplasmic localization of the b2 isoform,
therefore, appeared to be due to the combination of the EC2 domain alterations along with the absence of the EC3 domain. A similar phenomenon has been reported for the macrophage colony stimulating factor receptor, in which two separate mutations were silent when occurring individually, but changed the receptor function when combined (27). While apparent that the unique structure of FcγRI b2 results in its intracellular localization in the COS cell transfectants, the implications and significance of this observation remain to be investigated.

In this study we also analyzed the ability of b2 transfected in COS cells to bind IgG in rosetting assays. While the a1 COS cell transfectants displayed the expected IgG isotype specificity, the b2 transfectants did not appear to bind either human or murine Ig of any isotype. Therefore, we were unable to determine if the b2 isoform shares similar IgG specificity with FcγRII or FcγRIII, receptors that have a similar structure as FcγRI b2. The b2 protein did, however, bind rabbit IgG in rosetting analyses, supporting a role for this isoform as an FcγR. Although distinct, the rosettes formed on b2 transfectants were rare and inconsistently observed amongst assays. The small amount of the b2 isoform localized at the cell surface could explain the infrequent rosettes observed on the transfectants. Since most of the b2 isoform detected at the cell surface (by immunofluorescence analyses) did not form rosettes, this suggests there might be a minimum level of b2 surface expression required for efficient binding of immune complexes. It is possible that the surface expressed b2 did bind to both human and murine IgG, but was unable to form detectable rosettes in our analyses. The rosetting efficiency did not improve with cotransfection of γ chain with b2 in the COS
cells. Similar to our speculations pertaining to b2 surface expression, an additional associated subunit might also be required for efficient binding of IgG by this isoform.

To determine the structures within the b2 isoform responsible for inefficient IgG binding, the a1/b2 chimeras were analyzed in rosetting assays. We predicted that due to their structural similarities, a1-E3 would rosette similar to the b2 isoform while a1E2b2 would resemble the a1 isoform. Despite similar cell surface expression as the a1 COS cell transfectants, both chimeras appeared to bind IgG inefficiently. Interestingly, when the a1E2b2 chimera was analyzed by Western blot, it appeared slightly smaller in size than the a1 isoform (Fig. 9, lanes 2, 3). We speculated that since FcγR are glycoproteins, the b2-specific residues in the EC2 domain could be affecting glycosylation, resulting in the smaller size of the chimera. While none of the EC2 mutations in this chimera directly disrupt predicted N-linked glycosylation sites (10, 12), it is possible these residues altered protein conformation preventing either the addition or processing of glycosylation. The a1-E3F protein was similar in size to b2F, however both fusion proteins were detected as 45-50 kD doublets. The loss of the EC3 domain could also possibly affect the glycosylation state of these proteins. Similarly, glycosylation differences have been implicated in the size variations observed for the FcγRIII α chain (15). Deficient glycosylation of FcεRI results in improper folding and retention of the protein in the endoplasmic reticulum, however de-glycosylated FcεRI retains the ability to bind ligand (28). If alterations in glycosylation are responsible for the size differences observed with the chimeras, this would suggest that ligand binding rather than surface expression of FcγRI is dependent upon proper receptor
glycosylation. The potential for alterations in glycosylation and how such an occurrence would relate to the ligand binding of the b2 isoform remain to be investigated.

Although COS cell transfections represent an artificial environment, the cytoplasmic expression pattern observed with the b2 protein might actually reflect the true cellular localization of this isoform. FcγRI b2 could have an intracellular role, perhaps similar to that of the FcRn, the human homologue of the neonatal rat Fc receptor, FcRn. This receptor is structurally related to MHC Class I molecules (29) and binds IgG in a pH dependent manner, with high affinity at pH 6 and low affinity at physiological pH 7.5 (30, 31). FcRn is involved in IgG transport and has been postulated to have a role in regulating the catabolism of IgG (32, 33). Although this receptor is predicted to localize at the cell surface, it is thought that IgG passively enters cells and is bound by FcRn within the endosomes as the pH becomes acidic. Association with FcRn protects the IgG from degradation allowing for its return to circulation. Similar to our postulations for FcγRI b2, the α chain of FcRn requires an associated subunit (the extracellularly associated β2-microglobulin) for IgG binding (26, 34-36).

