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High level expression and characterization of a truncated soluble murine Fc gamma receptor

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HIGH LEVEL EXPRESSION AND CHARACTERIZATION OF A TRUNCATED SOLUBLE MURINE Fcγ RECEPTOR

by

Zhengxing Qu

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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ABSTRACT

HIGH LEVEL EXPRESSION AND CHARACTERIZATION OF A TRUNCATED SOLUBLE MURINE Fcγ RECEPTOR

by

Zhengxing Qu

Adviser: Professor Jay C. Unkeless, Ph.D.

An expression vector encoding a truncated secreted moFcγRIIβ (tFcγRIIβ) protein was constructed by deletion of transmembrane and cytoplasmic domains from an FcγRIIβ2 cDNA clone, and insertion of the truncated cDNA into the eukaryotic expression vector, pcEXV-3. The DNA construct was transfected into a dihydrofolate reductase-minus CHO cell line along with a *dhfr* minigene, and the production of FcγRIIβ was then amplified by gradual addition of methotrexate. The resulting cell line, D1959, secretes 2-3 μg/ml/24 h of truncated FcγRIIβ, which has a M_r of 30,000-35,000 on SDS-PAGE and reacts with anti-FcγR antibodies on immunoblots. The truncated FcγRIIβ was purified to homogeneity by affinity chromatography on IgG-Sepharose.

The truncated FcγRII purified from the medium conditioned by D1959 cells was further characterized. The truncated FcγRIIβ is glycosylated and sialylated and shows a high degree of size and charge heterogeneity on SDS-PAGE and IEF. Titration with Ellman's reagent revealed that the 4 cysteine residues in the protein form 2 disulfide

bonds. As dose intact FcγRII, the truncated FcγRII was shown to be capable of binding IgG1, IgG2a and IgG2b immune complexes. The binding of the immune complexes was somewhat better at more acidic pH. The glycosylation of tFcγRII is not required for the binding activity since both aglycosylated and deglycosylated tFcγRII bind with IgG-coated beads.

Crosslinking of the truncated FcγRII with a heterobifunctional crosslinker, SASD, provided evidence that, although tFcγRII exists as monomer in solution, there is interaction between tFcγRII molecules. Upon irradiation to activate the azido moiety, SASD derivatized FcγRII monomers form very large complexes. The extent and rate of formation of these complexes is independent on the concentration of tFcγRIIβ in the range of 3 μM to 0.3 μM. Complex formation does not occur after denaturation of the protein with SDS.

The truncated FcγRII was reactive with mAbs elicited by immunization with intact FcγRII, with the exception of 6B7C. Since the tFcγRII is lacking 8 amino acid residues proximal to the transmembrane domain, we hypothesized that the 6B7C epitope encompassed in the deleted sequence. By binding of mAb 6B7C to a synthetic peptide conjugate, the 6B7C epitope was mapped to residues 169-183 of the intact FcγRIIβ.

With the availability of a large quantity of purified tFcγRII protein, we have attempted to crystallize the truncated FcγRII for X-ray crystallography. The fully glycosylated tFcγRII crystallized very poorly from an oil. However, the partially deglycosylated protein crystallized readily. Single crystals were grown to approximate 1.0 x 0.2 x 0.2 mm in size and diffracted to 3 Å resolution. Further studies to obtain

high quality crystals for deglycosylated and neuraminidase-treated tFcγRII are underway.

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CHAPTER ONE

HISTORICAL INTRODUCTION

A. *The Receptors for the Fc Portion of IgG (Fc$_\gamma$ Receptors).*

The binding by monocytes and macrophages of antigen-antibody complexes (Ag-Ab) in the absence of serum was observed more than 20 years ago (LoBuglio *et al.*, 1967). During that period of time, cytophilic antibodies that are bound primarily to monocytes and macrophages were also characterized (Boyden and Sorkin, 1960; Berken and Benacerraf, 1966). That lymphocytes can bind aggregated IgG without the involvement of complement was later shown by Brown *et al.* (1970), and the binding of IgG to lymphocytes via the Fc portion of the molecule was further demonstrated by many investigators. Basten *et al.* (1972a,b,c) detected the binding on murine B lymphocytes by treating cells with antibody followed by the corresponding radioiodinated antigen, indicating that the interaction is through the C-terminal end of the molecule. Cline *et al.* (1972) shown that B lymphocytes have a trypsin-resistant receptor for IgG and IgG-Ag complexes and that an intact Fc portion is necessary but that serum complement is not required for binding. The binding of IgG was also demonstrated on murine T cells (Grey *et al.*, 1972; Yoshida and Andersson, 1972) and human B cells (Dickler and Sache, 1972). Paraskevas *et al.* (1972a,b) provided evidence that the isolated Fc fragment derived from rabbit IgG, but not Fab fragment, could bind to lymphocytes from mouse spleen and thus suggested that the membrane sites that bind IgG through the Fc portion be designated as Fc receptor (Paraskevas *et al.*, 1972a).

