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BIOLOGICAL STUDIES OF THE HUMAN IgG Fc RECEPTORS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of the Ohio State University

By

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ABSTRACT

Fc receptors for IgG (FcγR/FCGR) are major mediators of antibody action within the human immune system. Two different aspects of FcγR biology have been investigated. Namely, the organization and evolution of the FcγRI gene family and the involvement of the inositol 5-phosphatase SHIP in FcγR signal transduction. The FcγRI gene family encodes the high affinity receptor for IgG and is represented by 3 genes in the human genome. Initial results, through the characterization of FcγRI-containing YAC clones and analysis of the FcγRI gene family with long-range restriction endonucleases, demonstrated that the FcγRI genes are not tightly linked either to each other or the 5 FcγR genes clustered at 1q23. It was then shown through both fluorescence in situ hybridization analysis of human cells and Southern analysis of cell lines containing 1p and 1q that the 3 FcγRI genes flank the centromere of chromosome 1 at bands 1p12 and 1q21. FcγRIB was found at 1p12 whereas both FcγRIA and FcγRIC were localized to 1q21. This placed the FcγRI gene family within a large pericentric linkage group, which is conserved between humans and mice. We hypothesize that the 3 FcγRI
genes were separated by a pericentric inversion known to have occurred on human chromosome 1, which relocated FcγRIA and FcγRIC to the long arm and left FcγRIB positioned on the short arm. To investigate this hypothesis we determined the location of FcγRI in several ape species by FISH analysis. In both baboons (Papio papio) and monkeys (Macaca mulatta) FcγRI was localized to a region adjacent to the centromere of chromosome 1 on what is homologous to the human 1p chromosomal arm. This correlates the appearance of FcγRI on the 1q arm with the time period that the pericentric inversion is proposed to have occurred, supporting the idea that this inversion translocated FcγRI to 1q. FcγRI gene copy number experiments were also performed which indicate the existence of 3 genes within the human genome. Additionally, FcγR signal transduction was investigated and it was ascertained that the inositol 5-phosphatase SHIP is phosphorylated upon FcγR crosslinking in monocytes. Specifically, it was found that crosslinking of FcγRII or FcγRI leads to phosphorylation of the inositol 5-phosphatase SHIP in both U937 and THP1 monocytes. This phosphorylation occurs by 30 seconds is enhanced in monocytes pretreated with the cytokine γ-IFN. It was also determined that FcγR crosslinking in monocytes induces SHIP to associate with the SH2-containing adapter protein p52 Shc.
Dedicated to my mother Gloria Jean
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>1.1</td>
<td>Fc Receptors</td>
<td>3</td>
</tr>
<tr>
<td>1.1.1</td>
<td>FcγRI (CD64)</td>
<td>5</td>
</tr>
<tr>
<td>1.1.2</td>
<td>FcγRII (CD32)</td>
<td>6</td>
</tr>
<tr>
<td>1.1.3</td>
<td>FcγRIII (CD16)</td>
<td>7</td>
</tr>
<tr>
<td>1.2</td>
<td>Gene families</td>
<td>8</td>
</tr>
<tr>
<td>1.3</td>
<td>Cell Signaling</td>
<td>14</td>
</tr>
<tr>
<td>2</td>
<td>Genomic Mapping studies of the human \textit{FCGR1} gene family encoding FcγRI</td>
<td>18</td>
</tr>
<tr>
<td>2.1</td>
<td>Introduction</td>
<td>17</td>
</tr>
<tr>
<td>2.2</td>
<td>Experimental Procedures</td>
<td>20</td>
</tr>
<tr>
<td>2.3</td>
<td>Results</td>
<td>23</td>
</tr>
<tr>
<td>2.3.1</td>
<td>Characterization of 5 FCGRI-containing YAC clones through PCR analysis</td>
<td>23</td>
</tr>
<tr>
<td>2.3.2</td>
<td>Characterization of homologous regions shared between the FCGRI-containing YAC clones</td>
<td>25</td>
</tr>
<tr>
<td>2.3.3</td>
<td>Analysis of FCGRI-containing YAC clones for the presence of genes located on 1q</td>
<td>27</td>
</tr>
</tbody>
</table>
2.3.4 Analysis of the FCGRI gene family with long-range restriction endonucleases ....................................................... 28

2.4 Discussion .................................................................. 29

3 The three genes of the human FCGRI gene family encoding FcγRI flank the centromere of chromosome 1 at 1p12 and 1q21 ..................... 46

3.1 Introduction ............................................................................. 46
3.3 Experimental Procedures ...................................................... 48
3.3.1 Localization of the FCGRI gene family to 1p12 and 1q21 by FISH analysis .............................................. 52
3.3.2 Characterization of 4 FCGRI-containing YAC clones ........ 54
3.3.3 Hybridization of 4 FCGRI-containing YAC clones to 1p and 1q through FISH analysis ..................... 55
3.4 Genomic Southern analysis of 1p and 1q containing cell lines indicate that the FCGRI genes flank the centromere of chromosome 1 .......................................................................... 56
3.4.1 Further evidence that the FCGRI genes flank the centromere of chromosome 1 using gene specific oligonucleotides ........................................................................................................ 58
3.4.2 Quantification of FCGRI copy number within humans ........ 59
3.5 Discussion .............................................................................. 61

4. Localization of the FCGRI gene family encoding FcγRI in Apes, further evidence of a pericentric inversion of human chromosome 1 .......... 86

4.1 Introduction .......................................................................... 86
4.2 Experimental Procedures ........................................................ 88
4.2.1 Fluorescence in situ hybridization and chromosomal localization ......................................................... 88
4.3 Results .................................................................................. 89
4.3.1 Localization of FCGRI in 3 species of apes by FISH .................................................................................... 89
4.4 Discussion ............................................................................... 91

5. Involvement of the Inositol 5-Phosphatase SHIP in FcγR signaling in monocytes .................................................................................................. 98

5.1 Introduction ............................................................................ 98
5.2 Experimental Procedures ....................................................... 101
5.3 Results ................................................................................... 104
5.3.1 FcγRII crosslinking in U937 induces phosphorylation of the inositol 5-phosphatase SHIP ................................. 104

5.4.2 FcγRII crosslinking in THP1 also induces phosphorylation of SHIP ................................................................. 106

5.4.3 FcγRII-induced SHIP phosphorylation is greater in U937 Cells ................................................................. 107

5.4.4 FcγRII activation induces SHIP to associate with the adapter protein Shc ............................................. 108

5.4.5 Crosslinking of the high affinity FcγRI also induces SHIP phosphorylation ........................................... 109

5.5 Discussion ............................................................................. 110

6 Summary ................................................................................ 141

6.1 Studies of the FcγRI gene family ........................................ 141

6.2 Studies of FcγR Signal Transduction ................................ 147

Bibliography ................................................................................. 150
LIST OF TABLES

<table>
<thead>
<tr>
<th>Tables</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 Hybridization fragments generated by analyzing human genomic DNA which had been digested with long-range restriction endonucleases with an FCGRI cDNA probe</td>
<td>45</td>
</tr>
<tr>
<td>3.1 Localization of Hybridization Signals on Human Chromosome after FISH with sequences from 4 FCGRI-containing YAC clones</td>
<td>73</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>$FCGRI$-containing YAC clones measure between 190-310 Kb in size</td>
<td>33</td>
</tr>
<tr>
<td>2.2</td>
<td>Southern analysis of $FCGRI$-containing YAC clones reveals they do not contain $FCGR2$</td>
<td>35</td>
</tr>
<tr>
<td>2.3</td>
<td>Southern analysis of $FCGRI$-containing YAC clones reveals they do not contain $FCGR3$</td>
<td>37</td>
</tr>
<tr>
<td>2.4</td>
<td>Southern analysis YAC clones 1-5 and 7 ensures they contain YAC inserts</td>
<td>39</td>
</tr>
<tr>
<td>2.5</td>
<td>Southern analysis of $FCGRI$-containing YAC clones reveals they do not contain genes of the $CD1$ gene family</td>
<td>41</td>
</tr>
<tr>
<td>2.6</td>
<td>Analysis of human genomic DNA with long-range restriction endonucleases separates the $FCGRI$ gene family into 3 large DNA fragments</td>
<td>43</td>
</tr>
<tr>
<td>3.1</td>
<td>Chromosomal Localization of the hFCGR1 loci to 1p12 and 1q21</td>
<td>67</td>
</tr>
<tr>
<td>3.2</td>
<td>Human G-banded chromosomes 1</td>
<td>69</td>
</tr>
<tr>
<td>3.3</td>
<td>Characterization of YAC clones each containing a single gene</td>
<td>71</td>
</tr>
<tr>
<td>3.4</td>
<td>Schematic representation of human chromosome 1 and the portions of it contained within 5 human-rodent hybrid cell lines</td>
<td>74</td>
</tr>
</tbody>
</table>
3.5 Southern analysis of genomic DNA from cell lines containing portions of chromosome 1 localize the FCGRI genes to p and q arms of chromosome 1 .............................................................. 76

3.6 Southern analysis of PCR fragments amplified from cell lines containing portions of chromosome 1 using oligonucleotide probes specific for FCGRIA or FCGRIB/C ................................................. 78

3.7 Analysis of FCGRI copy number in 8 individuals using the single copy X-linked DMD gene as a standard ................................................................. 80

3.8 Comparison of DMD hybridization (D counts) of the FCGRI/DMD plasmid (pGCC) to the amount of pGCC ........................................................ 83

3.9 Comparison of FCGRI hybridization (F counts) of the FCGRI/DMD plasmid (pGCC) to the amount of pGCC ........................................................ 85

4.1 Evolution of the FCGRI gene family .................................................. 94

4.2 Localization of FCGRI to chromosome 1 in apes ............................. 96

5.1 Fc crosslinking on human monocytes induces tyrosine phosphorylation of a 145 kDa protein ................................................................. 115

5.2 FcyRII crosslinking in monocytes results in tyrosine phosphorylation of the 5'-inositol phosphatase SHIP .................................................. 117

5.3 Resting and activated U937 cells contain comparable amounts of SHIP ........................................................................................................ 119

5.4 FcyRII crosslinking in human monocyte cell line THP1 also induces phosphorylation of a p145 kDa protein .................................................. 121

5.5 FcyRII crosslinking in monocytes cell line THP1 induces tyrosine phosphorylation of SHIP ................................................................. 123

5.6 SHIP is phosphorylated within 30" of FcyRII crosslinking .................. 125

5.7 U937 cells at different points of FcgRII activation contain comparable amounts of SHIP ................................................................. 127
5.8 FcyRII crosslinking in monocytes treated with γ-IFN results in increased tyrosine phosphorylation SHIP......................... 129

5.9 (γ-IFN)-treated human monocytes U937 cells contain increased amounts of SHIP.......................................................... 131

5.10 A p145 kDa tyrosine phosphorylated protein associates with Shc upon FcyRII-crosslinking........................................... 133

5.11 SHIP associates with Shc upon FcyRII crosslinking......................... 135

5.12 Crosslinking of the high affinity Fc receptor FcyRI on human monocytes induces tyrosine phosphorylation of a 145 kDa protein .. 137

5.13 FcyRI crosslinking in human monocytes results in tyrosine phosphorylation of SHIP.................................................. 139
CHAPTER 1

INTRODUCTION

This dissertation focuses on the Fc gamma receptors (FcγR/FCGR) of the human immune system, specifically, the organization and evolution of the FcγR1 gene family (Chapters 2,3,4), and the involvement of the inositol-phosphatase SHIP in FcγR signal transduction (Chapter 5). Within this thesis, the data are presented in manuscript format (Chapters 2-5), which leads to terminology reflecting the group effort behind these projects. To clearly demonstrate that the majority of research described within this thesis is a result of my own efforts I clearly identify within the summary (Chapter 6) which findings are the result of my own work and which contributions that have been made by outside collaborators.

Within this introduction chapter I will first present a brief overview describing both the role that FcR play in the immune system and the characteristics of the different FcγR. Next, in order to provide a basis for my research, I will present general information regarding gene families and immune
receptor signal transduction. Specifically, I will describe the categories, creation, and regulation, of gene families, and the characteristics of the FcγR gene family, after which I will present aspects of the signal transduction of multi-domain immune recognition receptors (MIRRS) with special emphasis put on FcγR cell signaling. More detailed information regarding the specific aims of my research will be presented within the introduction sections of each chapter.
Fc Receptors

Within the human immune system the variable regions of antibodies (Ig) bind to antigen essentially serving only to tag the antigen as pathogen. To trigger the immune system to eliminate antigen, Ig molecules must additionally bind and activate immune cells. They achieve this by engaging, with their constant regions (Fc regions), protein receptors on the surfaces of the immune cells termed Fc receptors (FcR). As Ig-antigen complexes bind Fc receptors they cluster them into aggregates rousing the FcR to initiate a signal cascade. This signal cascade "activates" the immune cell inducing it to execute a number of functions aimed at the elimination of antigen such as phagocytosis, degranulation and Antibody-Dependent Cellular Cytotoxicity (ADCC). (1)

There are Fc receptors for each type of Ig molecule (IgG, IgA, IgM, IgD, IgE) which primarily occur as integral membrane proteins with Ig-like extracellular domains. FcR are present in different combinations on all of the cells of the immune system and their expression is regulated by cytokines. The particular process that FcR initiate is dependent upon the cell type that they are being clustered on. For example, in platelets FcγRIIA induces degranulation whereas in macrophages it induces phagocytosis. (1)
The Fc receptors that bind Immunoglobulin G (IgG) are termed Fc gamma receptors (FcγR/FCGR). They are organized into 3 classes (FcγRI, FcγRII, FcγRIII) based on their cell distribution, affinity for specific IgG, and recognition by anti-FcγR monoclonal antibodies (Mabs). FcγR are represented by 8 genes in humans which, based on their sequence homology and gene organization, appear to have arisen by gene duplication (2). Their expression is upregulated by the cytokine γ-IFN and once expressed, FcγR mainly activate cells to induce antigen-killing responses such as inflammatory mediator release, ADCC, phagocytosis, and superoxide generation. One exception to this is FcγRIIB, which has been shown to inhibit immune cell responses (3). It is possible to trigger FcγR artificially with anti-FcγR Mabs. This and cell transfection studies have allowed the independent study of FcγR, which have revealed that many of them induce redundant effector functions in immune cells. Nonetheless, in combination FcγR are major mediators of the immune system. This was most clearly demonstrated by experiments which “knocked out” FcγR expression in mice, revealing their central role in the initiation of immunocomplex-triggered inflammation and lymphocyte regulation (4).
Fc\(\gamma\)RI (CD64)

The Fc\(\gamma\)RI class is represented by 3 genes in humans (A,B,C) which are >96% homologous (5), although Fc\(\gamma\)RIA is considered the *bona fide* Fc\(\gamma\)RI. It is given this designation, as it is the only member of this gene family to exhibit the main characteristics previously attributed to Fc\(\gamma\)RI in the literature. Specifically, a size of 70 kDa, recognition by anti-Fc\(\gamma\)RI Mabs and an ability to initiate antigen-killing effector functions. Fc\(\gamma\)RIA encodes a 70 kDa molecule that is unique among the Fc\(\gamma\)R, as it has 3, rather than 2, Ig-like extracellular domains and binds IgG with high affinity. Fc\(\gamma\)RIA is expressed on monocytes, macrophages, and (\(\gamma\)-IFN)-treated neutrophils, and can initiate all of the functions attributed to Fc\(\gamma\)R (3). Fc\(\gamma\)RIA transduces cell signals through an Immunoreceptor Tyrosine-based Activation Motif (ITAM)-containing subunit, the \(\gamma\)-chain homodimer (whose gene designation is FCERIG), which it associates with noncovalently (6). Fc\(\gamma\)RIB and Fc\(\gamma\)RIC in contrast, each encode soluble receptors due to their containing premature stop codons. It has been found though that Fc\(\gamma\)RIB additionally produces an alternatively spliced transcript (Fc\(\gamma\)RIB2) which encodes a 40 kDa integral receptor with 2 extracellular domains, that is expected to be bind IgG with low affinity. (5)
**FcγRII (CD32)**

FcγRII is also represented in humans by 3 genes (A,B,C). All 3 FcγRII genes encode 40 kDa integral membrane glycoproteins with 2 Ig-like domains that bind IgG with low affinity. The extracellular domains of the FcγRII receptors are >95% homologous and there are presently no Mabs available which can distinguish them (7). The FcγRIIA cytoplasmic regions, in contrast, are diverse. The FcγRIIA and FcγRIIC receptors each have an ITAM signaling motif in their cytoplasmic tail regions that can activate cells to induce antigen-killing processes. FcγRIIB in contrast has an Immunoreceptor Tyrosine-based Inhibition Motif (ITIM) in its cytoplasmic region, which mediates a signal that abrogates the activation signal of other receptors (negative signal). FcγRIIA is expressed on monocytes, macrophages, and neutrophils whereas FcγRIIB is expressed predominantly in B cells but also in monocytes, macrophages, and mast cells (7;8). FcγRIIC mRNA has been found in monocytes, macrophages, and B cells, although the existence of its protein product in these cells has not yet been confirmed (7;8). Alternative splice products of the FcγRII transcripts include a soluble form of FcγRIIA. For FcγRIIB there are 3 alternate splice forms, 2 of
which (FcγRIIB2 and FcγRIIB3) differ only within their leader peptide sequences, and a third (FcγRIIB2) which has an extra 19 amino acids in its cytoplasmic tail. All 3 of the FcγRIIB receptors contain the ITIM inhibitory motif. (3;7)

FcγRIII (CD16)

FcγRIII is represented by 2 genes in humans (FcγRIIIA, FcγRIIIB), that are >95% homologous in nucleotide sequence (9). They each encode receptors with 2 extracellular domains that have low affinity for IgG and range in size from 50-80 Kd due to glycosylation variations. The FcγRIIIA receptor is an integral membrane protein that is expressed on NK cells, macrophages, and at low levels on monocytes (10). It can initiate antigen-killing functions and, similar to FcγRIA, associates noncovalently with ITAM-containing subunits. Specifically, it associates with the γ-chain homodimer in monocytes and with different combinations of the γ-chain and ζ-chain molecules in NK cells (11). FcγRIIIB in contrast, due to a serine to phenylalanine change in its cytoplasmic tail, is cleaved after translation and is expressed as a tailless molecule that is anchored to the membrane by a glycosyl-phosphatidyl inositol (GPI) link (12). The function of this unique GPI-linked FcγR is not yet fully understood.
II Gene Families

As cloning efforts in mice had only detected a single FcγRI gene it was curious that there were 3 FcγRI genes present in humans. Also of interest was the high degree of homology found between the 3 hFcγR1. Their coding regions differed by only approximately 12 nucleotides and this high homology appeared to persist into their intron and flanking regions. As mentioned previously the analysis of the hFcγR1 gene products clearly showed that FcγRIA was the bona fide FcγRI (5), yet there still remained compelling questions regarding the FcγRI gene family. Namely, what was the significance of FcγR1B and FcγR1C genes and the high homology of the FcγR1 gene family, and for what reasons had FcγRI evolved into a gene family in humans?