In addition to FcRn, there could be other proteins such as FcγRI b2 involved in the regulation and transport of IgG. This hypothesis is plausible since many FcR functions are redundant, presumably due to their importance in the immune system (37). There have been reports of b2-sized 45-50 kD intracellular IgG binding proteins in cells that also express FcγRI. An early study characterizing the bona fide high
affinity FcγRII reported the presence of a 50 kD protein in U937 cells detected with metabolic labeling but not with a surface labeling method, indicating intracellular but not cell surface localization (38). This protein was not specifically recognized by antibodies, but rather was purified by binding the murine IgG1 antibodies as ligand. In an unrelated study, an intracellular IgG binding protein was detected in a range of human cell types after induction with the cytokine IFN-α (39). While the FcγRI genes are induced with IFN-γ treatment (40-42), little is known about the effect of IFN-α on the expression of the genes. Similar to the previous report, this intracellular protein also bound to murine IgG1 as well as IgG2b, showing similar isotype specificity as the low affinity FcγR, FcγRII. This report is intriguing since we speculate that due to structural similarities FcγRI b2 might bind IgG similar to the low affinity FcγR. Although these studies did not investigate the function or identity of the intracellular IgG binding proteins, it is exciting to postulate that they actually represent FcγRI b2, a possibility we are currently pursuing.

While more extensive analyses will be required to fully characterize the ligand binding of the b2 isoform, the findings in this report were useful in identifying potential IgG binding sites within the a1 isoform. Parallels can be drawn between our results and studies of the IgG binding sites on the low affinity FcγR. The major binding site on both FcγRII and FcγRIII was shown to be contained within the EC2 domain of the α chains, although the site resides in different regions of the domain on each receptor (43-48). Single amino acid substitutions made within these sites reduced or abolished the affinity of the receptors for IgG. Additionally, the EC1 domain of
FcγRII was shown to be required for IgG binding, presumably to stabilize the α chain structure for proper binding via the EC2 domain (43). Since the a1E2b2 chimera differs from the a1 isoform by only three residues in the EC2 domain, the inability of this construct to rosette could imply that the major IgG binding site on the FcγRI a1 α chain is within the EC2 domain. The three b2-specific residues could be disrupting the binding site directly, or may be causing structural alterations to mask the site. The inability of the a1-E3 chimera to form rosettes suggests that like the EC1 domain of FcγRII, the presence of the EC3 domain could be required to stabilize the α chain for proper IgG binding. Alternatively, the EC3 domain, unique to FcγRI a1 and postulated to have a role in the high affinity binding of IgG (12, 14), could also contain a separate binding site.

The a1/b2 chimeras were also analyzed with anti-FcγRI extracellular domain-directed antibodies. Although the antibody epitopes were not specifically identified, the chimera results indicated that these sites were sensitive to the b2-specific structural alterations. Having lost the EC3 extracellular domain, it was not surprising that the b2 isoform and the a1-E3 chimera were not detected by the antibodies. The lack of anti-FcγRI extracellular antibody binding to the a1E2b2 chimera again pointed to the significance of the EC2 domain alterations and their potential to impact on protein structure.