Now it has been established that Fc receptors for IgG (Fcγ receptors or FcγRs) are a family of cell membrane glycoproteins. FcγRs are members of immunoglobulin superfamily, and they are expressed on a number of hematopoietic mammalian cell types and they play important roles in the immune system. At least three subclasses of Fcγ receptors exist on leukocytes of both humans and mice. These receptors differ in their antigenic determinants as well as binding specificity and affinity for IgG subclasses. The cell types that express FcγR include monocytes, macrophages, NK cells, neutrophils, eosinophils, basophils, platelets, trophoblasts, B cells and some subsets of T cells. Human receptors have been assigned as huFcγRI (or CD64), huFcγRII (or CD32) and huFcγRIII (or CD16), and mouse's as moFcγRI, moFcγRII and moFcγRIII (Unkeless *et al.*, 1988).

B. *Detection of FcγRs on Murine Cells.*

In early studies, FcγRs on different cell types were detected by measuring rosette formation, the binding of antibody-coated erythrocytes to lymphocytes (LoBuglio *et al.*, 1967; Cline *et al.*, 1972; Yoshida and Andersson, 1972), or by measuring the binding to cells of antigen-antibody complexes or aggregated IgG labeled with radioisotopes (Basten *et al.*, 1972b; Anderson and Grey, 1974) or fluorescence probes (Forni and Pernis, 1975; Dickler, 1974; Abbas and Unanus, 1975; Winchester *et al.*, 1975b; Dickler and Sache, 1972; Schwartz *et al.*, 1976).

Even in early results, there was a suggestion that there might be more than one FcγR. Kossard and Nelson (1968) tested both normal and trypsin-treated macrophages

sensitized with either "early" or hyperimmune anti-serum to sheep red blood cell (SRBC) for the ability to form rosettes with SRBCs. They found that treatment of mouse macrophages with trypsin did not abolish their ability to take up cytophilic antibodies from the hyperimmune sera, however, such treatment profoundly reduced their ability to take up cytophilic antibodies from "early" sera.

The trypsin-sensitive receptor was further characterized by other investigators. By fractionating "early" sera and typing the fractionated cytophilic antibodies, together with results of their inhibition study, Askenase and Hayden (1974) suggested that the subclass of mouse IgG that bound to the trypsin-sensitive site was IgG2a. With ^{125}I-labeled monomeric mouse myeloma proteins, Unkeless and Eisen (1975) provided direct evidence that IgG2a proteins were strongly bind to a trypsin-sensitive FcγR on mouse macrophages and macrophage-like cell lines in an exothermal fashion and an apparent Ka of 2×10^7 to 10^8 M^{-1}.

In addition to trypsin sensitivity, the presence on murine leukocytes of distinct trypsin-resistant and trypsin-sensitive FcγRs was shown by their specificity for different subclasses of IgG. Walker (1976) demonstrated that ^{125}I-labeled myeloma IgG2a in either non-aggregated or aggregated form, bound rapidly to a macrophage cell line, IC21, whereas IgG2b did so only as aggregated complexes. Furthermore, IgG2a and IgG2b did not compete with one another. These results led to the conclusion that IC21 macrophages possess distinct FcγRs for these two subclasses of antibody. To evaluate subclass specificity and aggregated size requirements of FcγRs on mouse cells, Heusser *et al.* (1977) measured binding of radiolabeled monomeric and bis-diazotized benzidine-

aggregated mouse myeloma proteins fractionated into various sizes. They found that macrophages and macrophage-like cells preferentially bound monomeric IgG2a and also bound aggregated IgG1, IgG2a and IgG2b with equal avidity. In contrast, some lymphoid lines, such as D2N and S49 bound only aggregated forms of IgG1, IgG2a and IgG2b, and showed no capacity to bind IgG2a monomers. The binding of monomeric IgG2a was only inhibited by monomeric IgG2a but not by aggregated IgG2a and binding of aggregated IgG1 or IgG2b was inhibited by all three subclasses of IgG.