Gene families are defined as multiple regions of DNA (primarily genes) which exhibit to varying degrees high sequence homology, close linkage, and shared function (13). In the evolutionary development of organisms, gene families first appeared in eukaryotic organisms (14). Evolution is considered to work either by altering the nature of superficial developmental processes or by superadding new functions upon conserved older ones (15). The duplication of a single gene into a gene family is one of the major mechanisms by which nature
adds new functions upon older ones, allowing organisms to react to their
environment and evolve (14). Once a gene duplication event occurs, certain
members of the gene family quickly begin to accumulate mutations (14). These
mutations are then tested out in the population by positive Darwinian selection,
which, at times leads to a gene with a novel structure, function, or regulation
pattern (14).

Leroy Hood organized gene families into 3 categories (13). 1) Simple-
sequence families consist of short segments of DNA that share 80-100%
sequence homology and are represented by \(10^2-10^7\) copies per genome (13). The
human specific Alu repeat is an example of a simple-sequence family. Alu repeats
are not transcribed and do not serve any known function yet they are present
approximately every 5 Kb within the human genome (16). 2) Multiplicational
families, in contrast, appear to have evolved to meet an organisms need for a vast
amount of a particular gene product. Multiplicational families exist as identical
repeating gene units that are represented by 10-10,000 copies per genome (13).
Examples of multiplicational families are the 5S RNA gene family (~1000
genes/genome) and the tRNA gene family (~ 400 genes/genome) (13).
Multiplicational families are unique as they do not diverge over time and instead
appear to be selected for functional uniformity (14). 3) Informational families
are also made up of genes but the individual members of these families can differ
markedly from one another in their sequences yet their homology is substantial enough to exhibit their common ancestry (13). Often their homology is concentrated to a particular functional domain (13). An example of an informational gene family is the notable Ig-superfamily that comprises several diverse members including Ig, FcR, the T cell antigen receptor (TCR), and the B cell antigen receptor (BCR). All Ig-superfamily members contain at least a single 100 amino acid domain termed an "Ig-like" domain that exhibits a very specific structure (17).

Of the 4 mechanisms by which genes are likely to be duplicated, 2 involve unequal chromatid exchange (18). Unequal chromatid exchange occurs during mitosis or meiosis when homologous chromotids mistakenly line up offset from one another and undergo a crossing over event (19). Specifically, the chromatids misalign, break, exchange uneven pieces, and reassociate, producing one chromatid which contains a duplicated region and another that contains a deleted region. These duplicated or deleted regions are usually ≥40 Kb in length (19).

Nonhomologous chromosomal breakage and reunion is due to an unequal chromatid exchange occurring at a non-homologous sequence whereas the more common Homologous Recombination Between Repeated Elements is due to an unequal exchange at a homologous region (13). Unequal chromatid exchanges primarily produce informational gene families (13). Additionally any type of gene
duplication event will increase the amount of homologous sequences in an area, making that area prone to unequal chromatid exchange (19). This often leads to certain individuals in a population having ±1 member of a gene family (19).

Of the 2 common mechanisms of gene duplication which do not involve unequal chromatid exchange, one is **RNA-Mediated Transposition** (13). RNA-mediated transposition occurs when an RNA molecule is reverse transcribed into dsDNA and then the dsDNA fragment is inserted into the genome. This creates what is referred to as a processed pseudogene. Processed pseudogenes are easily recognized as they lack introns and contain poly A regions. Many simple-sequence gene families are thought to have arisen by RNA-Mediated Transposition (13). The fourth common mechanism of gene duplication is **Gene amplification Through Over-Replication** that occurs due to multiple initiation events at a single origin during replication (13). This yields many repeated units of a gene and is the mechanism by which multiplication families develop (18).

Once a gene family is created, a general pattern of divergence ensues (with the exception of the multiplication gene families) (14). Usually, a member of the gene family, not necessarily the original gene, retains the sequence and function of the original gene, evolving at a rate comparable to single copy genes (14). In contrast, the remaining members of the gene family begin to quickly acquire mutations, a high percentage of which lead to amino acid substitutions (14).
Eventually these genes diverge to a point where they either become transcriptionally silent (pseudogenes) or they acquire a new function (14). Genes that do acquire new functions often encode proteins that regulate or interact with the molecule resembling the original protein (13).

The most interesting features of gene families are the mechanisms of coordinate regulation that some of them have evolved. Coordinate transcriptional regulation is mediated by a DNA element termed a locus control region (LCR) (20). The LCR lies several Kb away from the gene family where it mediates gene expression by altering the structure of the DNA region (chromatin) encompassing the genes (21). Primarily the LCR will direct the gene chromatin to be tightly wound, causing the genes to be transcriptionally silent, although in certain tissues the LCR will direct the chromatin to assume a looser configuration, enabling transcriptional activators to bind the genes and induce their expression (21).

The most thoroughly studied example of coordinate regulation is the intriguing mechanism by which the human β-globin gene family is regulated (22). The LCR of the β-globin family is located approximately 65 Kb from a region 50 Kb in length containing the 6 β-globin genes (22). It confers erythroid specific expression on the β-globin genes by controlling the structure of a 100 Kb chromatin region encompassing the β-globin loci (21). The β-globin LCR additionally acts as an enhancer (21). Specifically, it has been found to act in
combination with various regulatory proteins to confer individual expression to each of the β-globin genes. This causes different subsets of the β-globin genes to be expressed at different times of human development (21). LCR have also been identified for several other genes including those of the TCR, Ig-chains, and the MHC class II (23-25), although essentially the ability of long-range DNA elements to regulate gene expression and the complex organization of the interphase nucleus are just beginning to be understood. As each of the 8 FcγR genes contain Ig-like domains they are considered to be a subfamily of the Ig-superfamily (17). Their small number and possession of introns also identifies them as an informational family that most likely arose by unequal chromatid exchange. Comparisons of the FcR genes suggest that the FcγRIII genes most closely represent the ancestral FcγR gene (2). The FcγRI genes are closely related to the FcγRIII genes, with the exception that they each contain a third Ig-like domain that must have been inserted into the ancestral FcγRI (2). Alternatively, the FcγRII genes have diverged considerably from the other FcγR and are considered to be the most recently evolved. This is interesting in light of the fact that the FcγRII genes each encode proteins with ITAM or ITIM motifs located directly within their cytoplasmic tails, causing some to suggest that there is a selective pressure for simpler receptor complexes (2).
All 8 FcγR genes in humans are located on chromosome 1 and it has often been speculated that they may be coordinately regulated (2;26). Five of the FcγR genes are linked at 1q23 (26). They are organized into 2 clusters 1 that contains FcγRIIB and FcγRIIA and spans approximately 50 Kb, and another that contains FcγRIIB, FcγRIIC, and FcγRIIIA, and spans approximately 120 Kb (26). Additionally, the genes for the FcεR (FCER) and the γ-chain homodimer (FCERIG) are also located on 1q23 (9;27). At the time I began my graduate work, it was not yet known where on chromosome 1 the FcγRI genes were located. There was considerable interest in determining if they were located in close proximity to each another or to the FcγR gene clusters at 1q23 since it was thought that they were good candidates for studying coordinate regulation mechanisms.

III Cell signaling

For those who have studied multi-chain immune recognition receptors (MIRR), which encompasses the TCR, BCR, and the majority of the FcR, it has long been a question as to how clustering of the extracellular regions of these receptors initiates a signal cascade within the immune cell. Some believed that
clustering induced conformational changes in the cytoplasmic tail of the MIRR receptor, which in turn initiated signal transduction. Many scientists found this explanation unlikely, the most notable of which was Metzger who reasoned quite convincingly that the biochemistry of the cell membrane made it unable to conduct such subtle changes (28). Fortunately, advances in signal transduction research have provided insight into not only this conundrum but also many others regarding MIRR receptor signaling.

Many of the discoveries in MIRR receptor cell signaling arose primarily through the study of the BCR and TCR, although it was soon discovered that all MIRR receptors transduce signals in a similar manner (29,30). Specifically, MIRR receptors associate in the resting state with src tyrosine kinases through a tyrosine motif, termed the Immunoreceptor Tyrosine-based Activation Motif (ITAM), which is present in the intracellular region of the MIRR receptor complex (31,32). As the MIRR receptors are clustered, these src kinases phosphorylate the MIRR ITAM and undergo an increase in their catalytic activity (29,30). It has been theorized, by Cambier, that extracellular ligand clustering initiates signaling by additionally clustering these ITAM-bound src kinases up against the ITAM motifs of adjacent MIRR receptors, causing them to trans-phosphorylate the adjacent MIRR ITAM motif and initiate receptor signaling (30).
Once the ITAM is phosphorylated, a signal cascade ensues. Specifically, the phosphorylated ITAM proceeds to bind various SH2-containing signaling molecules such as the src-tyrosine kinases, the Syk/ZAP70 tyrosine kinases, and inositol kinases (33). These kinases interact at the ITAM, often leading to their being phosphorylated and undergoing an increase in their catalytic activity. They then perpetuate the MIRR receptor signal by binding and phosphorylating subsequent signaling molecules, such as the inositol kinases Phosphoinositol 3-kinase (PI3-K), Phospholipase C γ1 (PLCγ1), Phospholipase C γ2 (PLCγ2), and the adapter molecule Shc (29;30). This in turn induces inositol phosphate turnover, Ras activation, and an increase of intracellular Ca\(^{2+}\), which culminates in the induction of the various effector responses mediated by the MIRR receptors.

Recent advances regarding MIRR signaling have led to the discovery that certain integral molecules negatively regulate the responses of the MIRR receptors. The most thoroughly explored example of this is the regulation of the BCR by FcγRIIB. In certain instances, where the BCR is co-clustered with FcγRIIB, there has been found to be an inhibition of Ras activation, inositol phosphate turnover, and Ca\(^{2+}\) flux. This inhibition has been shown to be dependent upon a motif present within the tail of FcγRIIB termed the
Immunoreceptor Tyrosine-based Inhibitory motif (ITIM), which upon co-clustering engages both an inositol phosphatase (SHIP) and a tyrosine phosphatase (SHP-1) (34;35).

The signal transduction mechanisms of the remaining FcγR are consistent with that of MIRR receptors. As mentioned, both FcγRI and FcγRIII oligomerize with an ITAM-containing subunit (4) whereas FcγRII (FcγRIIA/FcγRIIC) has an ITAM motif located directly within its cytoplasmic region (7;36). Technically FcγRII is not a multi-chain receptor but it is still considered to be part of the MIRR family. Studies have confirmed that FcγRI, FcγRII, and FcγRIII each engage src kinases, the tyrosine kinase Syk and the inositol PI-3 kinase (37). There is also evidence that FcγRIII is negatively regulated by an ITIM-containing molecule in NK cells (34;35). Despite these notable advancements in understanding FCR receptors signaling questions still remain which need to be addressed such as identifying subsequent signaling molecules involved with the FcγR signaling and determining if FcγRI and FcγRII are regulated by ITIM-containing receptors.
CHAPTER 2

GENOMIC MAPPING STUDIES
OF THE HUMAN FCGR1 GENE FAMILY ENCODING FcγRI

Introduction

Receptors for the Fc region of immunoglobulin G (FCGR) serve as important links between immunoglobulin molecules and immune cells, and are considered to be central mediators of the human immune system. These receptors are organized into 3 classes FCGR1, FCGR2 and FCGR3 that are represented by 8 genes in humans. Five of these genes are closely linked on 1q23 (38). They are organized into 2 clusters (9), one that contains FCR3B and FCGR2A and spans approximately 50 Kb, and another which contains FCGR2B, FCGR2C, and FCGR3A, and spans approximately 120 Kb. Digestion of human genomic DNA with the long-range restriction endonuclease Nol yields a 3 Mb DNA fragment.
that contains both gene clusters (26). All 3 remaining human FCGR genes are members of the FCGR1 gene family, which encodes the high affinity FCGR. These genes are also present on chromosome 1 and it is of interest to determine if they are tightly linked to one another, to the FCGR gene clusters at 1q23, or to the FCER1G gene, which encodes a sub unit of FCGR1, that is also at 1q23 (27).

The 3 FCGR1 genes termed FCGR1A, B and C are highly homologous (>96%) and are most easily distinguished by characteristic endonuclease fragments rendered by digestion with the enzymes HindIII and NcoI (5). The organization and high homology of the FCGR1 genes suggest that they were created by 2 recent gene duplications. If this were the case they would be expected to be located in close proximity of one another (18). By examining human genomic DNA that had been digested with the long-range cutting restriction endonucleases, through Southern analysis, others had shown that the FCGR1 gene family localized to a single XhoI or SalI hybridization DNA bands measuring 50 or 60 Kb respectively (39).

Here we present evidence, through the characterization of FCGR1-containing Yeast Artificial Chromosome (YAC) clones and analysis of human genomic DNA, digested with long-range restriction endonucleases, that the FCGR1 genes are not tightly linked to one another (<300 Kb) but instead can be easily separated into 3 large genomic fragments. Our data implies that the high
homology found between the FCGRI genes also extends many Kb past the perimeter of the genes, accounting for why they contain similar restriction endonuclease patterns even with some long-range cutting restriction endonucleases. We also demonstrate that the FCGRI genes are not tightly linked to the FCGR2, FCGR3 and FCER1G genes at 1q23 nor are they in close proximity to the CD1 gene family, at 1q21 (40).

Experimental Procedures

Reagents. HindIII, NruI, ClaI, SmaI, NaeI, and EcoRI were purchased from Boehringer Mannheim (Indianapolis, IN) and NcoI, NotI, SfiI, SacII, and BssHII were purchased from New England Biolabs (Beverly, MA). HaeIII DNA fragments of PhiX174 and HindIII DNA fragments of Lambda DNA were acquired from GIBCO, BRL (Grand Island, NY) Yeast chromosomes from Sacchromyces cerevisiae and λ DNA concatemers were purchased from Pharmacia, (Piscataway, NJ). PBR322 DNA was acquired from Boehringer Mannheim.

DNA Clones. FCGRI-containing genomic DNA phage clones (phage 1 phage 2 and phage 3) (6) and the FCER1G cDNA clone (5) have been previously described. Brian Seed (Massachusetts General Hospital, Boston, MA),
generously provided the FCGR1 cDNA p135 (41). FCGR1-containing YAC clones 1-5 were identified by Dr. A Greg Chinault (Baylor College of Medicine, Houston TX) through PCR screening of the St. Louis human genomic library (42). Dr. Ing Ming Chui (The Ohio State University, Columbus, OH) donated YAC6, which also originated from this YAC library. The FCGR2 (43) and FCGR3 (9) cDNA clones were kindly provided by Dr. Petra Warmerdam (University of Utrecht, Utrecht, The Netherlands) and Dr. Gary Peltz (University of California, San Francisco, CA). The 1278 bp CD1A cDNA clone and the 14 cosmid clones spanning the CD1 loci (44) were generously provided by Dr. Yung Yu (The Ohio State University, Columbus, OH).

**PCR analysis of YAC clones.** A portion of the FCGR1 gene spanning the 5' HindIII and NcoI FCGR1 polymorphisms was PCR amplified with FCGR1 specific primers. Specifically, Pr1 (5'-GACAGATTCT_CACTGCTCC-3') which hybridizes to the 3' end of the S1 exon and Pr19 (5'-GATCACTGCCTTTGTGGTGT-3') which hybridizes to the 5' end of the EC1 exon, from genomic yeast DNA, and human genomic DNA, (which had been isolated by standard methods from yeast clones (45) and human leukocytes (46) respectively). Amplified fragments were digested with the restriction endonucleases HindIII and NcoI after which they were electrophoretically
separated in an agarose gel and visualized by ethidium bromide staining. DNA fragments were sized in comparison to *HindIII* fragments of λ-phage DNA and *HaeIII* fragments of PhiX174 molecular markers.