This report presents the initial steps in the characterization of a novel FcγRI isoform, FcγRI b2. The results of these preliminary analyses suggest several potential roles for this isoform in the immune system. Future studies will require the
development of methods to differentiate the FcγRI isoforms since it appears cells that endogenously express b2 also express a1. Such techniques will assist in the determination of the true cellular localization and ligand binding capabilities of this isoform, as well as identify any additional associated subunits.
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Summary of analyses with FcγRI α1/β2 chimera constructs transfected into COS cells
Table 4.1: Summary of analyses with FcγRI α1/β2 chimera constructs transfected into COS cells. A series of FcγRI α1/β2 chimeras (columns 2-7) were constructed so that α1 was mutated to be more like β2 (column 8), and β2 mutated to be more like α1 (column 1). The cDNAs were transfected into COS cells and analyzed by immunoadsorption and immunofluorescence microscopy assays with anti-FcγRI cytoplasmic mAb CT6 or a series of anti-FcγRI extracellular mAbs (22, 32, 197) as described earlier. The transfections were also studied for ligand binding capacity in rosetting assays with rabbit, human and murine IgG coated sheep erythrocytes as previously described. Diagrams depicting the FcγRI constructs are above each column. Closed circles, α1-specific residues: T, threonine, V, valine, D, aspartic acid; open circles, β2-specific residues: M, methionine, Q, glutamine, Y, tyrosine, N, asparagine; EC, extracellular domains; TM, transmembrane region; CT, cytoplasmic region; *, cDNAs also engineered to express as N-terminal FLAG-tagged fusion proteins; +, positive result; -, negative result; +/−, inconclusive or inconsistent result.
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Figure 4.1: Diagram depicting the construction of FcγRI chimera cDNAs using shared restriction sites. FcγRI cDNAs a1 and b2 (A) or a1 and b2 (B) were digested with restriction enzymes, separated on agarose gels, and the appropriate fragments isolated and religated to create the chimera cDNAs a1CTb2 and b2CTa1 (A) and a1E2b2 and b2+E3 (B). SnaBI, Nsi I, restriction endonuclease sites; S, sequences from the S1 and S2 domains; EC1-3, extracellular domains; TM/C, transmembrane/cytoplasmic domain; T, threonine; V, valine; D, aspartic acid; M, methionine, Q, glutamine; Y, tyrosine; N, asparagine; term*, termination codon within EC3 of the b1 cDNA.
Figure 4.1 continued
Figure 4.2: Diagram showing the construction of an FcγRI a1 cDNA mutant lacking the EC3 domain. a1, FcγRI a1 cDNA; b2, FcγRI b2 cDNA; a1-E3, FcγRI cDNA lacking the EC3 domain; S1, S2, EC1, EC2, EC3, TM/C, FcγRI domains; p1A, p82, p37A, p2A, oligonucleotide primers; *, a1-specific sequence.
Figure 4.2 continued
Figure 4.3: Anti-FcγRI mAb CT6 recognizes the cytoplasmic region of both FcγRI a1 and FcγRb2. COS cells were transfected (COS Tf) with cDNAs for FcγRI a1 (a1), FcγRI chimera containing the cytoplasmic domain of FcγRI b2 (a1CTb2), or no cDNA (mock). (A) The COS cell transfectants were iodinated, lysed in Triton-X100 and adsorbed by sepharose beads coated with anti-FcγRI mAbs CT6, 32, or an IgG isotype control, 3G8. The eluates were analyzed by SDS-PAGE and visualized with autoradiography. (B) The eluates from transfected COS cells lysed in Triton-X100 and adsorbed with anti-FcγRI mAb CT6 coated sepharose beads were analyzed by Western blot with rabbit anti-FcγRI antiserum and ECL detection. kD, kilodaltons
Figure 4.4: Evidence for FcγRI b2 protein expressed in a human monocytic cell line, U937. U937 cells cultured in IFN-γ were lysed in Triton-X100 lysis buffer and preadsorbed with beads coated with the anti-FcγRI extracellular domain mAb 22. The lysates were re-adsorbed (IA) with an IgG isotype control (3G8; lane 1), anti-FcγRI cytoplasmic domain mAb (CT6; lane 2), or with a series of anti-FcγRI extracellular domain mAbs (22, 32, 10.1, 197; lanes 3-6). The eluates were analyzed by Western blot with rabbit anti-FcγRI antiserum and ECL detection. The arrow indicates the FcγRI b2-sized protein. kD, kilodaltons.