These results were consistent with that of Diamond *et al.* who employed a different approach to study subclass specificity. Mouse anti-SRBC monoclonal antibodies (mAbs) of all subclasses were isolated. By cross-inhibition, they showed that on all macrophage cell types tested there were three distinct Fcγ receptors, one, moFcγRI for IgG2a (Diamond *et al.*, 1978), which was trypsin sensitive, one, moFcγRII for IgG1 and IgG2b (Diamond *et al.*, 1978; Diamond and Scharff, 1980), and the third one, moFcγRIII for IgG3 (Diamond and Yelton, 1981).

Recently, characterization of Fcγ receptors of both humans and mice has been facilitated by isolation of anti-FcγR monoclonal antibodies. The first anti-FcγR mAb, designated 2.4G2 (Unkeless, 1979) was derived from the fusion of the HPRT$^-$ mouse myeloma P3U1 with the spleen cells of a rat immunized with the mouse macrophage cell lines J774 and P388D1. The supernatants of resulting hybridoma clones were screened for the ability to inhibit rosette formation by erythrocytes coated with an IgG2b anti-SRBC mAb. MAb 2.4G2 is binds to moFcγRII with an extremely high avidity, 10^9 M^{-1} at 4°C (Poglitsch and Thompson, 1990), and recognizes an epitope near or within the

binding site of the receptor. Therefore, it is a powerful tool in studying the structure and functions of FcγRs.

C. *Detection of FcγRs on Human Cells and Anti-FcγR mAbs.*

The Fcγ receptors on human cells are able to bind mouse IgGs and are readily isolated by chromatography on IgG-coupled column. Several species of huFcγR were thus purified from human monocytes (Anderson, 1982; Cohen *et al.*, 1983), neutrophils and platelet (Kulczycki, 1984). All of these species appeared on SDS-PAGE as broad bands of distinctive mobility and all are glycosylated.

HuFcγRIII, was identified by mAbs 3G8 and 4F7 (Fleit *et al.*, 1982), which were generated by immunizing CD2F1 mice with viable PMN. The binding site for 3G8 was found on neutrophils, mature chronic myelogenous leukemia cells and eosinophils. There is no site on U937, HL-60, Raji, Daudi K562 or blood monocytes.

Looney *et al.* (1986) immunized CAF1 mice with K562 cells and isolated an anti-huFcγII mAb, IV.3, by screening for inhibition of rosette formation of K562 with mouse IgG1-coated erythrocytes. IV.3 immunoprecipitated a 40Kd FcγR from lysates of human monocytes, U937 and K562, but not lymphoma cells, Daudi or Raji. MAb IV.3 selectively inhibited the binding of mouse IgG1-RBC to U937. They also showed that intact IV.3 or its Fab fragments blocked stimulation of T lymphocytes by mouse IgG1 anti-T3 (Leu-4), but not by IgG2a anti-T3 (OKT3). Monomer human IgG1 blocked the stimulation of only OKT3. Therefore, human monocytes and U937 bear 2 classes of FcγR. They suggested that these two receptors are human homologue of moFcγRI and

moFcγRII. The antigen of IV.3 also exists on platelets (Rosenfeld *et al.*, 1985).

The first huFcγRI-specific monoclonal antibody, 32, was developed by Anderson *et al.* (1986). MAb 32 binds a site on the 72 kDa receptor distinct from the ligand binding site. MAb 197, an anti-FcγRI IgG2b devloped by Guyre *et al.* (1989) is capable of blocking ligand binding, perhaps through binding of its own Fc region to huFcγRI. MAb 10.1 can inhibit binding of immune complexes but not monomeric IgG to huFcγRI (Dougherty *et al.*, 1987). Therefore, it may recognize an epitope near the ligand binding site. Frey and Engelhardt (1987) isolated an mAb, FR51, that is able to inhibit the binding of both IgG monomer and aggregates to U937 cells and the myeloblast cell line, HL60.

A battery of mAbs directed against all three distinct subclasses of FcγRs has been developed. The reactivity with specific mAbs has been one of the criteria to differentiate huFcγRs, even within a subclass. For instance, mAbs 41H.16 (Vaughn *et al.*, 1985; Antoun *et al.*, 1989), KuFc79 and KB61 reacted with a neutrophil and macrophage huFcγRII molecule that is different from that recognized by mAb IV.3.