Inter-Alu DNA fragments were PCR amplified, with primers Pr 65 (5'-AAGTCGCGGCCGCTTGCAGTGAGCCGAGAT-3') and Pr33 (5'-CAGCGGATCTTTGGATTACAGGCGTGCAGCCA-3') (47) separately or in combination, from genomic yeast DNA and DNA of *FCGR1*-containing phage clones 1, 2 and 3. Inter-Alu fragments were then electrophoretically separated and visualized by ethidium bromide staining after which they were sized in comparison to *HindIII* fragments of λ-phage DNA and *HaeIII* fragments of PhiX174 DNA.

*Genomic Southern Analysis of YAC clones.* Genomic DNA isolated by standard methods from yeast clones (45) and human leukocytes (46) was digested with *HindIII* or *EcoRI*, after which it was electrophoresed through an agarose gel, transferred to a Hybond-N nylon filter (Amersham), and subjected to Southern Blot analysis (48). DNA probes were labeled with [α-32P]dCTP by the random primer method (Boehringer Mannheim). Hybridization and wash conditions of all probes matched those used previously (49). 32P-End-labeled-*HindIII* fragments of λ-phage DNA were used as molecular markers.
**PFGE analysis of YAC clone and human genomic DNA.** PFGE was performed according to standard protocols (50). Genomic DNA from human or yeast cells was formed into plugs and digested with long-range cutting restriction endonucleases after which the DNA was separated using an LKB Bromma 2015 Pulsaphor Electrophoresis unit, utilizing 70” pulses of 150V for 20 hours followed by 120” pulses of 150V for 14 hours. Once separated the DNA was prepared into a Southern blot (48) and probed with either the FCGRI cDNA, or PBR322 DNA (which makes up a region of the YAC vector), that had been labeled with $[^\alpha - ^{32}P]dCTP$ by the random primer method (Boehringer Mannheim). Hybridization and wash conditions matched those previously described. (49)

**Results**

**Characterization of 5 FCGRI-containing YAC clones through PCR analysis.**

Based on the findings that the FCGRI gene family hybridizes to a single genomic DNA fragment measuring either 50 or 60 Kb, our initial efforts to map the FCGRI gene family involved searching for a large genomic clone which contained all 3 FCGRI genes. PCR analysis of a YAC library identified 6 YAC
clones (1-5 and 7) that contained \textit{FCGR1} sequences. To identify which \textit{FCGR1} genes were present within each of the YAC clones we PCR amplified a 1,459 bp region which spanned the \textit{HindIII} and \textit{NcoI} polymorphisms of the \textit{FCGR1} gene from YAC clones 1-5 and 7, human genomic DNA, and DNA from phage clones containing \textit{FCGRIA}, \textit{FCGRIB}, and \textit{FCGRIC}. The amplified DNA fragments were digested with \textit{NcoI} and \textit{HindIII}, separated by gel electrophoresis, and visualized by ethidium bromide staining.

Surprisingly, each YAC clone yielded fragments of only a single \textit{FCGRI} gene (data not shown). Specifically, YAC1 yielded the 195, 513, 73 bp fragments specific for gene A. YAC2 and YAC3 yielded the undigested 1,459 bp fragment specific for gene B, and YAC4, YAC5, and YAC7 yielded the 703 bp and 736 bp fragments specific for gene C. Human genomic DNA yielded fragments of all 3 \textit{FCGRI} genes and the phage clones each yielded the fragments corresponding to the \textit{FCGRI} gene they contained. Southern analysis outlined in Chapter 3 confirms these findings and also shows each of the \textit{FCGRI} genes present within these YAC clones to be full length.

To further characterize the \textit{FCGRI}-containing YAC clones we sized YAC clones 1-5 and 7, by separating each of their chromosomes by PFGE and then analyzing these chromosomes by the Southern method with a YAC vector probe, as shown in Figure 2.1. This blot was then stripped and reanalyzed with an
FCGRI cDNA probe (data not shown). YAC1 was found to be 190 Kb in length, YAC2 310 Kb in length, YAC3 148 Kb in length, YAC4 215 Kb in length, and YAC5 245 Kb in length. YAC7 was found to contain 2 YAC clones and was considered unsuitable for our study.

Characterization of homologous regions shared between the FCGRI-containing YAC clones.

Still pursuing the idea that the FCGRI genes were tightly linked to one another we investigated if the FCGRI-containing YAC clones carried overlapping regions by searching for homologous regions shared between them. This was achieved by identifying the sizes of inter-Alu DNA fragments present within each of the YAC clones, as Alu is a repetitive sequence, which frequently occurs specifically in the human genome. Using primers that hybridize to the sense and antisense regions of the Alu consensus sequence, inter-Alu fragments were PCR amplified from YAC clones 1-5. In order to aid in the identification of Alu fragments originating from the duplicated regions shared between the genes inter-Alu fragments were also PCR amplified from genomic phage clones of FCGRIA,
FCGRIB, and FCGRIC. Inter-Alu fragments were then separated by gel electrophoresis, visualized by ethidium bromide staining, and sized by comparison with DNA markers.

A total of 68 Inter-Alu fragments were amplified, 13 from FCGRIA-containing clones, 30 from FCGRIB-containing clones, and 25 from FCGRIC-containing clones. The low number of fragments amplified from FCGRIA-containing YAC clone was presumed to be due to it being the smallest of the FCGRI-containing YAC clones. Combined, 25 different sized Alu fragments were amplified from the FCGRI-containing clones.

Fragments of 7 different sizes were amplified from clones containing all 3 FCGRI genes. This suggested to us that the areas of homology created when the genes were duplicated span past the perimeter of the genes. Fragments of 2 different sizes were amplified from clones of both FCGRIA and FCGRIB and fragments of 6 different sizes were amplified from clones of both FCGRIB and FCGRIC. The smaller number of fragments amplified from both FCGRIA and FCGRIC, as compared to those amplified in both FCGRIB and FCGRIC, may be due to the fact that the FCGRIA clones amplified a lower number of fragments overall.
Analysis of \textit{FCGRI}-containing YAC clones for the presence of genes located on chromosome 1q.

In an effort to determine if the \textit{FCGRI} genes were tightly linked to the \textit{FCGR} gene clusters at 1q23 we tried to detect these genes in our \textit{FCGRI}-containing YAC clones. Genomic DNA from \textit{FCGRI}-containing YAC clones 1-5, a YAC clone negative for \textit{FCGRI}, YAC6, and human DNA, was digested with \textit{HindIII} and prepared into Southern blots. These blots were probed with cDNA probes of either \textit{FCGR2}, as depicted in Fig. 2.2, \textit{FCGR3}, as depicted in Fig. 2.3, or \textit{FCER1G} (data not shown). In each of these Southern blots we did not detect the expected hybridizing fragments in the \textit{FCGRI}-containing YAC clones although they were present in human DNA. To ensure that each of the \textit{FCGRI}-containing YAC clone DNA preparations contained a sufficient amount of YAC DNA to yield a hybridization signal we examined them by Southern analysis with an \textit{FCGRI} cDNA as shown in Fig. 2.4. All \textit{FCGRI}-containing YAC clones yielded hybridization fragments corresponding to the \textit{FCGRI} gene they contained.

At this point, others reported that the murine \textit{FCGRI} was part of a conserved linkage group in mice of over 19 genes spanning over the pericentric region of chromosome 1 that was conserved in humans (51). This predicted that
the h*FCGR*1 would be closely linked to the h*CDI* genes that were present at 1q21. To investigate this possibility we analyzed the *FCGRI*-containing YAC clones for the presence of *CDI* that is represented by 5 genes in humans, *CDI* A-E. *FCGRI*-containing YAC clones 1-5, a YAC clone negative for *FCGRI*, YAC6, and human DNA, were digested with EcoRI and prepared into a Southern blot which was probed with the *CDI* cDNA, as shown in Fig. 2.5. None of the *FCGRI*-containing YAC clones yielded the expected hybridizing fragments of the *CDI* genes although hybridizing fragments corresponding to 4 *CDI* genes were detected in human DNA. We also analyzed DNA from 14 cosmid clones, which combined contain 190 Kb of DNA that spans the loci all 5 *CDI* genes, by digesting them with HindIII and then preparing the digested DNA into a Southern blot which was probed with the *FCGRI* cDNA, data not shown. We did not detect *FCGRI* sequences within any of the *CDI* cosmid clones.

**Analysis of the *FCGRI* gene family with long-range cutting restriction endonucleases.**

Next, to gain information on the how close the *FCGRI* genes were linked to one another, we digested human genomic DNA with long-range cutting restriction endonucleases, separated the digested DNA by PFGE and processed it
into a Southern blots which was probed with FCGR1 cDNA. Some enzymes separated the FCGR1 gene family into 3 large DNA fragments, as shown in Fig. 2.6. The summary of all of our data analyzing human genomic DNA digested with 9 different long-range restriction endonucleases is depicted in Table 2.1, including our observation that XhoI digestion does not yield a single 50 Kb signal but instead yields 2 hybridizing fragments 175 Kb and 50 Kb in length.

Discussion

Unlike others who concluded that all 3 FCGR1 genes were located within a single 50 Kb XhoI or 60 Kb SalI DNA fragment, we found that the FCGR1 genes are not this tightly linked. Our first indication of this was our identification of 5 FCGR1-containing YAC clones which range in size from 190-310 Kb yet each contain only a single FCGR1 gene. By Alu fingerprinting each of the FCGR1-containing YAC clones we found numerous regions of homology to be shared between YAC clones of all 3 FCGR1 genes. This suggests that the areas of homology, created when the FCGR1 genes were duplicated, extend many Kb past the perimeter of each of the genes. If this is indeed the case we surmise that it is a possibility that the FCGR1 genes may have similar restriction patterns even with some long-range restriction endonucleases.
We were then able to show, by analyzing human genomic DNA with 9 different long-range cutting restriction endonucleases, that the \textit{FCGRI} genes do span a region much larger than 50 Kb. Seven out of 9 enzymes separated the \textit{FCGRI} gene family into multiple hybridizing bands, with 4 enzymes separating it into 3 distinct DNA bands. \textit{SfiI} proved to be the most informative enzyme as it yielded 3 distinct hybridizing bands (of equal intensity) measuring 600, 500 and 300 Kb in length, demonstrating that the \textit{FCGRI} genes range over at least 300 Kb.

We did find that 2 long-range endonucleases yielded only a single hybridizing band. \textit{SmaI} yielded a band 50 Kb in length and \textit{NruI} yielded a band 700 Kb in length. Since the preponderance of our data support that the 3 \textit{FCGRI} genes span an area larger than 300 Kb, we suspect that enzymes, such as \textit{SmaI}, which yield only a single hybridizing band <100 Kb are actually cutting within the homologous flanking regions shared by the \textit{FCGRI} genes. This would cause these enzymes to appear to yield only a single hybridizing fragment when they in fact are producing 3 co-migrating hybridizing fragments. Enzymes yielding single hybridizing bands > 300 Kb may be generating a fragment containing all 3 \textit{FCGRI} genes or that they may in fact be additionally generating other very large (>1200 Kb) \textit{FCGRI}-containing DNA fragments, which would not be resolved by our methods.
It is also of note that we found XhoI to yield 2 hybridizing bands, 175 and 50 Kb in length, instead of only a single 50 Kb hybridizing band, as seen by others. We suspect this disparity is due to their not fully fragmenting their DNA after PFGE separation, a step necessary to enable the DNA to pass through the gel and be transferred onto the blotting membrane during Southern analysis. This would specifically prohibit the larger fragments from transferring, resulting in transfer and hybridization of only the smaller fragments. Another possibility is our XhoI digests were only partially digested, yielding a 175 Kb XhoI fragment artifact, although we did observe the 2 XhoI hybridizing fragments in 3 different experiments.

Through Southern analysis of the FCGRI-containing YAC clones we were also able to show that the FCGRI genes are not tightly linked with the FCGR2, FCGR3, FCERIγ genes at 1q23. New evidence shown by Oakey et. al. (51) makes it likely that the FCGRI gene family is located at human 1q21, adjacent to the CD1 and CACY genes. Although our Southern analysis of the FCGRI-containing YAC clones and the CD1-containing cosmid clones show that FCGRI genes are not tightly linked to CD1 it is still very possible that they are located in proximity to it.

Our next efforts will focus on exploring the possibility that FCGRI is present at 1q21 in humans. We plan to use In situ hybridization methods to
locate \textit{FCGRI} within human metaphases, which will also demonstrate if the \textit{FCGRI} genes are $>1000$ Kb from one another. If the \textit{FCGRI} genes were found located at 1q21, it would be of interest to analyze further human genomic DNA digested with long-range cutting restriction to investigate if they are linked to the \textit{CD71} and \textit{CACY} genes.
Fig. 2.1 *FCGRI*-containing YAC clones measure between 190-310 Kb in size. Chromosomes from YAC clones 1-5, and 7 were electrophoretically separated in agarose by PFGE and transferred to a nylon membrane. Membranes were then hybridized with $^{32}$P-labeled fragments of the YAC vector. Hybridization fragments were sized by comparison with *Saccharomyces cerevisiae* chromosomes (some of which are noted in the left margin). YAC chromosomes were found to range in size between 190-310 Kb. The clone YAC7 was found to contain two hybridizing YAC chromosomes.
Fig 2.2 Southern analysis of *FCGRI*-containing YAC clones reveals they do not contain *FCGR2*. DNA from human leukocytes (Hu) and YAC clones 1-5 and 7 were digested enzymatically with *HindIII*. Digested DNA was then separated electrophoretically in agarose and transferred to a nylon membrane that was hybridized with $^{32}$P-labeled fragments of the *FCGR2* cDNA. All expected fragments (left margin) were visible in Hu and were not detected in *FCGRI*-containing YAC clones. These findings are representative of 2 different experiments.
Fig 2.3  Southern analysis of *FCGRI*-containing YAC clones reveals they do not contain *FCGR3*. DNA from human leukocytes (Hu) and YAC clones 1-5 and 7 were digested enzymatically with *HindIII*. Digested DNA was then separated electrophoretically in agarose and transferred to a nylon membrane that was then hybridized with *^{32}P*-labeled fragments of the *FCGR3* cDNA. All expected fragments (left margin, no parenthesis) were visible in Hu and were not detected in *FCGRI*-containing YAC clones. An additional hybridization fragment (left margin, parenthesis), the expected size of the YAC vector, was detected in all YAC clones, although it was only barely visible in YAC2. These findings are representative of 2 different experiments.
Fig 2.4 Southern analysis YAC clones 1-5 and 7 ensures they contain YAC inserts. DNA from human leukocytes (Hu) and from the same preparations of YAC clones 1-5 and 7 used in the experiments of Fig 2.2 and 2.3 were digested enzymatically with HindIII. Digested DNA was then separated electrophoretically in agarose and transferred to a nylon membrane that was hybridized with $^{32}$P-labeled fragments of the FCGR1 cDNA. All expected fragments (left margin, no parenthesis) were visible in Hu and FCGR1-containing YAC clones. One lightly hybridizing fragment the expected size of the YAC vector (left margin, parenthesis) was detected in some YAC clones.
Fig 2.5 Southern analysis of FCGRI-containing YAC clones reveals they do not contain genes of the CD1 gene family. DNA from human leukocytes (Hu), FCGRI-containing YAC clones 1-5, and a YAC clone known not to contain FCGRI, YAC6, were digested enzymatically with EcoRI. Digested DNA was then separated electrophoretically in agarose and transferred to a nylon membrane that was hybridized with $^{32}$P-labeled fragments of the CD1 cDNA. Four hybridizing fragments of expected sizes (left margin, no parenthesis), each corresponding to a different CD1 gene (right margin), were visible in Hu and were not detected in FCGRI-containing YAC samples. A lightly hybridizing fragment was detected in all YAC clones but not in Hu DNA (left margin in parenthesis).
Fig. 2.6 Analysis of human genomic DNA with long-range restriction endonucleases separates the \textit{FCGRI} gene family into 3 large DNA fragments. DNA from human leukocytes were digested enzymatically with \textit{SfiI}, \textit{NruI}, \textit{SacII}, and \textit{BssHII}. Digested DNA was then separated electrophoretically in agarose by PFGE and transferred to a nylon membrane that was hybridized with $^{32}$P-labeled fragments of the \textit{FCGRI} cDNA. \textit{SfiI} and \textit{SacII} separated the \textit{FCGRI} gene family into 3 distinct fragments. These findings are representative of 2 different experiments.
Fig. 2.6
Table 2.1. Hybridization bands generated by analyzing human genomic DNA which had been digested with long-range restriction endonucleases with an FCGRI cDNA probe.