Figure 4.5: Diagram of FcyRI a1-FLAG and FcyRI b2-FLAG cDNA constructs. cDNAs were constructed to express amino-terminal FLAG epitope-tagged FcyRI a1 (FcyRI a1F) and FcyRI b2 (FcyRI b2F) fusion proteins. PCR amplified products of a1 and b2 cDNA sequences encoding from the EC1 domain through the termination codon of the isoforms were subcloned into the pFLAG-CMV-1 shuttle vector for E.coli and mammalian expression (Kodak). MPL, the murine preprotrypsin leader sequence preceding the FLAG sequence; FLAG, epitope-tag sequence; CMV, human cytomegalovirus promoter-regulatory region; poly A, polyadenylation sequence; SV40 ori, replication origin for efficient replication in COS cells; EC1-3, extracellular domains; TM/C, transmembrane/cytoplasmic domain; M, methionine; Q, glutamine; Y, tyrosine; D, aspartic acid; T, threonine; V, valine; N, asparagine.
Figure 4.6: Western analyses of FcγRI-FLAG COS cell transfectants. (A) Triton-X100 lysates of COS cells transfected (COS Tf) with pFLAG-CMV-1 vector (V; lane 1), FcγRI a1-FLAG (a1F; lane 2) or FcγRI b2-FLAG (b2F; lane 3) adsorbed with anti-FLAG mAb M2 coated sepharose beads. The eluates were analyzed by Western blot with anti-FLAG mAb M2 and ECL detection. (B) COS cells transfected with no cDNA (mock; lane 1), FcγRI a1-FLAG (a1F; lane 2), a1-FLAG with γ chain (a1Fγ; lanes 3, 4), b2-FLAG (b2F; lane 5), or b2-FLAG with γ chain (b2Fγ; lanes 6, 7) were immunoadsorbed (IA) with either anti-FLAG mAb M2 (>F; lanes 1, 2, 3, 5, 6) or anti-γ mAb 4D8 (>γ; lanes 4, 7) coated sepharose beads. The eluates were analyzed by Western blot with rabbit anti-γ chain antiserum and ECL detection. kD, kilodaltons.
Figure 4.7: Immunofluorescence and phase microscopy of FcyRI-FLAG a1 and b2 epitope-tagged COS cell transfectants. COS cells transfected with FcyRI a1-FLAG (a1F), FcyRI b2-FLAG (b2F), or pFLAG-CMV-1 vector (pFV) cDNAs were analyzed with immunofluorescent microscopy (Fluorescence) for cell surface (A) and intracellular (B) staining. The COS transfectants were incubated with anti-FLAG mAb followed by FITC-conjugated secondary antibody. Transfectants analyzed for intracellular staining were first fixed in methanol and permeabilized with 0.1% Tween. Phase microscopy depicts the cell morphology (Phase). Cells were visualized in fluorescent and phase microscopy with oil immersion at 1000x magnification. N, nucleus.
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Figure 4.8: FcγRI a1 and b2 FLAG constructs transfected in COS cells bind IgG in rosetting assays. COS cells transfected with FcγRI a1-FLAG (A) or FcγRI b2-FLAG (B) cDNAs were incubated with sheep erythrocytes coated with rabbit IgG, fixed in paraformaldehyde and visualized for binding with phase microscopy at 400x magnification. No rosettes were formed on mock transfected COS cells in the same assay.
Figure 4.9: Immunofluorescence microscopy of FcγRI α1/ β2-FLAG epitope-tagged chimeras transfected in COS cells. An α1 cDNA lacking the EC3 domain, in pFLAG-CMV-1 (α1-E3F), an α1 chimera containing the EC2 domain of β2, in pFLAG-CMV-1 (α1E2β2F), an α1-FLAG cDNA (α1F), a β2-FLAG cDNA (β2F) or pFLAG-CMV-1 vector (V) were transfected into COS cells and analyzed by immunofluorescent microscopy for cell surface (Surface) or intracellular (Intracellular) staining. The COS transfectants were incubated with anti-FLAG mAb followed by FITC-conjugated secondary antibody. Transfectants analyzed for intracellular staining were first fixed in methanol and permeabilized with 0.1% Tween. Cells were visualized in fluorescent and phase microscopy with oil immersion at 1000x magnification. N, nucleus.
Figure 4.9 continued
Figure 4.10: Western blot analyses of FcγRI-FLAG chimeras in COS cell transfections. Triton-X100 lysates of COS cells transfected (COS Tf) with cDNAs for pFLAG-CMV-1 vector (V; A, lane 1; B, lane 3), FcγRI a1-FLAG (a1F; A, lane 2), FcγRI a1 containing the EC2 domain of b2, in pFLAG-CMV-1 (a1-E3F; B, lane 1) or FcγRI b2-FLAG (b2-F; B, lane 3) were adsorbed with anti-FLAG mAb M2 coated beads. The eluates were analyzed by Western blot with anti-FLAG mAb M2 and ECL detection. kD, kilodalton.
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CHAPTER 5