D. *Molecular Cloning of FcγRs.*

Following the isolation of FcγR-specific mAbs, molecular cloning of the genes encoding FcγRs was achieved. By using anti-FcγR mAbs, mouse FcγRII protein was purified to homogeneity in several laboratories (Mellman and Unkeless, 1980; Hibbs *et al.*, 1986). Microsequencing of proteolytic fragments generated partial amino acid sequences and directed synthesis of corresponding oligonucleotide probes for cDNA

cloning. MoFcγRII genes were thus cloned and sequenced (Ravetch *et al.*, 1986; Lewis *et al.*, 1986; Hibbs *et al.*, 1986; Hogarth *et al.*, 1987).

Initial cloning of moFcγRII by Revatch *et al.* (1986) resulted in three cDNA clones, termed α, β1 and β2 from two distinct genes, α and β. These genes encode two distinct Fc receptors, moFcγRIIα and moFcγRIIβ. The β1 and β2 mRNAs are different splicing species of the moFcγRIIβ gene. The products of these two genes, moFcγRIIα and moFcγRIIβ, are 95% homologous in the extracellular domains (of 185 or 181 amino acids) due to an identical stretch of 171 amino acids. Neither the transmembrane domains (of 20 or 26 residues) nor the cytoplasmic domains (of 26, 47, or 94 residues) of moFcγRIIα and FcγRIIβ share any homology (Ravetch *et al.*, 1986).

The cloning of human and mouse cDNAs encoding FcγRs has been facilitated by a protocol of cDNA library screening for membrane antigens that was developed by Seed and Aruffo (1987). A eukaryotic expression shuttle vector was used to construct a library from human lymphocyte cDNA. This cDNA library was transfected into COS cells which express functional FcγRs. FcγR-expressing COS cells were then selected by panning. Hirt supernatants were then made and used to transform E.coli and the procedure cycled several times (Stengelin *et al.*, 1988; Simmons and Seed, 1988; Allen and Seed, 1989; Peltz *et al.*, 1989). The advantage of this procedure is that the protein encoded by the cloned gene is already expressed and is biologically active. Furthermore the affinity and specificity of a cloned FcγR is readily determined in the transient expression system.

Several genes encoding FcγRs have been cloned and sequenced. In addition to moFcγRII, these include the genes for moFcγRI (Sears *et al.*, 1990), huFcγRI (Allen and

Seed, 1989), huFcγRII (Stuart *et al.*, 1987; Stengelin *et al.*, 1988; Hibbs *et al.*, 1988; Seki, 1989; Stuart *et al.*, 1989; Brooks *et al.*, 1989), and huFcγRIII (Simmons and Seed, 1988; Scallon *et al.*, 1989; Ueda *et al.*, 1989, Ravetch and Perussia, 1989; Edberg *et al.*, 1989; Selvaraj *et al.*, 1989; Peltz *et al.*, 1989).

Three genes encoding huFcγRII proteins have been characterized (Stuart *et al.*, 1987; Stengelin *et al.*, 1988; Hibbs *et al.*, 1988; Seki, 1989; Stuart *et al.*, 1989; Brooks *et al.*, 1989). The three huFcγRIIs have homologous extracellular domains and are most homologous to moFcγRIIβ. At least two genes encode huFcγRIIIs. HuFcγRIII-1, unlike all other FcγRs, is anchored to the neutrophil cell membrane via a glycan-phosphatidylinositol (GPI) linkage and can be released from the membrane by a phosphatidylinositol-specific phospholipase C (Scallon *et al.*, 1989; Ueda *et al.*, 1989, Ravetch and Perussia, 1989; Edberg *et al.*, 1989; Selvaraj *et al.*, 1989).

The cloning of huFcγRI (Allen and Seed, 1989) and moFcγRI (Sears *et al.*, 1990) revealed a unique feature of these high affinity receptors. The extracellular domain of FcγRI is longer than that of FcγRII and FcγRIII, containing six cysteine residues, presumably disulfide linked to form three C2-set Ig-like structures (Williams and Barclay, 1988). In contrast, the extracellular domain of other FcγRs can form only two such structures.