<table>
<thead>
<tr>
<th>Number of Hybridization Fragments</th>
<th>Long-Range Restriction Endonucleases</th>
<th>Size of Hybridization Fragments (Kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SmaI, NruI</td>
<td>50, 750</td>
</tr>
<tr>
<td>2</td>
<td>ClaI, NaeI, XholI</td>
<td>650, 275, 560, 520, 175, 50</td>
</tr>
<tr>
<td>3</td>
<td>SfiI, SacII, ApaI, BssHII</td>
<td>600, 500, 300, 540, 300, 175, 270, 150, 50, 550, 300, 175</td>
</tr>
</tbody>
</table>

Human genomic DNA was digested with 9 different long-range restriction endonucleases. Digested fragments were then separated by PFGE and prepared into a Southern blot which was probed with the FCGRI cDNA. Four enzymes separate the FCGRI gene family into 3 large genomic fragments.
CHAPTER 3

THE THREE GENES OF THE HUMAN FCGR1 GENE FAMILY
ENCODING FcγRI FLANK THE CENTROMERE
OF CHROMOSOME 1 AT 1p12 AND 1q21

Introduction

Fc receptors for IgG (FCGR) are major mediators of antibody action. Present on the surface membranes of the various effector cells of the immune system, these receptors are clustered by immune complexes to initiate a number of diverse processes aimed at the elimination of antigen. Among the 3 classes of FCGR in humans, class I (FCGR1) is unique as it contains 3, rather than 2, extracellular Ig-like domains and exhibits relatively high affinity for IgG. It is expressed exclusively on phagocytic cells and is capable of mediating virtually all of the functions attributed to Fc receptors including endocytosis, cytolysis and inflammatory mediator release.
Three genes (A, B, C) make up the human FCGRI gene family. These genes are remarkably similar, differing by approximately a dozen nucleotides within their coding regions, but are readily distinguishable by characteristic endonuclease cleavage fragments rendered by digestion with the enzymes HindIII and NcoI (5). Of the 6 different transcripts found for FCGRI, 1 designated FCGRIa1 is considered to be the bona fide FCGRI, as it encodes a transmembrane molecule with 3 extracellular domains that is recognized by several anti-FCGR1 antibodies and binds IgG with high affinity (6). Recently FCGR1A has been shown to be a member of a multimolecular signalling complex comprised of an ITAM-containing homodimer (FCER1G), and at least 2 protein tyrosine kinases, including Syk (52).

Although all 3 FCGRI genes are known to be present on chromosome 1 (5), we have sought to define more precisely their location relative to one another and to the 1q23 region which contains the Fc receptor gamma chain subunit (FCER1G) and 5 other FCGR genes all tightly linked to one another (9;26). In contrast to others (53;54), we find through both fluorescence in situ hybridization (FISH) analysis of human cells and Southern analysis of 1p and 1q containing cell lines, that the 3 FCGRI genes flank the centromere of chromosome 1 at bands 1p12 and 1q21. Our gene dosage experiments suggest that there are no more than 3 FCGRI genes in humans.
Experimental Procedures

DNA Clones and Cell Lines. Preparation and characterization of the $FCGRI$ genomic DNA containing phage clones (phage 1, phage 2 and phage 3) and the subcloned $FCGRI$ genomic DNA plasmid, p7.4, have been previously described (5). The h$FCGRI$ cDNA, p135, was generously provided by Dr. Brian Seed (Massachusetts General Hospital, Boston, MA) (41). YAC clones 1-4 and 6 were identified through PCR screening by Dr. A. Craig Chinault (Baylor College of Medicine, Houston, TX) of the St. Louis human genomic YAC library (42). Human/hamster hybrid cell line 1099, which contains a full length human chromosome 1, was purchased from Bios Corp., (New Haven, CT). Human/mouse hybrid cell line 1492 (55), which contains a derivative chromosome formed by the translocation of an the entire human 1p chromosomal arm to a mouse chromosome, and human/hamster hybrid cell line 1173 (56), which contains a derivative chromosome X, t(X;1)(p21.2;p12), were acquired from the NIGMS Human Genetic Mutant Cell Repository (Coriell Institute, Camden, NJ). Human/hamster hybrid cell line CON5 (57), which contains a derivative chromosome, t(1;19)(1pter→1cen::19cen→(19qter), was kindly provided by Dr. Susan Povey (MRC University College London, London, U.K.), and the HAL26
human/mouse hybrid cell line (58), which also contains a derivative chromosome, der(6)t(1;6)(6qter→6pter::1q21→qter), was generously provided by Dr. Jeffrey M. Trent (National Center for Human Genome Research, Bethesda, MD). The mouse cell line NIH3T3 and the hamster cell line CHO were acquired from ATCC (Rockville, MD). Cosmid 2, a human genomic DNA cosmid clone, which contains exon 7 of the dystrophin gene (DMD) and surrounding flanking area (59), was kindly provided by Dr. Arthur Burghes (The Ohio State University, Columbus, OH).

**Fluorescence in situ Hybridization and Chromosomal Localization.**

Human metaphase chromosomes from phytohemagglutinin-stimulated normal lymphocytes were prepared on microscope slides by standard cytogenetic techniques (60). The FCGRI probe, an FCGRIA genomic DNA phage clone (phage 1), was labeled with biotin by nick translation (Amersham, Arlington Heights, IL) utilizing biotin-conjugated uracil dNTPs (Stratagene, La Jolla, CA), and after blocking of its repetitive elements with Cot1 and human placental DNA, was used for standard FISH analysis (Oncor Inc., Gaithersburg, MD.). Hybridization targets were made visible with FITC-conjugated avidin and FITC-conjugated anti-avidin mabs (Oncor) after which chromosomes were counterstained with propidium iodide. Chromosomes were then analyzed by fluorescence microscopy. Each fluorescence
hybridization signal was localized to a chromosomal band by approximating its position relative to a GTW-banded metaphase chromosome from the same individual. Human metaphase chromosomes were also subjected to FISH analysis utilizing as probes PCR amplified Alu-Alu sequences from each of the FCGRI containing YAC clones, by methods previously described (61).

*Genomic Southern Analysis of YAC clones and Human/Rodent Hybrid Cell Lines.* Genomic DNA was isolated by standard methods from yeast clones (45), human/rodent hybrid cell lines, and human leukocytes (46). Southern analysis of hybrid cell lines required 125 ug of DNA for each sample, sometimes employing hamster DNA as a negative carrier. DNA, digested with *HindIII* (Boehringer Mannheim, Indianapolis, IN) and *NcoI* (New England Biolabs, Beverly, MA), was electrophoresed through an agarose gel, transferred to a Hybond-N nylon filter (Amersham), and subjected to Southern Blot analysis (48). The hFCGRI S1 exon DNA probe was PCR amplified from the FCGRIA genomic DNA clone, phage 1. The probe was labeled with [α-32P]dCTP by the random primer method (Boehringer Mannheim). Hybridization and wash conditions for the S1 probe matched those used previously for FCGRI Southern analysis (49). 32P-End-labeled- HindIII fragments of λ-phage DNA were used as molecular markers.
Southern Analysis of PCR amplified products with Gene Specific Oligonucleotides. A portion of the FCGRI gene, consisting of the 3' region of the EC2 exon (150 bp), was PCR amplified with 2 FCGRI specific primers, Pr31 (5'-CCTCACCATTCTGAAAAC-3'), which hybridized to the 3' end of EC2, and Pr16 (5'-ATTATGATGGTCCTCAGG-3'), which hybridized to the 5' end of intron D. Amplified fragments were electrophoretically separated in an agarose gel, visualized by ethidium bromide staining, and subjected to Southern blot analysis with FCGRI cDNA (p135) and 2 FCGRI gene specific DNA probes. The blots were stripped of probe between hybridizations with boiling 0.1% SDS. Oligonucleotide 37A, which specifically hybridizes to gene FCGRIA, and oligonucleotide 39B/C, which specifically hybridizes to both genes FCGRIB and FCGRIC, along with hybridization and wash conditions for each, have been previously described (5).

Quantification of FCGRI Copy Number. The pGCC construct was prepared by cloning into the psp70 vector (Promega, Madison, WI) both an FCGRI 3.3 kb EcoRI genomic fragment (F), that was isolated from a subcloned HindIII fragment of FCGRIA phage 1 (p7.4), and 3 DMD EcoRI genomic fragments of the sizes 4.1 kb (D), 1.4 kb and 0.95 kb, which were isolated from a DMD genomic clone (Cosmid 2). pGCC DNA was added to
hamster carrier DNA and diluted into amounts that would mimic a range of genomic copy numbers (p0.5 - p6.0), based on the sizes of both the construct and the human genome (45).

Human DNA, isolated from the leukocytes of 8 women (G1-G8), and pGCC DNA samples (p0.5 - p6.0) were then digested with EcoRI (Boehringer Mannheim), separated in an agarose gel, and subjected to Southern analysis. Blots were hybridized simultaneously with exon 7 of DMD (d7, 110bp) and the 5' half of the FCGR1 EC3 exon (f3, 138 bp). Both the d7 and f3 probes were prepared by PCR amplification of these regions from the genomic DNA clones, Cosmid 2 and p7.4, respectively. Hybridized blots were then visualized and analyzed by phosphorimage scanning using a Molecular Dynamics phosphorimager.

Results

Localization of the FCGR1 gene family to 1p12 and 1q21 by FISH analysis.

To define the location of the 3 genes of FCGR1 within chromosome 1, we first analyzed chromosome spreads of human cells from 3 individuals by FISH. As the DNA sequences of the FCGR1 genes are more than 98%
identical, all probes of suitable length for FISH were ones that would hybridize to all 3 genes. The probe utilized was an 18 kb human genomic phage clone, phage 1, that contains the \textit{FCGRI} A gene (\textit{hFCGRI}A). Of the spreads examined, 44 chromosomes 1 from 27 cells were suitable for analysis. The hybridization signals for each selected chromosome 1 were noted and then positioned by comparison with GTW banded chromosomes.

Two different hybridization patterns were observed. 61% of analyzed chromosomes 1 displayed a hybridization signal solely at 1q21 whereas 39% showed hybridization at both 1p12 and 1q21. Photomicrographs of coincident 1p and 1q hybridization signals are shown in figure 3.1 and matching GTW banded chromosomes are shown in figure 3.2. The 1p12 signal was observed only in the presence of the 1q21 signal and was especially apparent on cell spreads in which the chromosomes were loosely compacted and the hybridization signal was intense. Despite the inconsistency of the 1p12 signal, it was observed on chromosomes 1 originating from all 3 individuals and was of considerable intensity within certain samples.
Characterization of 4 FCGR1 containing YAC clones.

Attempting to map the FCGR1 genes relative to one another we characterized several large (190 - 310 kb) FCGR1 containing genomic clones, Yeast Artificial Chromosome (YAC) clones, to determine if they contained more than 1 FCGR1 gene. FCGR1 specific primers were used to screen a human genomic YAC library for FCGR1 containing clones. Four YAC clones (1-4) were found to contain FCGR1 sequences and were characterized by Southern analysis.

In order to determine the specific FCGR1 gene present on each YAC clone, Southern analysis involving digestion with both NcoI and HindIII was performed, as these restriction endonucleases have been shown to yield a distinct fragment for each FCGR1 gene (5). Digested DNA of YAC clones 1-4 and YAC6, a YAC clone which is negative for FCGR1, were prepared into a Southern blot and probed with hFCGR1 S1 exon. As shown in the right panel of figure 3.3, YAC clones 1-4 each yielded hybridizing fragments of only a single FCGR1 gene. Specifically, YAC1 displayed hybridization to the 0.6 kb fragment distinctly associated with FCGR1A, YAC2 and YAC3 to the 1.9 kb fragment of FCGR1B and YAC4 to the 1.1 kb fragment of FCGR1C. The control YAC6 showed no specific hybridization. Southern analysis
involving HindIII digestion was also performed on the DNA samples probing with the hFCGRI cDNA in combination with the hFCGRI S1 exon (data not shown) as this type of Southern distinguishes a full length FCGRIA gene from a full length FCGRIB or FCGRIC gene (5). Each clone was found to contain 1 full length FCGRI gene and there was no evidence for multiple genes on a single YAC clone.

Hybridization of 4 FCGRI containing YAC clones to 1p and 1q through FISH analysis.

We then extended our FISH analysis using sequences of the FCGRI containing YAC clones as probes with the premise that if the flanking sequence surrounding each FCGRI gene were significantly unique, the YAC sequences would hybridize to regions specifically associated with each FCGRI gene. To acquire human genomic sequences from each YAC clone, we utilized a single primer that annealed to the distinctly human Alu sequence to PCR amplify inter-Alu DNA fragments from each of the 4 FCGRI containing YAC clones (1-4). These fragments were then used as probes in individual FISH experiments on human cell spreads. The data are summarized in table 3.1.
Probes from each of the 4 FCGRI YAC clones yielded hybridization signals at both 1p12 and 1q21. Once again the 1q21 signal was both stronger and more consistent than the 1p12 signal. Specific hybridization to areas not observed with the phage probe were also noted and were considered to be most likely due to chimeric inserts: YAC1 probes showed hybridization at 20q13.32-13.33 whereas YAC2 probes yielded hybridization at both 1q32 and 9q13-21.13. Strong hybridization was observed with the YAC4 probes at 1p36.13-36.22, implying that this YAC clone may contain either a U1 pseudogene or a tRNA gene, as members from each of these gene families are clustered at both 1q21 and 1p36 (62,63).

Genomic Southern analysis of 1p and 1q containing cell lines indicate that the FCGRI genes flank the centromere of chromosome 1.

Next, to localize each of the 3 FCGRI genes to the 1p12 and 1q21 loci, we examined human-hybrid cell lines that contain either the 1p or 1q arm but lack a full length chromosome 1. The portions of chromosome 1 contained within each of the cell lines are depicted in figure 3.4. DNA from 3
cell lines containing lp (1492, 1173 and CON5), and a cell line containing 1q (HAL26), were analyzed by Southern analysis for the presence of \textit{FCGRI} sequences.

To determine the particular \textit{FCGRI} genes present within the lp and 1q cell lines, the DNA samples were digested with both \textit{HindIII} and \textit{NcoI}, and probed with the \textit{hFCGRI} S1 exon. The data are shown in figure 3.5. All 3 lp-containing cell lines (1492, 1173 and CON5) exhibited specific hybridization at a band 1.9 kb in size, the hybridizing fragment characteristic of the \textit{FCGRI}B gene, whereas the 1q cell line (HAL26) yielded hybridization bands 1.1 kb and 0.6 kb in size, the hybridizing fragments associated with the \textit{FCGRI}C and \textit{FCGRI}A genes, respectively. Human DNA displayed all 3 \textit{FCGRI} fragments, namely, 1.9 kb, 1.1 kb and 0.6 kb. Mouse and hamster DNA showed no specific hybridization. DNA samples were also digested with \textit{HindIII} and probed with a mixture of \textit{hFCGRI} cDNA p135 and \textit{hFCGRI} S1 exon revealing that the \textit{FCGRI} genes within these clones were full length (data not shown).
Further evidence that the $FCGR1$ genes flank the centromere of chromosome 1 using gene specific oligonucleotide probes.

To gain further evidence as to which $FCGR1$ genes were present within the p and q regions of chromosome 1, we used an oligonucleotide probe specific for the $FCGR1A$ gene (37A) and an oligonucleotide probe specific for both the $FCGR1B$ and $FCGR1C$ genes (39B/C). The exon encoding the second extracellular domain of h$FCGR1$ was amplified by PCR from the DNA of 3 cell lines containing 1p (1492, 1173 and CON5), a cell line containing 1q (HAL26), and a cell line containing a full length chromosome 1 (1099). The DNA was analyzed by Southern analysis first applying the h$FCGR1$ cDNA (p135) probe, as shown in the top panel of figure 3.6, and then by applying the $FCGR1A$ specific oligonucleotide probe (37A), as shown in the middle panel of figure 3.6. Lastly, the $FCGR1B/C$ specific oligonucleotide probe (39B/C) was applied to the Southern blot, as depicted in the bottom panel of figure 3.6. All 3 1p containing cell lines (1492, 1173 and CON5) showed hybridization with both the h$FCGR1$ cDNA and the $FCGR1B/C$ specific oligonucleotide whereas the cell line containing 1q (HAL26) exhibited hybridization to all 3 probes. The cell line containing the full length chromosome 1 (1099) also yielded hybridization to all 3 probes.
Quantification of FCGR1 copy number within humans.

Next, we quantified FCGR1 gene copy number by Southern analysis of human genomic DNA using dystrophin (DMD), a single copy X-linked gene, as a standard. Specifically, we compared the hybridization intensity of an FCGR1 EcoRI genomic DNA fragment (F, 3.3 kb), probed with the 3' half of the FCGR1 EC3 exon (β, 138 bp), to the hybridization intensity of a single copy DMD EcoRI genomic DNA fragment (64) (D, 4.1 kb), probed with the DMD exon 7 (d7, 110 bp). To adjust for transfer and hybridization differences, a plasmid (pGCC) which contained both the F and D fragments was included within the analysis.

Genomic DNA, isolated from 7 cytogenetically normal women (XX, G1-G7) and 1 woman with 4 X chromosomes (4X, G8), pGCC plasmid DNA in mouse carrier DNA (P) and mouse DNA alone (Mo), were digested with EcoRI, electrophoretically separated in agarose, and processed into a Southern blot. Blots were then hybridized simultaneously with the β and d7 probes and analyzed by phosphorimage scanning. Genomic and pGCC samples yielded hybridizing fragments 4.1 kb (D) and 3.3 kb (F) in size, shown in figure 3.7. Mouse DNA showed no specific hybridization. pGCC
samples of various amounts were also analyzed and plotted, D counts (as depicted in figure 3.8) or F counts (as shown in figure 3.9) vs pGCC DNA amount (ug) and were each found to have a correlation coefficient of 0.95.