SUMMARY AND DISCUSSION

FcR, integral membrane cell surface receptors that bind the Fc portion of Ig, trigger an array of functions in the immune system after binding immune complexes (1). The three classes of FcR specific for IgG, FcγRI, FcγRII and FcγRIII, have rich structural diversity (2). FcγRI, the primary focus of my studies, is encoded in humans by three highly homologous genes (A, B and C) and six transcripts (3-5). FcγRI, consisting of a 70 kD ligand binding α chain noncovalently associated with the 20 kD homodimerized signaling subunit γ chain, binds monomeric IgG with high affinity (9). Before the studies described in this dissertation, the high affinity of FcγRI was attributed to the unique third extracellular domain of the α chain (10, 11), which was thought to bind IgG as a monomer (12). Characterization of the FcγRI transcripts indicated that a1, a product of gene A, represented the bona fide FcγRI α chain, while b2, an alternatively spliced transcript from gene B, predicted a two-extracellular domain α chain similar in structure to the low affinity receptors, FcγRII and FcγRIII (4). The reports presented in this composition reveal several unique observations, including a novel γ chain role in enhancing the FcγRI α chain affinity for monomeric
IgG, as well as evidence for homodimerization of the α chains, a unique finding among Ig-superfamily FcR. These studies also provide initial characterizations of the novel FcγRII b2 isoform.

**FcγRI Affinity for Monomeric IgG**

Our findings in chapter 2 suggested that besides functioning in signal transduction, the FcγRI γ chain subunit has a role in modifying the receptor-ligand interaction by enhancing the binding affinity for IgG. In previous reports, both the human and murine FcγRI α chains transfected in COS cells bound monomeric IgG with a five-fold lower affinity (Ka of $5.0 \times 10^7 M^{-1}$) (10-12) than that measured on cells that naturally express the receptors (Ka of $10^{8-9} M^{-1}$) (9,12), suggesting an additional factor may be participating in the high affinity binding. We determined through ligand binding assays and Scatchard analyses that the high affinity of both the human and the murine FcγRI for monomeric IgG was reconstituted in COS cells after cotransfection of the associated γ chain subunit with the ligand binding α chain. The affinity increase measured after γ chain cotransfection with the α chain in COS cells (human α+γ: Ka of $15.6\times10^7 M^{-1}$, murine α+γ: Ka of $9.1\pm0.3\times10^7 M^{-1}$) was shown to be statistically significant and similar to the affinity measured on cell lines that naturally express both FcγRI α and γ chains (human FcγRI: Ka of $19.0\times10^7 M^{-1}$; murine FcγRI: Ka of $10.0\times10^7 M^{-1}$).

Copurification of γ chain with the α chain after adsorption with anti-FcγRI antibody and/or monomeric IgG correlated the affinity increase with association of this subunit.

We also analyzed the ligand binding affinity of FcγRIIIa in chapter 2. This receptor, which requires association with γ chain for cell surface expression, binds
monomeric IgG with higher affinity than FcγRII and FcγRIIIb, but with lower affinity than FcγRI (13). The binding affinity of FcγRIIIa cotransfected with γ chain in COS cells was higher than that measured for a glycosyl phosphatidylinositol (GPI)-linked FcγRIIIa mutant which did not require γ chain association for surface localization, suggesting that the intermediate binding affinity of FcγRIIIa was a result of γ chain association.

Analyses of a truncated FcγRI α chain lacking its cytoplasmic region not only supported our hypothesis that γ chain associated with the transmembrane domain of the receptor (6), but also showed that the cytoplasmic region of the α chain was not required for the increased binding affinity conferred by association with γ chain. A slight increase in the binding affinity of this construct compared with the wild-type suggested that the α chain cytoplasmic region may actually have an inhibitory effect upon ligand binding affinity. Therefore, it appeared that cotransfection of γ chain with the FcγRI α chain overcomes any inhibitory effect of the α chain cytoplasmic region.

To explain the mechanism of this affinity increase, we postulated that through association γ chain association induces homodimerization resulting in two α chains binding to a single IgG molecule with high affinity. Since the Fc portion of Ig is itself a dimer (14), it is plausible that two FcγRI α chains could bind to a single IgG ligand.