From the cDNA sequencing data, it is evident that the common binding features of FcγRs result from the structural similarity in the extracellular domains. The predicted amino acid sequences of moFcγRIIα and moFcγRIIβ are nearly identical in this region and not surprisingly, the cloned human low-affinity FcγRs, huFcγRII and huFcγRIII, are

highly homologous to moFcγRII with about 30-60% amino acid identity. The first two N-terminal Ig-like domains in extracellular region of both huFcγRI and moFcγRI are closely related to the two Ig-like domains of FcγRII and FcγRIII. The third membrane-proximal domain is relatively unique and probably confers the high affinity that huFcγRI shows for human IgG1 and IgG3 (Allen and Seed, 1989).

However, neonatal rat Fcγ receptor, FcγRn, from neonatal intestinal epithelia differs from the structural motif of other FcγR's (Simister and Mostov, 1989). This receptor mediates the uptake of maternal IgG from milk and the affinity purified receptor is associated with β2-microglobulin (Simister and Ress, 1985). Translation of the cDNA sequence of FcγRn revealed a putative extracellular region consisting of three domains which are structurally related to class I MHC antigens and unrelated to leukocyte FcγRs.

E. *Multi-functions of Fcγ Receptors.*

Fcγ receptors play a central role in cellular immune defense as a link between the humoral and cellular pathways (Unkeless *et al.*, 1988). The binding of immune complexes to FcγRs on effector cell results in a variety of cellular immunological processes, including phagocytosis (Mellman *et al.*, 1983; Ralph *et al.*, 1980), clearance of immune complexes (Kurlander *et al.*, 1980; Leslie, 1985; Kurlander and Hall, 1986), antibody-dependent cellular cytotoxicity (ADCC, Herlyn and Koprowski, 1982; Matthews *et al.*, 1981; Nathan *et al.*, 1980), release of activated oxygen metabolites (Yomamoto and Johnson, 1984) and inflammatory mediators (Aderem *et al.*, 1986; Rouzer *et al.*, 1980), and secretion of hydrolytic enzymes (Nathan *et al.*, 1980). It is difficult to assign

a specific physiological function to a particular subclass of Fcγ receptor because of the structural homology between FcγRs, the existence of isoforms within a subclass, and, normally, the coexpression of multiple forms of the receptor on cells. The complexity is not only that an FcγR could mediate several different physiological functions but also that different subclasses of FcγR may play same function. For instance, all three FcγRs, with the exception of FcγRIII-1, are capable of ADCC toward target cells although the effect is dependent on the type and maturity of the effector cells as well as that of the target cells (Shen *et al.*, 1989; Fanger *et al.*, 1989; Graziano *et al.*, 1989).

The studies on the expression levels of FcγRs on the cells isolated from patients or animal models sheded light on the physiological importance of some FcγRs. Increasing evidence suggests that FcγR's play an important role in modulation of immune responses (Pillips and Parker, 1985) and expression of tumor immunity (Svennevig and Andersson, 1982; Ran *et al.*, 1984). A basic phenomenon established in the studies on *in situ* expression of tumor immunity was the *in vivo* localization of IgG with non-lymphoid tumor tissue of human (Ran *et al.*, 1973) and animal (Ran and Witz, 1970) origin. Further studies shown that metastatic breast cancer patients had significantly elevated FcγR levels on their peripheral blood mononuclear cells (PBMC) and that there was a direct correlation between increased FcγR levels on PBMC and tumor mass in colon, ovary and lung metastatic carcinoma patients (Ran *et al.*, 1988).

The decreased levels of huFcγRIII were found in some AIDS patients. A substantial percentage (25%) of neutrophils in HIV-infected men were negative for huFcγRIII-1 expression, while levels of other PI-G-linked proteins and huFcγRII were

normal (Boros *et al.*, 1990). The reason for the loss of huFcγRIII-1 and the clinical consequence are unknown. The levels of huFcγRIII in serum were reported to vary during the course of AIDS, with an initial rise and a subsequent fall in serum huFcγRIII levels in the terminal stage of the disease (Khayat *et al.*, 1990). Recently, it has been suggested that FcγRs may enhance the entry into FcγR-bearing cells of infective agents such as human immunodeficiency virus and dengue virus (Bolognesi, 1989).