$FCGRI$ copy number was then calculated for each genomic sample by comparing F counts with D counts, correcting for the hybridization differences of the 2 target fragments with a quotient derived from the pGCC sample. Averaging the $FCGRI$ copy numbers for each of the 8 individuals, in 2 duplicate experiments we found the number of $FCGRI$ genes within the human genome to be $3.2 \pm 0.5$, mean ± SD (n=8). The $FCGRI$ copy number of the 4X individual was also found to equal 3.2. Although not visibly apparent in the Southern blot pictured in figure 3.7, one individual (G3) had an estimated $FCGRI$ copy number of 4.1 genes and was statistically found with over 95% confidence to be an outlier (65). Recalculating the data omitting this outlier yields a copy number of $3.1 \pm 0.3$, mean ± SD (n=7), suggesting that the human genome contains 3 $hFCGRI$ genes.
Discussion

We have determined that the 3 genes for hFCGRI flank the centromere of chromosome 1, with FCGRIB located at 1p12 and both FCGRIA and FCGRIC located at 1q21. This finding, based on data derived from both FCGRFISH analysis of human lymphocyte chromosomes and genomic Southern analysis of cell lines containing either the p or q arm of chromosome 1, differs from previous reports which locate all 3 genes to 1q21 (S3;54).

FCGRFISH analysis utilizing either an FCGRIA DNA phage clone probe or FCGRI DNA YAC clone probes (Figure 3.1, Table 3.1) yielded a consistent signal at 1q21 and an inconsistent weaker signal at 1p12. The 1p12 locus may have been overlooked in earlier studies because of its weak hybridization by FCGRI probes. Possibly the 1p12 FCGRI locus yields a weak signal because there is only a single FCGRI locus within this region whereas the 1q21 region contains 2 tightly linked FCGRI loci.

Southern analyses which distinguish the 3 FCGRI genes by polymorphisms in either the 5' region or the EC2 exon were performed on DNA isolated from hybrid cell lines containing either the 1p or 1q chromosomal arm (Figures 3.5 and 3.6). This strategy confirmed the
existence of an *FCGRI* 1p locus. These experiments additionally showed that all 3 1p cell lines contain the *FCGRIB* gene and that the 1 1q cell line contains both the *FCGRIA* and *FCGRIC* genes. The *FCGRI* genes detected in these cell lines appeared to be full length, based on the sizes of the observed hybridizing fragments. Others have found all 3 *FCGRI* genes present within hybrid cell lines that lacked various segments of the 1q arm but still contained both the 1p12 and 1q21 regions (53); these data are consistent with our findings.

Our determination of the number of *FCGRI* gene copies in the human genome indicates that most individuals bear 3 genes (Figure 3.7). To measure *FCGRI* copy number we analyzed genomic DNA from 8 individuals by the Southern method, using the single copy X-linked *DMD* gene as a standard. Analysis of varying amounts of a plasmid clone containing the target fragments of both genes established that the relationship between DNA amount and hybridization intensity was linear. We found that the *FCGRI* copy number in a sample of 8 individuals was $3.2 \pm 0.5$ (mean ± SD). Removing the single outlier with an *FCGRI* copy number near 4 (3.8 and 4.4) from the sample yielded a copy number of $3.1 \pm 0.3$. An individual carrying 2 *DMD* genes (4X) was also found to have a copy number of 3 *FCGRI* genes. This demonstrated that our method found *FCGRI* copy number to equal 3.
from 2 different standards (1 or 2 DMD genes), supporting the validity of our method. We have not pursued the suggestion that some individuals may bear 4 FCGRI genes.

We estimate by the Jukes-Cantor method (66), using a human mutation rate of $1.3 \times 10^{-9}$ (67), that the ancestral FCGRI gene was duplicated approximately 2.4 to 3.4 million years ago. A likely scenario is that the ancestral FCGRI gene first duplicated into 2 genes, 1 of which was maintained as the *bona fide* FCGRI (FCGRIA), whereas the other soon acquired the 6 bp polymorphism in EC2 specific for FCGRI/B/FCGRI/C. Soon after, this second gene itself was then duplicated yielding 2 FCGRI genes with the 6 bp EC2 polymorphism (FCGRI/B and FCGRI/C). A comparison of the nucleotide differences of the FCGRI/B and FCGRI/C genes to the FCGRIA gene revealed twice as many differences which cause an amino acid change as those which cause no amino acid change, suggesting that FCGRI/B and FCGRI/C have evolved at rates comparable to noncoding regions (68), a common circumstance for duplicated genes developing into either pseudogenes or genes which will encode proteins with a new function (18). Since the FCGRI/B and FCGRI/C genes do contain nonsense mutations, one may reason that they indeed have developed into pseudogenes, but
evidence of alternate spliced transcripts of these genes suggests they may very well encode proteins with alternative, currently unknown, FCGRI1 functions (5).

Curiously, the *FCGRI* gene family is separated from the other *FCGR* genes at 1q23 and is also itself divided onto 2 different chromosomal arms. The *FCGRI* genes would be expected to be closely linked to one another since they duplicated recently and most likely by mechanisms that would place them closely in tandem (13;18;69). Reasons behind the location of the *FCGRI* genes become evident after considering how the pericentric region of chromosome 1 evolved.

Specifically, the *FCGRI* genes lie within a large pericentric linkage group of nearly 20 genes on human chromosome 1, which is conserved in order in mice, partially on mouse chromosome 1 and partially on mouse chromosome 3 (70-72). Within this linkage group there are 2 regions containing homologous genes, (*Fcgr2/Fcgr3, Bcm1, and Atpl1a2*) and (*Fcgr1, Cd2/Lfa3, and Atpl1a1*). These regions are considered to have evolved by the duplication of a chromosomal segment (51), suggesting that the creation of *FCGRI* involved the duplication of a lengthy region, causing it to be placed away from the other Fc receptors.
As humans and mice diverged the linkage group in mice split into 2 different chromosomes whereas in humans the centromere was inserted dividing this area into the p and q arms of chromosome 1 (73). It is our speculation that after the centromere inserted, the ancestral FCGRI gene, originally a part of the p arm of chromosome 1, eventually was duplicated into 3 FCGRI genes, all linked on lp. Then, as others have proposed, a pericentric inversion of human chromosome 1 occurred which translocated the chromosomal bands presently known as 1q21.1 and 1q21.2 from the p arm to the q arm (74). This inversion is considered to have occurred after the divergence of the human and chimpanzee lineages which, based on our calculations of when the FCGRI genes duplicated, correlates with the time period during which the FCGRI gene family would have been separated. Thus we surmise that the FCGRI genes were originally present on lp and the pericentric inversion of human chromosome 1 translocated the FCGRIA and FCGRIC genes to 1q but left the FCGRIB gene positioned on lp.

Others have suggested that a portion of the tRNA gene family was translocated from one region of chromosome 1 to another (75), as this gene family is located on 1q21 in humans but not in monkeys. It is possible that the
pericentric inversion proposed to have occurred on human chromosome 1 (74), translocated the human tRNA genes, together with the FCGRI genes, to 1q.

Our study leads us to suggest that the pericentric inversion of the human chromosome 1 occurred within the span of the FCGRI gene family less than approximately 3 million years ago. It also leads to several predictions; namely, that the linear orientation of the 1p12 FCGRIB gene would be opposite from that of the 1q21 FCGRIA and FCGRIC genes and that FCGRIA would be closer to the centromere than FCGRIC. In nonhuman primates FCGRI would be expected to be positioned at 1p12. Additionally, future physical mapping studies would presumably show that within the large pericentric linkage group of loci conserved between humans and mice, the loci along 1q21.1 and 1q21.2 in humans would be reversed from their order in mice.
Fig. 3.1 Chromosomal localization of the hFCGRI loci to 1p12 and 1q21. Human lymphocyte metaphase chromosomes were hybridized with an 18 Kb biotin-labeled hFCGRI A genomic phage clone and counterstained with propidium iodide. Hybridization was detected with avidin-FITC and fluorescence microscopy. The fluorescence signals which mapped to 1p12 and 1q21 have been indicated (white arrows) on paired chromosomes from 2 representative metaphases.
Fig. 3.2 Human G-Banded Chromosomes 1. A GTW-banded pair of human chromosomes 1 from the same individual shown figure 3.1 with a chromosome 1 idiogram to designate band locations (black arrows).
Fig. 3.2
Fig. 3.3 Characterization of Yeast Artificial Chromosome (YAC) clones, each containing a single FCGRI gene. DNA from 4 YAC clones (1-4), which were found to contain FCGRI specific sequences by PCR amplification and a YAC clone negative for FCGRI, YAC6 (6), were digested enzymatically with both HindIII and NcoI. The digested DNA was electrophoretically separated in agarose and transferred to a nylon membrane filter. Filters were then hybridized with $^{32}$P labeled DNA fragments of the 170 bp S1 exon of hFCGRI. Hybridizing fragments were sized by comparison with λ-Phage DNA that had been HindIII digested (left margin). The size ($\times 10^3$) and gene designation for each of the hybridizing fragments are noted at the right margin of each panel.
Table 3.1. Hybridizing bands of human chromosome 1 after fluorescence in situ hybridization with Alu fragments from 4 FCGRI-containing YAC clones.

<table>
<thead>
<tr>
<th>YAC Clone</th>
<th>FCGRI gene</th>
<th>Size (kb)</th>
<th>Chromosome 1 signal (intensity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>190</td>
<td>1q21 (3+) 1p12 (1+)</td>
</tr>
<tr>
<td>2</td>
<td>B</td>
<td>310</td>
<td>1q21 (4+) 1p12 (3+)</td>
</tr>
<tr>
<td>3</td>
<td>B</td>
<td>148</td>
<td>1q21 (2+) 1p12 (1+)</td>
</tr>
<tr>
<td>4</td>
<td>C</td>
<td>215</td>
<td>1q21 (3+) 1p12 (2+)</td>
</tr>
</tbody>
</table>

Inter Alu DNA sequences were PCR amplified from YAC clones which contained either FCGRIA (YAC1), FCGRIB (YAC2 and YAC3) or FCGRIC (YAC4). The Alu fragments were then used as probes for in situ hybridization of human metaphase cells. Fragments from all 4 YAC clones yielded hybridization signals at both 1p12 and 1q21 with a range of intensities (1+ - 4+).
Fig. 3.4 Schematic representation of human chromosome 1 and the portions of it contained within 5 human-rodent hybrid cell lines.

Several well characterized human-rodent cell lines containing either the full length chromosome 1 (1099), the p arm of chromosome 1 (1492, 1173 and CON5), or the q arm of chromosome 1 (HAL26), were employed. Loci of the FCER1G gene and the FCGR1, FCGR2 and FCGR3 gene families are depicted.
Fig 3.4
Fig. 3.5 Southern analysis of genomic DNA from cell lines containing portions of chromosome 1 localize the *FCGRI* genes to both p and q arms of chromosome 1. DNA from human (Hu), 3 hybrid cell lines containing the human 1p arm (1492, 1173 and CONS), and a hybrid cell line containing the human 1q arm (HAL26); mouse DNA (Mo) and hamster DNA (Ha) were digested with *HindIII* and *NcoI*. The digested DNA was then processed and analyzed as in figure 3.3. The size and gene designation for each of the hybridizing fragments are noted at the margin of each panel.
Fig. 3.5
Fig. 3.6 Southern analysis of PCR fragments amplified from cell lines containing portions of chromosome 1 using oligonucleotide probes specific for FCGRIA or FCGRIB/C. The FCGRI EC2 exon was PCR amplified from the DNA of human (Hu), a hybrid cell line containing chromosome 1 (1099), genomic phage clones of FCGRIA (A), FCGRIB (B) and FCGRIC (C), 3 hybrid cell lines containing the human 1p arm (1492, 1173 and CON5), a hybrid cell line containing the human 1q arm (HAL26); and also of mouse (Mo) and hamster (Ha) origins, along with a water control (W). The amplified fragments were then electrophoresed through agarose and transferred to a nylon filter membrane. Filters were first hybridized with $^{32}\text{P}$ labeled DNA fragments of either a FCGRI cDNA, p135 (top panel), then an oligonucleotide 37A specific for FCGRIA (middle panel), then an oligonucleotide 39B/C specific for FCGRIB and FCGRIC (bottom panel).
Fig. 3.6
Fig. 3.7 Analysis of *FCGRI* copy number in 8 individuals using the single copy, X-linked, *DMD* gene as a standard. Genomic DNA from 7 cytogenetically normal women (XX, G1-G7), and 1 woman with 4 X chromosomes (4X, G8), plasmid DNA from a construct (pGCC) which contained both target hybridization fragments in mouse carrier DNA (P), and mouse DNA alone (Mo) were digested with *EcoRI*. Digested DNA and molecular weight marker DNA, λ-Phage that had been *HindIII* digested, were then electrophoretically separated and subjected to Southern blot analysis. Blots were hybridized simultaneously with 2 radiolabeled probes, 1 for exon 7 of *DMD* (d7) and the other for the 5' half of the EC3 exon of *FCGRI* (F3). Blots were then visualized and analyzed by phosphorimage scanning (top panel), and the radioactivity associated with the hybridizing fragments, a 4.1 kb *DMD* fragment (D) and a 3.3 kb *FCGRI* fragment (F), were quantified into arbitrary phosphorescence units (counts) for each sample. *FCGRI* copy number was then calculated for each genomic sample by comparing F counts with D counts, correcting for the hybridization differences of the 2 target fragments with a quotient (D counts/F counts) derived from the pGCC plasmid, illustrated by the following equation: $X = \frac{F \times KG}{D}$, where $X$ is the number of *FCGRI* genes.
in each genomic sample, $K$ is the pGCC sample quotient ($D/F$), $G$ is the number of $DMD$ genes in the genomic sample (1 for the $XX$ women and 2 for the $4X$ woman), and $D$ is the amount of $DMD$ counts from the genomic sample.
Fig. 3.7
Fig. 3.8 Comparison of \textit{DMD} hybridization (D counts) of the \textit{FCGR1/DMD} plasmid (pGCC) to pGCC amount. In 2 different experiments, one of which is plotted here; PGCC samples of amounts correlating with different gene copy numbers (0, 2, 4, 6, 8) were analyzed by the Southern methods with the d7 \textit{DMD} probe. D counts (y axis) were then plotted against pGCC amount (x axis, \textit{depicted in gene copy numbers}) and found to result in a correlation coefficient of 0.95.
y = 12069.164x - 5657.026

D counts/pGCC DNA

pGCC DNA amount (copy number equivalents)

Fig. 3.8
Fig. 3.9 Comparison of FCGR1 hybridization (F counts) of the FCGR1/DMD plasmid (pGCC) to the amount of pGCC. In 2 different experiments, one of which is plotted here; PGCC samples of amounts correlating with those of different gene copy numbers (0, 2, 4, 6, 8) were analyzed by the Southern methods with the f3 FCGR1 probe. F counts (y axis) were then plotted against pGCC amount amount (depicted in gene copy numbers, x axis) and found to result in a correlation coefficient of 0.95.
Fig. 3.9

pGCC DNA amount
(copy number equivalents)
CHAPTER 4

LOCALIZATION OF FCGR1 ENCODING FcγRI IN APES,
FURTHER EVIDENCE OF A PERICENTRIC INVERSION DURING THE
EVOLUTION OF HUMAN CHROMOSOME 1

Introduction

Receptors for Immunoglobulin G (FCGR) play an important role linking the humoral and cellular portions of the immune system. As integral membrane proteins expressed on a variety of cells within the immune system, they are bound and clustered by immune complexes and in turn initiate a number of cellular processes aimed at the elimination of antigen. There are 3 classes of FCGR, represented by 8 genes in humans. Five of these genes of the FCGR2 and FCGR3 classes are clustered at 1q23 in humans (26). The remaining hFCGR genes are of the FCGR1 class, which encodes the high affinity FCGR. The 3 hFCGR1 genes (A,B,C) are remarkably similar (>95%) (5) and are estimated to have been created
by 2 gene duplication events occurring approximately 3 million years ago. We have previously shown that the FCGRI gene family flanks the centromere of chromosome 1 in humans, with FCGRIB present at 1p12, and both FCGRIA and FCGRIC present at 1q21 (76), placing the FCGRI gene family within a large pericentric linkage group that is conserved between humans and mice.

It is curious that the FCGRI gene family is dispersed on either side of the centromere since the FCGRI genes were duplicated recently and in their entirety (exons and introns), and therefore would be expected to be closely linked to one another (18). The reason behind this discrepancy becomes apparent though after considering the evolution of human chromosome. Specifically, it has been proposed that a pericentric inversion of human chromosome 1 occurred after humans diverged from apes, translocating the bands presently known as 1q21.1 and 1q21.2 bands over from the p arm to the q arm (74). It is our hypothesis, as depicted in Fig. 4.1, that the 3 FCGRI genes were all originally linked on 1p. Then, after humans diverged from apes, the pericentric inversion translocated the FCGRIA and FCGRIC genes to the q arm of chromosome 1 but left the FCGRIB gene positioned on 1p (76). Here we present evidence supporting this hypothesis by localizing FCGRI, through fluorescence in situ hybridization, in
both rhesus monkey (*Macaca mulatta*) and baboon (*Papio papio*) to a band adjacent to the centromere on what is equivalent to the human 1p chromosomal arm.

**Experimental Procedures**

*Fluorescence in situ hybridization and chromosomal localization*

Metaphase chromosomes from phytohemagglutinin-stimulated normal lymphocytes of chimpanzee (*Pan troglodytes*), baboon (*Papio papio*), and rhesus monkey (*Macaca mulatta*), which were kindly donated by the Laboratory for Experimental Medicine and Surgery in Primates (New York University, NY, NY), and humans, were prepared on microscope slides by standard cytogenetic techniques (77). The *FCGR1* probe consisted of a cocktail of 3 pBluescript (Stratagene, La Jolla, CA) *FCGR1A* DNA genomic clones (p1.9, p5.4, p7.4) which have been previously described (5). These 3 *FCGR1* plasmids were labeled with biotin by nick translation (Gibco, BRL, Grand Island, NY) and then, after blocking of their repetitive elements with Cot1 and human placental DNA, were used for standard FISH analysis (Oncor, Gaithersburg, MD). Hybridization signals were made visible with Texas Red-conjugated avidin and anti-avidin
monoclonal antibodies (Oncor) after which DNA was counter stained with DAPI II (Vysis Inc., Downers Grove, IL). Samples were analyzed by fluorescence microscopy.