*Homodimerization of FcγRI α Chains*

Although dimerization of ligand binding subunits is a common theme among many cell surface receptors (15, 16), it has not been demonstrated for Ig-superfamily FcR, which instead are thought to bind Ig through a monomeric α chain (17). In chapter 3 we presented evidence supporting homodimerization of the FcγRI α chains.
Iodinated U937 cell lysates adsorbed with anti-FcγRI antibodies and analyzed by both one and two dimensional SDS-PAGE indicated the presence of a dimer-sized complex that contained proteins of the appropriate sizes for an α chain monomer and for γ chain. This dimer-sized complex was reconstituted in COS cells transfected with FcγRI α chain cDNA, and confirmed to contain the α chain in Western blot analysis with anti-FcγRI antibodies. The dimer-sized band was detected both with and without γ chain cotransfection indicating that in COS cell transfections the formation of this complex was independent of γ chain association.

Two differentially epitope-tagged FcγRI α chain constructs, one containing an N-terminal FLAG tag and one with a C-terminal histidine tag, were cotransfected in COS cells and utilized to demonstrate homodimerization of the FcγRI α chains. The COS cell cotransfectants were lysed and the histidine-tagged α chain was affinity purified with nickel beads. When the eluates were examined by Western blot analysis with an anti-FLAG antibody, the FLAG-tagged α chain was detected copurifying with the histidine-tagged α chain in a receptor homodimer. Again, γ chain was not required for the homodimer formation. Our results suggested that instead of inducing dimerization, γ chain association might function to stabilize the α chain homodimers enabling a receptor conformation that binds IgG with high affinity.

Although our report in chapter 3 supplies the first evidence among classical FcR for two receptor subunits binding to a single ligand, many other cell surface receptors form such structures. While FcγRI α chain dimers were detected without IgG, many receptor tyrosine kinases (RTK) require ligand binding to induce dimerization, which
results in autophosphorylation and activation of the receptors (15, 16). Similar to our observations with FcγRI, receptors such as the insulin receptor and the type II transforming growth factor-β (TGF-β) receptor homodimerize in the absence of ligand (18-20). It is thought that ligand binding to these receptors results in conformational changes that yield an active dimer. The ligand-binding subunits of many cytokine receptors associate to form heterodimeric complexes that bind a single bivalent ligand with higher affinity than that measured for the monomeric receptor (21, 22). Since our studies do not include analyses of the FcγRI α chain homodimer in the presence of IgG, the receptor-ligand relationship of FcγRI will require further clarification to completely correlate the affinity findings in chapter 2 with the structural observations in chapter 3.

After adsorption of U937 cells with anti-FcγRI antibody, we also detected a larger complex in addition to the α chain dimers that appeared to contain both α and γ sized proteins. Due to its size, we referred to this complex as an α chain tetramer. In iodinated COS cell transfectants, the presence of this tetramer-sized band was dependent upon γ chain association, however this complex was not detected by Western blot analysis with anti-FcγRI antibodies even with γ chain cotransfection.

While further studies will be necessary to characterize the components of this complex, it is possible that this tetramer-sized structure actually represents a complex of an FcγRI α chain homodimer in association with γ chain and other signaling molecules. It has been suggested that receptor dimerization functions to mediate cell responses by concentrating signaling molecules (16, 23, 24). The binding of immune complexes activates FcγRI, which cluster and trigger tyrosine phosphorylation of various proteins,
including γ chain, resulting in the association of the 70 kD nonreceptor tyrosine kinase Syk (25-27). Potentially FcγRI was inadvertently activated during iodination, resulting in association of Syk or a Syk homologue in COS cells, which could explain why this larger complex was detected only with iodination.

In U937 cells, both the dimer and tetramer sized complexes contained a 30 kD iodinated protein that was not detected in iodinated COS cells, either with or without γ chain cotransfection with the FcγRI α chain. This 30 kD protein is similar in size to the β chain, an associated signaling subunit of the high affinity receptor for IgE, FceRI (28). Since FcγRI and FceRI are similar in that both bind monomeric ligand with high affinity and associate with the same γ chain signaling subunit (6, 8, 29), we inferred that FcγRI might also contain a β chain-like protein. In a recent report we identified a phosphorylated protein of similar size that associated directly with FcγRI in U937 cells (30). The β chain links FceRI to the Src kinase Lyn (31), which was also recently shown to associate with FcγRI (30, 32). This finding further supports the possibility for association of a β-like protein with FcγRI.