F. *The Interactions between of IgG and FcγR.*

The epitopes of IgG that interact with FcγR have been most intensively studied on human high-affinity receptor, FcγRI, because of its strong interaction with IgG monomer. The valency of huFcγRI was determined by O'Grady *et al.* (1986). The FcγRI on U937 cells was first saturated with a mixture of IgG1κ and IgG1λ, cell lysates were prepared, and immunoprecipitation of the IgG1-FcγRI complexes was performed with immobilized antibodies. If the receptor were multivalent, both IgG1κ and IgG1λ would be co-precipitated by either anti-IgG1κ or anti-IgG1λ antibody. In contrast, if the receptor were univalent, none of the anti-IgG1 antibodies alone would be able to co-precipitate both IgG1κ and IgG1λ, however, an anti-FcγRI antibody would do so. The experimental results led to the conclusion that the valency of the high-affinity FcR on U937 cells is one. This conclusion may be applied to low-affinity Fcγ receptors since all cloned functional FcγRs are single chain proteins. A remaining question is how many FcγRs can interact with one IgG molecule since an Fc of IgG consists of two identical peptide chains.

The binding site for huFcγRI on the Fc fragment of IgG has been mapped. Earlier studies localized the binding site for monocyte FcγRI on IgG to the Cγ2 domain of human IgG (Woof *et al.*, 1984; Partridge *et al.*, 1986). Burton *et al.* (1988) assayed mAbs directed against different epitopes on human IgG and IgGs from different species for their capacity to inhibit the binding of radiolabeled IgG to human monocytes. The amino acid sequences of these IgGs exhibiting relatively tight, intermediat or weak binding activity were compared. From these experiments, the monocyte FcR-binding site on human IgG was postulated to involve the lower hinge region of IgG, comprising residues Leu234-Ser239 (Woof *et al.*, 1986), with possible involvement of the nearby N-proximal bend and two β-strands (Gly316-Lys338). In particular, comparison of the sequences of two high-avidity ligands, human IgG1 and mouse IgG2a, with that of mouse IgG2b, which does not bind to huFcγRI, revealed an Leu235 in the former and Glu235 in the latter. Mutation of Glu235 in the hinge region of a mouse IgG2b to Leu235 by oligonucleotide-directed site-specific mutagenesis increased the avidity of IgG2b235Leu 100-fold relative to IgG2b (Duncan at al., 1988), indicating the Leu235 is one of the major determinants in the high avidity binding of IgG2a to FcγRI.

The studies to localize the sites of IgG2a and IgG2b that interact with macrophage moFcγRI and moFcγRII, respectively, were performed by Diamond *et al.* (1985). They showed, with cyanogen bromide fragments, that a fragment from the CH2 domain of IgG2b bound to the FcγRII and that two fragments from IgG2a, one from the CH2 domain and the other from the CH3 domain, specifically bound to FcγRI. They also demonstrated that the binding of the fragment from the CH3 domain of IgG2a to

moFcγRI augmented the binding of the fragment from the CH2 domain of IgG2a, but not that of the homologous fragment from IgG2b, indicating that two regions on IgG2a contribute to the high-affinity binding with the receptor. Comparison of the sequences of the two fragments from the CH2 domain of IgG2a and IgG2b showed that they differ each other in only four amino acid residues with only one definitive charge difference, a leucine residue in IgG2a and a lysine in IgG2b at same position.

These studies provide some indirect evidence to indicate the important elements that might influence or determine the specificity and affinity of FcγR interacting with IgG: electrostatic verses hydrophobic forces and single site verses multiple site interactions. Direct evidence must come from studies of FcγR proteins themselves.

The extracellular domains of murine and human FcγRs are homologous to other members of immunoglobulin superfamily (Williams and Barclay, 1988). Representative members in this large and complex group include proteins involved in adhesion or binding, cell-cell recognition, and antigen presentation, and in many cases trigger intracellular events following cell surface interaction. The external portion of deduced murine FcγRIIα and FcγRIIβ consists of two repeats with about 80 amino acid residues in length, each of which contains two cysteine residues that form a disulfide bridge with spacing of 42 to 45 amino acids, resulting in a C2-set Ig-like loop. By sequence homologies between different members of the Ig superfamily, three types of Ig-like structures have been defined: V-set, C1-set and C2-set (Williams, 1987; Williams and Barclay, 1988). From the cDNA predicted protein sequence we know that FcγRII share homology with the C2-set. However, the folding criterion for an Ig-like structure is

dependent on tertiary structural analysis by X-ray crystallography. Thus far, only the structures of IgV and IgC domains, members of V-set and C1-set, respectively, and the β2-m and α3 domains of MHC class I antigens which have structures related to IgC domain, C1-set (Becher and Reeke, 1985; Bjorkman *et al.*, 1987a,b), have been resolved. There is no report on the C2-set structure yet.