Results

Localization of FCGR1 in 3 species of apes by FISH analysis

To determine the location of the FCGR1 gene in apes we analyzed chromosome spreads from chimpanzee (Pan troglodytes), baboon (Papio papio), monkey (Macaca mulatta), and human (as a positive control) through FISH analysis. Two individuals of each species were analyzed in 2 different experiments. The probe utilized was a cocktail of 3 genomic plasmid clones that, in combination, contained a 14.7 Kb region spanning the human FCGRIA gene (which in humans would hybridize to all 3 FCGR1 genes). Signals of an intensity above background that were consistent in at least 3 chromosome spreads were noted from each sample.

In all species studied we only detected signals which were both consistent and of an intensity above background located adjacent to the centromere of chromosome 1, as shown in Fig. 4.2. Specifically, in baboons a total of 16
spreads were examined and 63% of 30 baboon chromosomes 1 considered
suitable for analysis yielded a strong hybridizing signal adjacent to the centromere,
on the chromosomal arm equivalent to the human 1p. We observed a similar
result with Rhesus monkeys. Out of the 11 monkey spreads examined, we found
that 55% of 20 chromosomes 1 considered suitable for analysis yielded a strong
hybridizing signal adjacent to the centromere on the chromosomal arm equivalent
to the human 1p.

In chimpanzee our results were ambiguous, possibly due to the
combination of \textit{FCGRI} lying very close to the centromere and our difficulty in
detecting the chimpanzee centromere, which is surrounded by only a small amount
of heterochromatin. We examined 26 chimpanzee chromosome spreads. Thirty-
eight chimpanzee chromosomes 1 were considered suitable for analysis. Of these
34% yielded a signal which appeared to be located at the centromere, 26% yielded
a signal adjacent to the centromere on the equivalent of the human 1p arm, and
32% yielded a signal adjacent to the centromere on the equivalent of the human
1q arm.

In humans we analyzed 16 chromosome spreads. Of 28 chromosomes 1
that displayed signals, 18% yielded a signal solely at 1q21 whereas 82% yielded
signals at both 1p12 and 1q21. This is greater than our last analysis in which only
39% of chromosomes 1 that displayed signal yielded a dual signal at both 1p12
and 1q21. It was also noted that 7 human chromosomes 1 yielded 4 signals at
1q12. The somewhat different \textit{FCGRI} human hybridization pattern of this study,
as compared to our last study, is most likely due to the fact that slightly different
\textit{FCGRI} probes were used for each. Specifically, a cocktail of 3
\textit{FCGRI}-containing DNA plasmid clones were utilized as the \textit{FCGRI} probe for
this study whereas an \textit{FCGRI}-containing phage clone was utilized as the \textit{FCGRI}
probe for our past study. Since both of these probes span approximately the same
region we suspect that the difference in hybridization may actually be due to the
DNA of our \textit{FCGRI}-containing plasmid clones being of a higher quality than the
DNA of the \textit{FCGRI}-containing phage clone.

\textbf{Discussion}

We have determined, through FISH analysis of chromosome spreads with
a human \textit{FCGRI} probe, the location of \textit{FCGRI} in several ape species. In both
baboons (\textit{Papio papio}) and monkeys (\textit{Macaca mulatta}) we find \textit{FCGRI} to be
located adjacent to the centromere on the equivalent of the human 1p arm. In
chimpanzees (\textit{Pan troglodytes}) we localized \textit{FCGRI} to a region adjacent to the
centromere but were unable to discern which chromosomal arm it is located on.
Our data in humans supports our past finding that the 3 \textit{hFCGRI} genes are
located at both 1p12 and 1q21, and also revealed that in certain samples FCGRIA and FCGRIC (both at 1q21) yield 2 signals, implying that they lie approximately 500-1000 Kb apart.

Our data localizing FCGRI in baboons and rhesus monkey is molecular evidence that before the divergence of humans and apes FCGRI was located on the equivalent of the human 1p chromosomal arm. This correlates the appearance of FCGRI on the human 1q arm with the time period during which the pericentric inversion is proposed to have occurred, supporting our idea that this inversion translocated hFCGRIA and hFCGRIC to 1q but left hFCGRIB on 1p. Others have shown that a cluster of tRNA genes lies adjacent to the centromere on the equivalent of the human 1p chromosomal arm in 2 types of Old World Monkeys, macaque (Macaca fascicularis) and the African Green monkey (Cercopithecus aethiops) whereas it is located at 1q21 in humans (75). This makes it likely that this tRNA gene cluster was also translocated by the pericentric inversion of human chromosome 1, although unexplainably a tRNA gene cluster was also found at 1q21 in orangutans (Pongo pymaeus).

It would be of interest to further analyze FCGRI in apes. Our next efforts will be to localize FCGRI to a specific arm of chromosome 1 in chimpanzees utilizing 2-color florescence simultaneously hybridizing with probes of FCGRI and the chromosome 1 centromere (Oncor). It may also be worthwhile to
determine if, like the tRNA gene cluster, FCGRI is located at 1q21 in orangutans, and also to analyze ape chromosomes with human subchromosomal paint probes of chromosome 1 (78) to further define the region translocated during the pericentric inversion. Additionally it is of interest to determine how many FCGRI genes there are in apes, which could possibly be achieved by Southern analysis utilizing the HindIII and NcoI restriction endonucleases that distinguish the 3 hFCGRI genes. Since we have estimated that the hFCGRI genes were duplicated 3 million years ago it is our prediction that there will only be 1 gene found in apes and that it will resemble the bona fide FCGRI, FCGRIA.
Fig. 4.1 Evolution of the *FCGRI* gene family. The original *FCGRI* gene is considered to have originated from the duplication of a chromosomal segment of chromosome 1. After the centromere was inserted between these duplicated areas we speculate that the ancestral *FCGRI* gene itself duplicated into 3 *FCGRI* genes, all linked on 1p. Then, as others have proposed, a pericentric inversion of human chromosome 1 occurred, which we suggest translocated the *FCGRIA* and *FCGRIC* genes to 1q21 but left the *FCGRIB* gene positioned at 1p12. This inversion is considered to have occurred after the divergence of the human and chimpanzee lineages which, based on our calculations of when the *FCGRI* genes duplicated, correlates with the time period they would have been separated.
Fig. 4.1
Fig 4.2 Localization of \textit{FCGRI} to chromosome 1 in apes. 2 sets of Lymphocytes metaphase chromosomes 1 of \textit{(from left to right)} Baboon (\textit{Papio papio}), Rhesus Monkey (\textit{Macaca mulatta}), Chimpanzee (\textit{Pan troglodytes}), and Human (\textit{Homo Sapiens}), were hybridized with 3 \textit{FCGRIA} plasmid clones probes spanning 14.5 Kb combined and counter stained with DAPI. Hybridization was detected with avidin-Texas Red and fluorescence microscopy. In apes the florescence signals where found located adjacent to the centromere of chromosome 1 \textit{(white dashes)} on what would be homologous to the human 1p arm. In humans florescence signals were detected at both 1p12 and 1q21.
Baboon (Papio papio)  Rhesis Monkey (Macaca mulatta)  Chimpanzee (Pan troglodytes)  Human (Homo Sapiens)

Fig. 4.2
CHAPTER 5

IN Volvement of the Inositol 5-phosphatase Ship in FcyR Signaling in Monocytes

Introduction

Immunoglobulin G (IgG) molecules bind antigen and then initiate immune responses on monocytes by also binding and crosslinking integral membrane proteins termed Fc gamma receptors (FcyR). Four FcyR are expressed on monocytes (7;8;79). Three of these receptors (FcyRIA, FcyRIIA, FcyRIIIA) transduce signal through an Immunoreceptor Tyrosine-based Activation Motif (ITAM) and activate processes aimed predominantly at the elimination of antigen (80;81). Alternatively, the fourth FcyR (FcyRIIB) transduces signal through an Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM), found to negatively regulate immune responses (82;83).
ITAM activation motifs are required for the signal transduction of FcγR, the TCR, and the BCR, where they operate in a primarily consistent manner (29;30). FcγRIIA and FcγRIIIA, like most ITAM signaling receptors, associate noncovalently with an ITAM-containing molecule in monocytes; namely, the γ-chain homodimer. Crosslinking of either FcγRIIA or FcγRIIIA leads the γ-chain to be phosphorylated on the 2 conserved tyrosines of its ITAM motif, present in its cytoplasmic region (84;85). FcγRIIA in contrast contains an ITAM motif (2 tyrosines) and a third tyrosine residue directly in its own cytoplasmic region. All 3 of these tyrosines are phosphorylated upon FcγRIIA crosslinking (7;36).

Evidence at this point suggests that FcγRIIA, FcγRIIA and FcγRIIIA crosslinking induce indistinguishable signal cascades. First the ITAM motif is phosphorylated (most likely by a src kinase) after which various SH2 domain-containing signaling molecules bind the phosphorylated ITAM, namely the src tyrosine kinases Lyn and Hck (86), the tyrosine kinase Syk (85;87;88), and the inositol PI 3-Kinase (89). These kinases interact at the ITAM and undergo an increase in their catalytic activity. They then perpetuate the FcγR signal by binding and phosphorylating subsequent signaling molecules (29;30) specifically, PLCγ1, PLCγ2 (90-92), and many proteins of the Ras pathway namely Shc, Vav, GAP, Raf-1, and MAPK (91-93). The result of FcγR ITAM-mediated signal transduction in monocytes is the initiation of a variety of cellular processes aimed
at the elimination of antigen (3;94). The role of PLCγ1/PLCγ2-mediated Ca²⁺ flux during FcγR activation has not yet been clearly established although PI3-kinase has been shown to have a central role in the initiation of FcγR-mediated phagocytosis (95). The Ras pathway in contrast appears to be involved in the induction of respiratory burst and inflammatory mediator release (93;96).

As opposed to these 3 ITAM-signaling FcγR, the function of the ITIM-signaling FcγRIIB receptor in monocytes is not yet understood, although in B cells and mast cells it is involved in negative signaling (34;97). When crosslinked FcγRIIB does not induce protein tyrosine phosphorylation or antigen killing processes although it does have the ability to endocytose immune complexes (98). Recently it has been shown that when the BCR or the mast cell FceR are co-crosslinked with FcγRIIB their ability to activate cells is inhibited (34;99).

Although many of the details of FcγRIIB inhibition are unknown, this effect has been found to require the ITIM motif present in its cytoplasmic regions (34). Co-crosslinking causes the ITIM to be phosphorylated and bind the inositol 5-phosphatase SHIP correlating with SHIP being tyrosine phosphorylated and associating with the adapter protein Shc (82;83).

In this study we present evidence that SHIP is also involved in the signal transduction of FcγR on monocytes. Our observations show that crosslinking of
either FcγRIIA and FcγRII induces phosphorylation of SHIP in both U937 and THP1 monocytes. This response is upregulated when monocytes are treated with γ-interferon. We have also found that FcγR-mediated monocyte activation induces SHIP to associate with adapter protein Shc.

Experimental Procedures

Cells: The human monocytic cell line U937 was maintained in RPMI Complete: RPMI 1640 medium (Life Technologies, Gaithersburg, MD), supplemented with 10% heat-inactivated fetal calf serum, (Hyclone, Logan, UT) 2mM L-Glutamine, 100 μg/ml penicillin and 100 μg/ml Streptomycin. The THP-1 monocytic cell line was a gift from Dr. Paul Guyre, Dartmouth University, Lebanon, NH) grown in RPMI Complete supplemented with 5.5 x 10^2 mM 2-mercaptoethanol. When indicated cells were treated with 100 u/ml recombinant human γ-interferon (γ-IFN) (Genentech, CA) for 48-72 hours.

Antibodies: Whole IgG anti-FcγRII mouse monoclonal antibody IV.3 (IgG2b), whole IgG anti-FcγRI mouse monoclonal 197, and purified F(ab')2 fragments of anti-FcγRII monoclonal antibody 32 were supplied by Mederex (Annandale, NJ). F(ab')2 fragments of goat anti-mouse IgG (GAM) were purchased from Pierce (Rockford, IL). Anti-SHIP polyclonal antibody against
SHIP amino acid residues 874-941 and normal rabbit serum were kind gifts from
Dr. K. M. Coggeshall, (the Ohio State University). An anti-phosphotyrosine
Mabs cocktail was utilized which consisted of mouse monoclonals Py20 (IgG2b),
purchased from Santa Cruz Biotechnology Incorporated (Santa Cruz, CA),
Py72.10.5, obtained from Bart Sephton (of Salk Institute) and then purified from.ascites, and 4G10 (IgG2b), purchased from (UBI) Upstate Biotechnology
Incorporated (Lake Placid, NY). These 3 Mabs were used in a 30:30:1 ratio
respectively. MOPC 141, Isotype control for mouse IgG2b, was purified from.ascites fluid by ion exchange chromatography after procuring the cells from
ATCC. Anti-Shc rabbit polyclonal was purchased from UBI. Anti-Mouse IgG
consisting of Horseradish peroxidase linked to F(ab')2 anti-mouse fragments from
sheep and anti-Rabbit IgG consisting of Horseradish peroxidase linked to F(ab')2
anti-mouse fragments from donkey were purchased from Amersham (Arlington
heights, IL).

*Monocyte activation and Immunoadsorbtions:* For FcγRII activation 1 x
10^7 cells per sample were washed twice and then resuspended in either 1XPBS or
Hanks buffer saline solution (HBSS) at 1 x 10^6 cells/ml. Cells were then incubated
with whole IgG molecules of IV.3 (3 µg/ml) first at 4°C for 30 minutes and then
at 37°C for 10' after which F(ab')2 fragments of goat anti-mouse (GAM) IgG
were added (30 µg/ml) to initiate activation. Activation proceeded for 3 minutes
unless indicated otherwise. The cells were then lysed with Triton Lysis Solution made with Triton Lysis Buffer (1XPBS, 10mM Hepes/Ph 7.4, 10mM EDTA/Ph 8.0, 1% Triton-X-100), that had been recently supplemented with 3 mM Sodium orthovanadate, Ph 8.0, 20 µg/ml Aprotinin (Sigma), 40 µg/ml Leupeptin Calbiochem (La Jolla, CA) and 2 µg/ml Pepstatin (Sigma). Insoluble material was removed by centrifugation at 16,000g for 20 minutes and the supernatant immunoadsorbed overnight with specific Mabs at 4°C with 25 µl of Protein G or Protein A Sepharose (Pharmacia, Piscataway, NJ). Unbound proteins were removed with 4 washes of Triton Lysis Buffer plus 10mM Sodium orthovanadate. FcγRI activation 2 x 10^7 proceeded as previously described (84) using F(ab')_2 fragments of 32 (10 µg/ml), or whole IgG fragments of 197 followed by incubation at 37°C with F(ab')_2 fragments of goat anti-mouse (GAM) IgG at (20 µg/ml) for 3 minutes.

**Western Blot Analysis:** After elution by boiling in Laemmli sample buffer containing 5% 2-mercaptoethanol (2-ME), immunoadsorbed proteins, Rainbow protein markers (Amersham) and monoclonal antibodies alone (Mabs) were separated by 7.5%-8.0% SDS-polyacrylamide gel electrophoresis (PAGE), and electrophoretically transferred to nitrocellulose membranes (Amershan). Membranes were incubated either 1 hour at room temperature or overnight at 4°C in TBS-Tween (10mM Tris-HCL, Ph 7.5, 150mM NaCL, 0.1% Tween-20)
containing 5% Bovine Serum Albumin (BSA). Blots were then incubated sequentially with the immunoblotting antibody and peroxidase conjugated anti-mouse or anti-rabbit Mabs for 1 hr each at room temperature with 4 15 minute washes of TBS-Tween after each step. Bound antibodies were then visualized by Enhanced Chemiluminescence (ECL, Amersham)

Results

FcyRII crosslinking in U937 induces phosphorylation of the inositol 5-phosphatase SHIP.

Activation of FcyRII receptors through crosslinking has been shown to induce tyrosine phosphorylation of several cellular proteins including FcyRII, Syk, and PLC-γ1 (36;87;88;90;100). To identify other proteins tyrosine phosphorylated during FcyRII signal transduction we activated human monocyte U937 cells by crosslinking FcyRII with anti-FcyRII Mabs (IV.3) (79;101) and goat anti-mouse F(ab')2 fragments (GAM). Cells were then prepared into lysates that were analyzed with anti-phosphotyrosine Mabs. Tyrosine phosphoproteins of
approximately 180, 150, 145, 70, 60, 50, and 40 kDa in size, were detected in FcγRII-crosslinked cells (Fig. 5.1, Lane 4) but were either not present or present only in very low levels without receptor crosslinking (Fig. 5.1, Lanes 1-3).