The results in chapter 3 led us to speculate that the other members of the FcγR class might also have homodimerized α chains. Although there is no direct structural evidence for homodimerization of the α chains of FcγRII or FcγRIII, as members of the Ig-superfamily (33) these receptors are potentially capable of self-associating. Since the binding affinity of FcγRIIIa is also enhanced by γ chain association, the α chains of this receptor might also homodimerize similarly to FcγRI. In addition, some indirect evidence supports homodimerization of FcγRII. Detection of FcγRII with both IgG and an Fab
fragment of an anti-FcγRII mAb suggested a 2:1 receptor to ligand ratio (34), similar to our dimerized FcγRI structural model. The implications of homodimerization of the FcγR α chains include the possibility of heterodimerization between the FcγR α chains. Comparably, many cytokine receptors share common ligand binding subunits, which is thought to explain the redundant functions of these receptors (21, 22). Heterodimerization of the FcγR α chains could also explain some of the redundancy among this family of receptors.

In addition to analyzing the other FcγR, future structural studies of FcγRI might also include crystallization of the ligand binding α chains. Since crystallization analyses of integral membrane proteins requires removal of the hydrophobic sequences of the transmembrane region, we have engineered a soluble FcγRI α1 α chain cDNA construct. Crystallization of the soluble α chains might not accurately reflect the structure of the intact membrane proteins, however, and the contributions of γ chain cannot easily be studied by this approach since the subunit associates with the α chain through the transmembrane region. Therefore, along with crystallization studies, structural analyses of other FcγRI isoforms might assist in determination of the structural requirements for association of the α chains.

While one would predict that loss of an entire α chain extracellular domain might inhibit dimer formation, we detected bands the appropriate size for receptor dimers when analyzing the FcγRI b2 isoform by Western blot analysis in chapter 4 (Fig. 2), suggesting
that the EC3 domain does not contain a site required for dimerization. Further studies of the b2 isoform will be required, however, to determine definitively if this isoform homodimerizes.

Characterization of FcγRI b2 Isoform

An N-terminal FLAG epitope-tagged FcγRI b2 construct transfected in COS cells and analyzed by Western blot analyses confirmed that b2 was a 45 kD protein (chapter 4). Immunofluorescence microscopy of the COS cell transfectants indicated that unlike FcγRI a1, the b2 isoform was predominantly intracellularly localized and surface expression was not rescued with γ chain cotransfection. While this cytoplasmic localization could indicate that the b2 isoform is retained in the endoplasmic reticulum and targeted for degradation, detection of the b2 protein in U937 cells, a human monocyte cell line that expresses both the a1 and b2 transcripts (4), suggested that this isoform was potentially functional. In addition, the b2 protein associated with the FcγRI signaling subunit γ chain in COS cell cotransfectants, which provided further evidence that this isoform was capable of function.

Preliminary ligand binding data from rosetting assays indicated that the FcγRI b2 isoform can bind IgG, however in an inefficient manner. A series of FcγRI a1/b2 mutants and chimeras studied in COS cell transfections indicated that the b2-specific extracellular domain differences disrupted ligand binding. When analyzed in separate chimeras, either the loss of the EC3 domain or the three b2-specific EC2 domain residues resulted in inefficient IgG binding, despite abundant cell surface localization. These results suggested the IgG binding site could reside in the EC2 domain of the FcγRI α chain, similar to both FcγRII and FcγRIII, and pointed to the importance of the unique EC3 domain in the
structure of this receptor. These chimeras further indicated that the cytoplasmic localization of FcγRI b2 expressed in COS cell transfectants was a result of the collective differences in the extracellular domains.

The inefficient surface expression could indicate that like FcγRIIIa and FceRI, the FcγRI b2 isoform might require association with an additional subunit to enable correct folding, processing and cellular transport. For surface expression in COS cell transfectants, both FcγRIIIa and the human FceRI require γ chain cotransfection, while the murine FceRI needs association of both γ and β chains (29, 35-37). Since neither cell surface localization nor ligand binding of FcγRI b2 was rescued by γ chain cotransfection, we postulated that this isoform might associate with a β chain homologue. In addition to γ chain, FcγRIIIa has been found to associate with β chain in mast cells (38), which supports the possibility of such an association with FcγRI. Although the FceRI β chain is not expressed in the same cells as FcγRI (39), similar tetraspan molecules have been characterized in these cells (40). In chapter 3 a 30 kDa β chain-sized molecule was observed associating with the FcγRI dimer complex in U937 cells (Fig.), but was not detected in COS cell transfectants. While the FcγRI a1 α chain does not require associated subunits for cell surface expression in COS cells, it is possible that due to structural differences, the b2 isoform depends upon interactions with this 30 kDa protein to rescue cell surface localization.