We suspected the 145 kDa protein phosphorylated upon FcγRII activation to be the inositol-5 phosphatase SHIP, which is phosphorylated when FcγRIIB1 is co-crosslinked with either the BCR or the FceR (82;83). To investigate this we immunoadsorbed lysates of FcγRII-crosslinked U937 cells with an antibody specific for SHIP and then analyzed these immunoadsorbates with anti-phosphotyrosine Mabs. A phosphorylated protein equal to the size of SHIP was found in SHIP immunoprecipitates of FcγRII-activated U937 cells (Fig. 5.2, Lane 4), but was either not detected or detected only a very low levels without crosslinking (Fig. 5.2, Lanes 1-3), or in FcγRII-activated lysates immunoadsorbed with normal rabbit serum (NRS) (Fig. 5.2 Lane 5) or mock adsorbates containing only monoclonal antibodies without cells (Mabs) (Fig. 5.2, Lane 6). Re-analysis of these immunoadsorbates with anti-SHIP revealed SHIP to be present in comparable amounts in both resting and activated samples (Fig 5.3 Lanes 1-4). To ensure that the 145 kDa phosphorylated protein in SHIP immunoadsorbates of FcγRII-activated cells was indeed SHIP and not a phosphoprotein of similar size associated with SHIP, we immunoadsorbed FcγRII-activated U937 cell lysates with anti-phosphotyrosine Mabs and analyzed
these immunoadsorbates with anti-SHIP. Phosphorylated SHIP was found in FcγRII crosslinked cells but was not present without crosslinking nor in NRS or Mabs negative controls (data not shown).

**FcγRII crosslinking in THP1 also induces phosphorylation of SHIP.**

To investigate if FcγRII signaling induces SHIP phosphorylation in additional cells we repeated our experiments with the human monocyte cell line THP1. Tyrosine phosphoproteins from FcγRII-crosslinked THP1 cells were immunoprecipitated and analyzed with anti-phosphotyrosine Mabs. As compared to U937 (Fig. 5.4, Lane 7), THP1 FcγRII activation induced enhanced tyrosine phosphorylation of similar sized proteins including one of 145 kDa, and also induced phosphorylation of additional proteins approximately 120, 80, 37, and 35 kDa in size (Fig. 5.4, Lanes 4,5). These phosphoproteins were either not present or present only in low levels either without crosslinking (Fig. 5.4, Lanes 1-3,6) or in the Mabs negative control (Fig 5.4, Lane 8). Next we immunoprecipitated SHIP from FcγRII-activated THP1 cells and analyzed these immunoadsorbates with anti-phosphotyrosine Mabs. FcγRII activation of THP1 induced SHIP...
phosphorylation (Fig. 5.5, Lane 4) to an extent comparable to that found in U937 (Fig. 5.5, Lane 7). SHIP was not phosphorylated without crosslinking (Fig. 5.5, Lane 1-3,6) nor in NRS or Mabs negative controls. (Fig. 5.5, Lanes 5,8)

In order to determine the point at which SHIP is involved in FcγRII signal transduction we also analyzed SHIP phosphorylation at different timepoints of FcγRII activation. U937 cells were stimulated by FcγRII crosslinking from 30 seconds to 8 minutes and prepared into lysates that were immunoadsorbed with anti-SHIP. Immunoadsorbates were then analyzed with anti-phosphotyrosine Mabs. We found that upon FcγRII crosslinking SHIP phosphorylation is induced by 30 seconds, peaks at 1-2 minutes, and then decreases, returning to basal level by 8 minutes (Fig. 5.6, Lanes 4-8). Re-analysis of these adsorbates with anti-SHIP detected a comparable amount of SHIP in all FcγRII-activated cells (Fig 5.7, Lanes 4-8).

FcγRII-induced SHIP phosphorylation is greater in U937 cells treated with γ-IFN.

To determine the effect of the cytokine γ-Interferon (γ-IFN), a potent upregulator of FcγR expression, on FcγR-induced SHIP phosphorylation, we also analyzed (γ-IFN)-cultured monocytes. U937 cells were treated with γ-IFN for 48-72 hours and activated by FcγRII crosslinking. Cells were then prepared into
lysates that were immunoadsorbed with anti-SHIP after which these adsorbates were analyzed with anti-phosphotyrosine Mabs. FcγRII activation of γ-IFN-treated cells (Fig. 5.8, Lane 2) induced 5X the amount of SHIP phosphorylation as untreated cells (Fig. 5.8, Lane 4). Re-analysis of these adsorbates with anti-SHIP revealed that γ-interferon treated U937 cells contained at least 5X the amount of SHIP (Fig 5.9, Lanes 1-2) as U937 cells that had not been treated with γ-IFN (Fig 5.9, Lanes 3-4).

FcγRII activation induces SHIP to associate with the adapter protein p52 Shc.

We next examined if FcγRII signaling induces SHIP to associate with the adapter protein p52 Shc, as this association occurs in many of the instances where SHIP is phosphorylated (82; 102-105). U937 cells were activated by FcγRII crosslinking and prepared into lysates which were immunoadsorbed with an antibody specific to Shc. Analysis of these adsorbates with anti-phosphotyrosine Mabs revealed that in FcγRII-crosslinked cells Shc co-purifies with a 145 kDa protein (Fig. 5.10, Lane 6) which co-migrates with phosphorylated SHIP (Fig.
5.10, Lane 2). The p145 was not present without crosslinking nor in NRS or Mabs negative controls (Fig. 5.10, Lanes 3-5,7,8). Shc immunoadorbates were also analyzed with anti-SHIP. We observed SHIP to be associated with Shc only in FcγRII-activated cells (Fig 5.11, Lane 6) but not without crosslinking or in other negative controls (Fig. 5.11, Lanes 3-5,7,8).

Crosslinking of the high affinity FcγRI also induces SHIP phosphorylation.

We also investigated if SHIP is phosphorylated during signaling of the high affinity FcγR, FcγRI. U937 cells were activated by FcγRI crosslinking and prepared into lysates that were analyzed with anti-phosphotyrosine Mabs. Tyrosine phosphorylated proteins, including one of 145 kDa, were present in FcγRI-crosslinked cells (Fig. 5.12, Lane 4) but were not present, or present only in very low levels, without receptor crosslinking or in the Mabs negative control (Fig. 5.12, Lanes 1-3,5). We then crosslinked FcγRI on U937 cells, utilizing either F(ab')₂ for whole IgG anti-FcγRI Mabs, prepared these cells into lysates and analyzed them for the presence of phosphorylated SHIP. FcγRI crosslinked cells contained phosphorylated SHIP of comparable amounts irrespective of whether or not they had been activated with an anti-FcγRI Mab that contained an
Discussion

We have found that crosslinking of FcγRI or FcγRII leads to phosphorylation of the inositol phosphatase SHIP in U937 or THP1 monocytes. This phosphorylation occurs by 30 seconds, peaks at 1-2 minutes and returns to basal level by 8 minutes. SHIP phosphorylation is enhanced in monocytes pretreated with the cytokine γ-IFN which also increases the overall amount of SHIP. Additionally we observed that FcγR crosslinking in monocytes induces SHIP to associate with the SH2-containing adapter protein p52 Shc. These findings raise questions regarding how SHIP is phosphorylated upon FcγR crosslinking and more importantly how it is involved in FcγR-mediated monocyte activation.

Presently the information regarding SHIP is substantial even though it has only recently been identified (103;105). Its most unique attribute is that it contains both an inositol phosphatase domain and an SH2 domain. SHIP
additionally contains three NXXY phosphotyrosine binding sites and a proline rich sequence, which is able to bind to SH3 domains. It can remove the phosphate from the 5' position of either inositol (1,3,4,5)P$_4$ or PtdIns (3,4,5)P$_3$ creating the products inositol (1,3,4)P$_3$ and PtdIns (3,4)P$_2$ respectively but is unable to hydrolyze PtdIns (1,4,5)P$_3$ (83;103;105). SHIP has been found to be tyrosine phosphorylated when FcγRIIB is co-crosslinked with either the BCR or FcεR (82;83), or when the IL4 or LPS receptors are engaged (102). This phosphorylation does not appear to affect the activity of SHIP (83) and may instead enable it to bind certain effector molecules such as the adapter protein Shc, which has been found associated with SHIP wherever it is phosphorylated (82;102;103;105). SHIP additionally has been reported to bind PLCγ1 (103), Grb2 (103;105) and Syk (102) as well as phosphorylated peptides of ITAM motifs originating from γ-chain, ζ-chain, and CD3 (103) and the ITIM motif originating from FcγRIIB (83;106).

Upon FcγR crosslinking a likely scenario by which SHIP is phosphorylated is that it associates with an ITIM or ITAM motif within the FcγRIA and FcγRII receptor complex and then upon receptor crosslinking is acted upon by a src kinase. The src kinase Lyn, which is known to associate with both FcγRIA and FcγRIIA, has already been shown to be able to phosphorylate SHIP (103).
During FcγRII signaling there are several possible mechanisms by which SHIP could associate with the FcγR receptor complex. The ability to discern between these mechanisms is hindered by the fact that there are no Mabs available which will specifically bind to FcγRIIA or FcγRIIB (107). Our experiments, crosslinking FcγRII, utilized the Mab IV.3 (79). IV-3 binds to both FcγRIIA and FcγRIIB, although it has been shown to adhere 4 times more strongly to FcγRIIA (107;108). As crosslinking of FcγRIIB alone is already known not to induce SHIP phosphorylation, there remain 3 possible mechanisms by which SHIP could be phosphorylated upon FcγRII-crosslinking. The most likely is that, consistent with its function during BCR and FceR signal transduction, SHIP associates with FcγRIIB and is phosphorylated as these receptors co-crosslink with the ITAM-containing FcγRIIA. Alternatively FcγRIIA crosslinking could induce SHIP phosphorylation independently of FcγRIIB either directly through its ITAM or indirectly by associating noncovalently with one of the other integral ITIM-containing receptors known to be present in monocytes (109).

FcγRIA-mediated SHIP phosphorylation in contrast does not involve FcγRIA being co-crosslinked with FcγRIIB. We surmise this since we utilized Mabs whose variable regions bind specifically to FcγRIA and also because our experiments crosslinking FcγRIA with either whole IgG or F(ab')2 anti-FcγRIA
Mabs yielded equivalent amounts of SHIP phosphorylation, demonstrating that
SHIP is not being phosphorylated as a result of the Fc portion of the Mabs
engaging Fc receptors, such as FcγRIIB. This leaves the possibility that FcγRIIA-
mediated SHIP phosphorylation occurs either through the ITAM of its γ-chain
homodimer, which has been shown to engage SHIP (103), or by it associating
noncovalently with an ITIM-containing molecule.

Once SHIP is phosphorylated it could effect a variety of FcγR-mediated
responses. In B cells and mast cells the phosphorylation of SHIP correlates with
an inhibition of receptor-mediated Ca\(^2+\) flux, Ras activation and cytokine
production (99;110). We surmise that in monocytes SHIP will also inhibit these
pathways during FcγR activation, decreasing FcγR-mediated effector functions.
Specifically, the ability of SHIP to suppress PI3-Kinase activity (111) should
inhibit FcγR-mediated phagocytosis (97). Additionally, the putative ability of
SHIP to inhibit Ras activation, theorized to occur by it binding Shc and blocking
Shc-GRB2-SOS complexing (110), would be expected to inhibit FcγR-mediated
respiratory burst (93) and cytokine production (112). Alternatively, SHIP may
aid in FcγR-mediated activation as its products can activate certain molecules as
the kinase AKT (113) and it can link the FcγR ITAM motif to molecules such as
Shc, Syk, or PLCγ.
The finding that SHIP is involved with FcγR-mediated monocyte activation raises many questions worthy of investigation. For instance it should be determined if FcγRIIIA crosslinking induces SHIP phosphorylation. Primarily our next efforts will be directed towards furthering our understanding of how SHIP associates with the FcγR complex and ascertaining what effect it and FcγRIIB have on FcγR-mediated activation. We have preliminary data showing that SHIP binds the phosphorylated ITIM motif in both resting and FcγR-activated monocytes and we are currently discerning if SHIP can additionally bind the FcγRIIA ITAM. We also plan to engage in transfection studies analyzing the effect of FcγRIIB and SHIP on FcγR downstream responses such as phagocytosis and cytokine production. If SHIP does prove to be a negative regulator of FcγR on monocytes it may be of interest to determine how this inhibition is regulated. Perhaps it will be found that the immune system curbs the activity of monocytes by upregulating the expression of certain inhibitory molecules such as FcγRIIB and SHIP.
Fig. 5.1 FcγRII crosslinking in U937 cells induces tyrosine FcγRII phosphorylation of a 145 kDa protein. U937 cells (1 x 10⁷ cell equivalents/lane) were left resting or were incubated with Mab IV.3 (3 μg/ml) and F(ab')² goat anti-mouse, GAM (30μg/ml) for 2 minutes either separately or combined, to crosslink FcγRII receptors. Cells were then lysed in Triton solution and lysates were immunoabsorbed with anti-phosphotyrosine Mabs. Immunoabsorbed proteins and protein markers (noted at left margin of the panel in kDa) were sized on an 8% SDS-PAGE gel under reducing conditions, transferred to a nitrocellulose filter and probed with anti-phosphotyrosine Mabs. Phosphoproteins were then visualized by enhanced chemiluminescence (ECL) and sized by comparison with protein markers. A phosphorylated 145 kDa protein (arrow) was detected in FcγRII activated cells. These data are representative of 4 different experiments.
Activation:

Immunoadsorption: p145

Anti-Phosphotyrosine

Immunoblot: Anti-phosphotyrosine

Fig. 5.1
Fig. 5.2 FcγRII crosslinking in U937 cells results in tyrosine phosphorylation of the 5-inositol phosphatase SHIP. U937 cells (1 x 10^7 cell equivalents/lane) were activated by FcγRII crosslinking and prepared into lysates which were immunoadsorbed with either anti-SHIP or normal rabbit serum (NRS). Immunoadsorbed proteins, Mabs alone (Mabs) and protein markers (noted at left margin of the panel in kDa) were separated by SDS-PAGE and analyzed with anti-phosphotyrosine Mabs. Methods used matched those described in Fig 5.1. Phosphorylated SHIP (arrow) was detected only in FcγRII-activated cells. These data are representative of 4 different experiments.
Activation:

Immunoadsorption:

<table>
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<tr>
<th>Resting</th>
<th>GAM</th>
<th>IV3</th>
<th>IV3/GAM</th>
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<tbody>
<tr>
<td>Anti-SHIP</td>
<td>NRS</td>
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<td></td>
</tr>
</tbody>
</table>

Immunoblot: Anti-phosphotyrosine

Fig. 5.2
Fig. 5.3 Resting and FcγRII-activated U937 cells contain comparable amounts of SHIP. The nitrocellulose filter of Fig 5.2 was reanalyzed with anti-SHIP by methods described in Fig. 5.1. Protein markers are noted at left margin of the panel in kDa. Phosphorylated SHIP (arrow) was found to be of comparable amounts in both resting and activated cells. These data are representative of 4 different experiments.
Activation; Immunoadsorption:

Immunoblot: Anti-SHIP

Fig. 5.3
Fig. 5.4 FcγRII crosslinking in THP1 cells also induces tyrosine phosphorylation of a 145 kDa protein. THP1 and U937 cells (1 x 10⁷ cell equivalents/lane) were activated by FcγRII crosslinking and prepared into lysates which were immunoadsorbed by anti-phosphotyrosine Mabs. Immunoadsorbed proteins, Mabs alone (Mabs) and protein markers (noted at left margin of the panel in kDa) were separated by SDS-PAGE and analyzed with anti-phosphotyrosine Mabs. Methods used matched those described in Fig. 5.1. Several tyrosine phosphoproteins, including one of 145 kDa (arrow), were detected in FcγRII-activated cells. These data are representative of 2 different experiments.
Cell Type: THP1  U937

Activation:

<table>
<thead>
<tr>
<th></th>
<th>Resting</th>
<th>GAM</th>
<th>IV.3</th>
<th>IV.3/GAM</th>
<th>IV.3</th>
<th>IV.3/GAM</th>
<th>Mabs</th>
</tr>
</thead>
</table>

Immunoadsorption: Anti-Phosphotyrosine

Immunoblot: Anti-phosphotyrosine

Fig. 5.4
Fig. 5.5 FcγRII crosslinking in THP1 cells induces tyrosine phosphorylation of SHIP. THP1 and U937 cells (1 x 10^7 cell equivalents/lane) were activated by FcγRII crosslinking and prepared into lysates which were immunoadsorbed with anti-SHIP or normal rabbit serum (NRS). Immunoadsorbed proteins, Mabs alone (Mabs) and protein markers (noted at left margin of the panel in kDa) were separated by SDS-PAGE and analyzed with anti-phosphotyrosine Mabs. Methods used matched those described in Fig. 5.1. Phosphorylated SHIP (arrow) was detected in all FcγRII-activated cells. These data are representative of 2 different experiments.
<table>
<thead>
<tr>
<th>Activation</th>
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<th>U937</th>
</tr>
</thead>
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<tr>
<td>Resting</td>
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</tr>
<tr>
<td>IV.3/GAM</td>
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<tr>
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<td></td>
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<tr>
<td>IV.3/GAM</td>
<td></td>
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</tr>
</tbody>
</table>

**Immunoadsorption:** Anti-SHIP, NRS, SHIP, Mabs

**Immunoblot:** Anti-phosphotyrosine

Fig. 5.5
Fig 5.6 SHIP is phosphorylated within 30 seconds of FcγRII
crosslinking. U937 cells (1 x 10^7 cell equivalents/lane) were activated
by FcγRII crosslinking for 30 seconds to 8 minutes, and prepared into
lysates which were immunoabsorbed with anti-SHIP or normal rabbit
serum (NRS). Immunoabsorbed proteins and protein markers (noted at
left margin of the panel in kDa) were separated by SDS-PAGE and
analyzed with anti-phosphotyrosine Mabs. Methods used matched those
described in Fig. 5.1. The presence of phosphorylated SHIP (arrow)
increased by 30 seconds and returned to basal level by 8 minutes. These
data are representative of 2 different experiments.
Activation:

|-----------------|------------|--------|--------|--------------|--------------|-------------|-------------|-------------|

Immunoadsorption:

- Anti-SHIP
- NRS

220
SHIP
97
66
46

Immunoblot: Anti-phosphotyrosine

Fig. 5.6
Fig. 5.7 U937 cells at different points of FcγRII-activation contain comparable amounts of SHIP. The nitrocellulose filter from Fig. 5.6 was reanalyzed with anti-SHIP by methods described in Fig. 5.1. Protein markers are noted at left margin of the panel in kDa. A comparable amount of SHIP (arrow) was present in all FcγRII-activated cells. These data are representative of 2 different experiments.
Activation:

|-----------------|------------|-------------|-------------|--------------|-------------|-------------|-------------|-------------|-------------|

Immunoadsorption: Anti-SHIP NRS

Immuno blot: Anti-SHIP

Fig. 5.7
Fig. 5.8 FcγRII crosslinking in monocytes treated with γIFN results in increased tyrosine phosphorylation of SHIP. U937 cells (1 x 10^7 cell equivalents/lane) either treated or untreated with γ-IFN were activated by FcγRII crosslinking and prepared into lysates which were immunoadsorbed with anti-SHIP. Immunoadsorbed proteins, Mabs alone (Mabs) and protein markers (noted at left margin of the panel in kDa) were separated by SDS-PAGE and analyzed with anti-phosphotyrosine Mabs. Methods used matched those described in Fig 5.1. γ-IFN treated FcγRII-activated cells contained at least 3x more phosphorylated SHIP (arrow) than untreated cells. This data is representative of 2 different experiments.
W

Cells:  

| \( \gamma \)-IFN |  
| treated | untreated |
| IV.3 | IV.3/GAM | IV.3 | IV.3/GAM |

Activation:

Immunoadsorption:  

| Anti-SHIP | Mabs |

Immunoblot: Anti-phosphotyrosine

Fig. 5.8
Fig. 5.9 γIFN-treated human monocytes U937 cells contain increased amounts of SHIP. The nitrocellulose filter from Fig. 5.8 was reanalyzed with anti-SHIP using methods described in Fig 5.1. Protein markers are noted in left margin of panel in kDa. γ-IFN treated cells contained at least 4X more SHIP than untreated cells. These data are representative of 2 different experiments.
Cells: \( \gamma\text{-IFN treated untreated} \)

Activation: IV.3 IV.3/GAM IV.3 IV.3/GAM

Immunoadsorption: Anti-SHIP

\( \gamma\text{-IFN treated untreated} \)

Immunoblot: Anti-SHIP

Fig. 5.9
Fig. 5.10 A 145 kDa tyrosine phosphoprotein associates with Shc upon FcγRII-crosslinking. U937 cells (1 x 10^7 cell equivalents/lane) were activated by FcγRII crosslinking and prepared into lysates which were immunoadsorbed with anti-SHIP, anti-Shc, or normal rabbit serum (NRS). Immunoadsorbed proteins, Mabs alone (Mabs) and protein markers (noted at left margin of the panel in kDa) were separated by SDS-PAGE and analyzed with anti-phosphotyrosine Mabs. Methods used matched those described in Fig 5.1. In FcγRII activated cells alone Shc associated with a 145 kDa tyrosine phosphoprotein (arrow) which comigrated with SHIP.
**Activation**

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</table>

**Immunoadsorption:**

<table>
<thead>
<tr>
<th>SHIP</th>
<th>Anti-Shc</th>
<th>NRS</th>
<th>Mabs</th>
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</thead>
</table>

**Immunoblot:** Anti-phosphotyrosine

**Fig. 5.10**
Fig. 5.11 SHIP associates with Shc upon FcγRII-crosslinking. U937 cells (1 x 10^7 cell equivalents/lane) were activated by FcγRII crosslinking and prepared into lysates which were immunoadsorbed with anti-SHIP, anti-Shc, or normal rabbit serum (NRS). Immunoadsorbed proteins, Mabs alone (Mabs) and protein markers (*noted at left margin of the panel in kDa*) were separated by SDS-PAGE and analyzed with anti-SHIP. Methods used matched those described in Fig. 5.1. SHIP (arrow) was detected associated with Shc only in FcγRII activated cells.
**Fig. 5.11**

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<td>SHIP →</td>
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136
Fig. 5.12 Crosslinking of the high affinity Fc receptor FcγRI on human monocytes induces tyrosine phosphorylation of a 145 kDa protein. γ-IFN-treated U937 cells (2 x 10^7 cell equivalents/lane) were left resting or were incubated with anti-FcγRI Mab 197 (10 μg/ml) and F(ab')^2 goat anti-mouse, GAM (20 μg/ml) for 2 minutes either separately or combined, to crosslink FcγRI receptors. Cells were prepared into lysates which were immunoadsorbed with anti-SHIP or normal rabbit serum (NRS). Immunoadsorbed proteins, Mabs alone (Mabs) and protein markers (noted at left margin of the panel in kDa) were separated by SDS-PAGE and analyzed with anti-phosphotyrosine Mabs. Methods used matched those described in Fig 5.1. A tyrosine phosphoprotein of 145 kDa (arrow) was detected in FcγRI-activated samples. Material lateral to Lanes 3 and 4 was cropped out.
Activation:

Immunoadsorption: Anti-Phosphotyrosine

Immunoblot: Anti-phosphotyrosine

Fig. 5.12
Fig. 5.13 FcγRI crosslinking in human monocytes results in tyrosine phosphorylation of SHIP. γ-IFN-treated U937 cells (2 x 10⁷ cell equivalents/lane) were left resting or were incubated with anti-Fc(RI Mab 197 whole IgG molecules (10 μg/ml) or anti-FcγRI Mab 32 F(ab')² fragments (10 μg/ml) either separately or combined with F(ab')² GAM (20 μg/ml) for 2 minutes, to crosslink FcγRI receptors. Cells were then prepared into lysates which were immunoadsorbed with anti-SHIP or normal rabbit serum (NRS). Immunoadsorbed proteins and protein markers (noted at left margin of the panel in kDa) were separated by SDS-PAGE and analyzed with anti-phosphotyrosine Mabs. Methods used matched those described in Fig 5.1. Phosphorylated SHIP (arrow) was detected only in FcγRI-activated samples.
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Immunoblot: Anti-phosphotyrosine

Fig. 5.13
SUMMARY

Within this chapter I will summarize my graduate research studying the immune Fc receptors for IgG. I will discuss my studies of the organization and evolution of the FcγRI gene family after which I will describe my analysis of FcγR signaling in monocytes. Specifically, I will present my findings, interpretations of these findings and also considerations for extending these studies.

Studies of the FcγR1 gene family

As the 5 FcγRII, FcγRIII, FceR genes and the FceRIγ gene, encoding the FcγR1 subunit, were all located at 1q23 in humans (9;26), it was of interest to determine if the related 3 FcγRI genes were also clustered at this position. This was because it was speculated that these functionally related genes may be coordinately regulated (2;26). The 3 FcγRI genes had already been located to chromosome 1 (5). There were also 2 pieces of evidence, obtained by analyzing the hFcγRI gene family with long-range restriction endonucleases, that all 3
FcyRI genes hybridized to single bands measuring 50 and 60 Kb in length (39). It therefore became an enticing idea to investigate if the FcyRI genes were tightly linked to one another or to the FcyR genes clustered at 1q23.

My initial studies showed that the FcyRI genes were not closely linked to each another or the FcyR genes at 1q23. By both PCR and Southern analysis I characterized 5 FcyRI-containing YAC clones, which ranged in size from 190-310 Kb yet each contained only a single FcyR1 gene. I also determined through Southern analysis that the FcyRII, FcyRIII, FceRIγ and CD1 genes were not present on these FcyR1-containing YAC clones. Somewhat informative was my analysis of the FcyRI-containing YAC clones by Alu fingerprinting, which revealed there to be numerous regions of homology shared between YAC clones of all 3 FcyR1 genes. These data implied that the area of homology created when the FcyR1 genes were duplicated extended many Kb past the perimeter of the genes. This suggested to me that the 3 FcyR1 genes could falsely appear to co-localize to 50 - 60 Kb bands when in fact they may be exhibiting similar restriction patterns, even with long-range restriction endonucleases.

To explore this possibility I analyzed human genomic DNA with 9 different long-range cutting restriction endonucleases. I found that 2 enzymes yielded single hybridizing bands but 7 enzymes separated the FcyR1 gene family into either 2 or 3 multiple hybridizing bands. One particular enzyme, Sfil, yielded
3 distinct hybridizing bands (of equal intensity) measuring 600, 500 and 300 Kb in length, clearly demonstrating that the FcγRI genes spanned a DNA region at least 300 Kb in length.

With a more realistic understanding of the FcγRI gene family organization, I proceeded to determine the location of the 3 FcγRI genes on chromosome 1 by fluorescence in situ hybridization (FISH) of human cells. Through collaborations with Dr. Karl Theil of OSU and Dr. Uta Francke of Stanford, FISH was performed on human metaphases utilizing either an FcγRIA DNA phage clone probe or probes derived form each of the FcγRI-containing YAC clones. All of our FcγRI FISH studies yielded a consistent signal at 1q21 and an inconsistent weaker signal at 1p12.

At this time, 2 different reports were published which identified an FcγRI FISH signal in humans at 1q21 but concluded that all 3 FcγRI genes were located at 1q21 (53;54). Despite this, we were confident that the FcγRI signal we were observing at 1p12 was genuine. To confirm the existence of the FcγRI 1p locus, I performed Southern analyses which distinguish the 3 FcγRI genes by polymorphisms in either the 5' region or the EC2 exon on DNA isolated from 4 human-hamster hybrid cell lines containing portions of human chromosome 1.
found the FcγRIIB gene was indeed present in 3 cell lines containing the human 1p chromosomal arm and also identified both the FcγRI and FcγRIC genes in a cell line that contained the human 1q chromosomal arm.

It is curious that the FcγRI gene family is separated from the other FcγRs at 1q23 and is also itself divided onto 2 different chromosomal arms. I expected the FcγRI genes to be closely linked, since they appeared to have been duplicated recently and most likely by mechanisms that would place them closely in tandem (13;18;69). To understand the reasons behind these discrepancies, I began to explore the evolution of the FCR gene family and of human chromosome 1.

I surmised that our data, localizing the FcγRI gene family in humans, supported a plausible hypothesis, by others, which explained why the FcγRI genes were separated from the other FcγR genes at 1q23. Specifically, the positioning of FcγRI to the pericentric region of chromosome 1 in humans had placed it within a large pericentric linkage group of ~20 genes conserved in order in mice, partially on mouse chromosome 1 and partially on mouse chromosome 3 (70-72). Within this linkage group there are 2 extended regions containing homologous genes, (FcγRII/FcγRIII, Bcm1, and ATP1a2) and (FcγRI, CD2/LFA3, and ATP1a1). These regions were considered by others to have evolved by the
duplication of a chromosomal segment (51) suggesting that the creation of the ancestral FcγRI involved the unequal chromatid exchange of a very large DNA fragment, placing FcγRI away from the 1q23 FCR genes.

As humans and mice diverged the linkage group in mice split into 2 different chromosomes whereas in humans the centromere inserted, dividing this area into the p and q arms of chromosome 1 (73). It is my speculation that after the centromere inserted the ancestral FcγRI gene was originally a part of the p arm of chromosome 1. This gene then duplicated into 3 FcγRI genes, resulting in 3 FcγRI genes all linked on 1p. By comparing the sequences of the FcγR1 genes, by the Jukes-Cantor method (66), I determined that the genes were duplicated approximately 2.4 to 3.4 million years ago.

After the divergence of the humans and chimpanzee lineages, a pericentric inversion of human chromosome 1 occurred, as others have proposed, translocating the bands presently known as 1q21.1 and 1q21.2 bands over from the p arm to the q arm (74). I surmise that this inversion translocated the FcγRIA and FcγRIC genes to the q arm of chromosome 1 but left the FcγRIB gene positioned on 1p.

To investigate this hypothesis I determined the location of FcγR1 in several ape species. In both baboons (Papio papio) and monkeys (Macaca mulatta) I located FcγR1, by FISH analysis, to a region adjacent to the
centromere on what is homologous to the human 1p arm. This correlates the appearance of FγR1 on the human 1q arm with the time period during which the pericentric inversion is proposed to have occurred, supporting my idea that this inversion translocated hFγR1A and hFγR1C to 1q but left hFγR1B on 1p.

My analysis of the human FγRI gene family also included FγRI gene copy number experiments, initiated to investigate reports by others that there were actually 9 FγR1 genes in humans. To measure FγRI copy number I analyzed genomic DNA from 8 individuals by the Southern method, using the X-linked dystrophin gene as a standard. My analysis of varying amounts of a plasmid clone containing the target fragments of both genes established that the relationship between DNA amount and hybridization intensity was linear. I found that a copy number of $3.1 \pm 0.3$. is present in most humans. The FγRI copy number of an individual carrying 2 dystrophin genes (4X) was also found to equal 3 FγRI genes, supporting the validity of my method.

It was at this point that I concluded this project although there were aspects regarding the organization of the FγRI gene family which could be studied further. Namely, to determine the distance between the FγRIA and FγRIC genes on 1q21 and to also determine if, consistent with conserved human–mice linkage group of chromosome 1, FγRIA/FγRIC lie adjacent to the CACY and CD1 genes in humans. Further analysis in apes should include localizing
FcγR1 to a specific chromosomal arm in chimpanzees, further defining the region which translocated during the pericentric inversion of human chromosome 1, and determining the number of FcγR1 genes in apes. Since the hFcγR1 genes appear to have been duplicated 3 million years ago it is my prediction that only 1 gene will be found and that it will resemble the bona fide FcγR1, FcγR1A.

Studies of FcγR Signal Transduction

I next seized an opportunity to study a different aspect of FCR biology, namely, the signal transduction of FcγR in monocytes. There are 4 FcγR in monocytes, 3 of which (FcγRIA, FcγRIIA, FcγRIIIA) are classified as multi-domain immune recognition receptors (MIRR) along with the TCR and BCR. Consistent with MIRR receptors, these 3 FcγR transduce signals through an ITAM motif present within the intracellular region of their receptor complex and activate processes aimed at the elimination of antigen. In contrast, the fourth FcγR on monocytes (FcγRIIB) transduces a negative signal that down regulates cells. FcγRIIB-mediated inhibition is dependent upon a sequence motif present in the tail of FcγRIIB termed the ITIM, which has been found to engage both the inositol 5-phosphatase SHIP and the tyrosine phosphatase SHP-1 (34;35). As it had just been found that FcγRIIA signaling led to its engagement with the tyrosine
kinase Syk (87,88) and the inositol kinase PI3-K (89), I began my studies of FcγR signal transduction with the aim of identifying subsequent signaling molecules involved in FcγRIIA signal transduction.

Through a collaboration with Dr. K. M. Coggelshall of OSU, I ascertained that crosslinking of FcγRIA or FcγRII leads to phosphorylation of the inositol-5-phosphatase SHIP in both U937 and THP1 monocytes. This phosphorylation occurs by 30 seconds, peaks at 1-2 minutes and returns to basal level by 8 minutes. I also found that SHIP phosphorylation is enhanced in monocytes pretreated with the cytokine γIFN, which appears to increase the overall amount of SHIP. Additionally I determined that FcγR crosslinking in monocytes induces SHIP to associate with the SH2-containing adapter protein p52 Shc.

These findings raise questions regarding how SHIP is phosphorylated upon FcγR crosslinking and more importantly how it is involved in FcγR-mediated monocyte activation. A likely scenario by which SHIP is phosphorylated is that it associates with an ITIM or ITAM motif within the FcγRIA and FcγRII receptor complex and is acted upon by a src kinase. The src kinase Lyn, which associates in monocytes with both FcγRIA and FcγRII, has already been shown to have the ability to phosphorylate SHIP (103). Once SHIP is phosphorylated it could effect a variety of FcγR-mediated responses. In B cells and mast cells its phosphorylation correlates with an inhibition of receptor-mediated Ca^{2+} flux, Ras
activation and cytokine production (99). I expect that these pathways will also be inhibited when SHIP is phosphorylated during FcγR-mediated activation leading to the downregulation of FcγR effector functions, such as phagocytosis and cytokine production.

The finding that SHIP is involved with FcγR-mediated monocyte activation raises many questions worthy of investigation. Further study should focus on broadening our understanding of how SHIP associates with the FcγR complex and determining what effect it and FcγRIIB have on FcγR-mediated activation. I have preliminary data showing that SHIP binds the phosphorylated ITIM motif in both resting and FcγR-activated monocytes and it would be interesting to next ascertain if SHIP can additionally bind the FcγRIIA ITAM. It would also be informative to perform transfection studies analyzing the effect of FcγRIIB and SHIP on FcγR downstream responses, such as phagocytosis and cytokine production. If SHIP does prove to be a negative regulator of FcγR on monocytes it may be of interest to determine how this inhibition is regulated. Perhaps it will be found that the immune system curbs the activity of monocytes by up regulating the expression of certain inhibitory molecules such as FcγRIIB. The close positioning of FcγRIIB with both FcγRIIA and FcγRIIC (<120 Kb) causes me to speculate once again that these genes are controlled by coordinate regulation.
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