While FcγRI b2 might associate with additional subunits, it is possible that the intracellular expression of this isoform in COS cell transfectants reflects its true localization. FcγRI b2 could be similar to the erythropoietin receptor (EPO-R), which is
sequestered in the endoplasmic reticulum due to inefficient folding, with few receptors reaching the cell surface to bind ligand (41). This inefficient folding and cellular transport have been proposed as a regulatory mechanism for controlling the number of EPO-R expressed at the cell surface.

It is also feasible that the FcγRI b2 isoform may function differently from other FcγR. As we speculated in chapter 4, b2 could have an intracellular role, perhaps similar to FcRn which functions in IgG transport and/or catabolism (42). Previous reports of intracellular IgG binding proteins of the same size as the b2 isoform lend support to this hypothesis, as discussed in chapter 4, and we are currently pursuing these studies.

Alternatively, the meager binding of IgG by FcγRI b2 could suggest that this isoform binds an alternate ligand. Recently FcγRI b2 has been implicated in functioning as the receptor for C-reactive protein (CRP). CRP is produced by the liver early in an acute infection and non-specifically opsinizes antigen surfaces to trigger phagocytic cells (43). While the receptor for CRP (CRP-R) has not yet been identified, it is thought to be approximately 45 kD (44), the size of the FcγRI b2 isoform, and it also appears to associate with γ chain (45). Membrane proteins have been isolated from U937 cells, which express FcγRI b2, by their ability to bind CRP. Additionally, CRP has been shown to bind to the *bona fide* FcγRI α chain at a site homologous to the FcγR binding site on IgG (46).

Preliminary evidence from our laboratory (not previously presented) suggests that anti-CRP-R antibodies and CT6, our anti-FcγRI cytoplasmic antibody, both recognize the same 45 kD protein in THP-1 cells, a human monocyte cell line (Fig. 5.1). After adsorbing
cell lysates with either the anti-FcγRI mAb (CT6), two anti-CRP-R antibodies (RC10, R>CRP-R), or Ig isotype controls (3G8, IgMC), the eluates were analyzed by Western analysis with anti-CRP-R mAb. The initial evidence suggesting FcγRI b2 as the CRP-R is encouraging, and we are continuing to investigate the intriguing possibility that the FcγRI b2 isoform actually represents the CRP-R.

The studies described in this dissertation reveal the quaternary structure of FcγRI to be more complex than anticipated. A novel function was identified for the associated γ chain subunit in enhancing receptor affinity for IgG, suggesting that signaling subunits may also regulate receptor-ligand interactions. Our evidence also supports the possibility for additional subunits associating with FcγRI, indicating that the complete quaternary structure of this receptor remains to be defined. The homodimerization of the FcγRI ligand binding α chains is a unique observation among Ig-superfamily FcR and suggests reevaluation of current FcγR structural models. Our preliminary characterizations of the novel FcγRI b2 isoform reveal the potential for many functional capabilities for this receptor which we are currently pursuing.
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Figure 5.1: Drawing of FcγRI a1 and b2 integral membrane proteins. FcγRI a1 depicted as an α chain homodimer. Both FcγRI a1 and b2 α chains associate with the homodimerized γ chain signaling subunit. EC, extracellular domains, TM, CYT, transmembrane and cytoplasmic domains.
Figure 5.2: Anti-CRP-R antibodies and anti-cytoplasmic domain antibodies recognize the same 45 kD protein. THP-1 cells cultured with IFN-γ were lysed and immunoadsorbed (IA) with anti-cytoplasmic domain (CT6) mAb, and isotype control (3G8) antibodies, or a CRP-R mAb (RC10), an isotype control (IgMC) and a rabbit anti CRP-R (R > CRP-R). Western blot was analyzed with mAb anti-CRP-R. M, molecular weight marker.
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