CHAPTER TWO

EXPRESSION OF A TRUNCATED MOUSE Fcγ RECEPTOR

Introduction

Fc receptors for IgG (FcγR) on macrophages, polymorphonuclear leukocytes, and B and T lymphocytes perform a central role in immune defenses since these receptors link the humoral and cellular effector limbs of the immune system and thus confer an element of humoral specificity on the effector cells that bear FcγR. Recently, two murine FcγR genes, FcγRIIα and FcγRIIβ, have been cloned and sequenced (Ravetch *et al.*, 1986; Lewis *et al.*, 1986; Hibbs *et al.*, 1986; Hogarph *et al.*, 1987). Both the FcγRIIα and FcγRIIβ genes encode proteins containing a leader sequence, N-terminal extracellular domains, one putative transmembrane spanning domain, and a cytoplasmic domain. Transfection of the FcγRIIβ2 gene into melanoma cells results in the appearance, on the cell surface, of the epitope recognized by monoclonal antibody 2.4G2, an anti-FcγR mAb (Unkeless, 1979; Mellman and Unkeless, 1980), and the FcγR binding activity.

The most striking characteristic of Fcγ receptors is their ability to interact specifically with the Fc domain of IgG. The nature of the interaction is still poorly understood. To reveal the molecular structure of FcγRs and to study their biochemical and biophysical properties, it is essential to have a large quantity of pure receptor protein. Our initial efforts were directed toward the production of a secreted soluble form of FcγR in cultured cells. To make the secreted form of an FcγR, the coding sequence

for the transmembrane and cytoplasmic domains of FcγRIIβ was deleted from the cDNA, and a termination codon introduced. This truncated FcγRIIβ cDNA in a eukaryotic expression vector was then transfected into a dihydrofolate reductase (DHFR)-negative CHO line along with a *dhfr* minigene. Production of the truncated FcγRIIβ was then amplified by addition of methotrexate in medium. The resulting cell line secretes 2-3 μg/ml/d of truncated FcγRIIβ. We also succeeded in expression of the truncated FcγRII in the Sf9 insect cell line by using baculovirus expression vector and in *E. coli* by using the pINIII-ompA vectors.

Materials and Methods

A. *Chemicals, Stains and Cell Lines.* Unless otherwise specified, chemicals were obtained from Sigma (St Louis, MO). The CHO mutant cell line, DG44, which has a deletion of the dihydrofolate reductase (DHFR) gene (Urlaub *et al.*, 1986), was obtained from Dr. Chasin, (Columbia University, NYC, NY). The *S. frugiperda* insect cell line, Sf9 was from ATCC (Rockville, MD) and *Autographa californica* Nuclear Polyhedrosis Virus (AcNPV) was provided by Dr. Summer (Texas A&M University, College Station, TX). The *E. coli* stains used in the studies were MM294 (F^-, *endo*A1, *thi*$^-$, *pro*$^-$, *hsd*R$^-$, *sup*E44), W620 *rec*A (F^-, *thi*I, *pyr*D36, *glu*A6, *gal*K30, *str*129, λ^-, *sup*E44, *rel*A, *rec*A) (Wurtzel *et al.*, 1981).

B. *Cell Cultures.* DG44 cells were maintained in DME medium (Gibco

Laboratories, Grand Island, NY) supplemented with 0.016 mM thymidine, 0.1 mM hypoxanthine, 0.3 mM proline and 10% fetal calf serum (FCS) (Armour Pharmaceutical Company, Kankakee, IL). Hypoxanthine was omitted and dialyzed FCS used in medium for selection of transfectants. The S49.1 cell line was grown in suspension culture in α-MEM (Gibco) supplemented with 5% FCS. In some experiments, cells were cultured in serum-free medium, supplemented with a hormone mixture containing transferrin (5 μg/ml), epidermal growth factor (50 ng/ml), bovine insulin (10 μg/ml), glucagon (1 ng/ml), somatotropin (6.5 ng/ml), and hydrocortisone (3.5 μM) (Sells *et al.*, 1985).

S. frugiperda cell line, Sf9, was cultured in Grace's insect medium (Hazleton Research Products, Denver, PA) with supplements of Yeastolate (Difco Laboratories, Detroit, MI), lactalbumin hydrolysate (Difco) and 10% FCS.

C. *Plasmid Construction.* The enzymes used were purchased from New England Biolabs (Beverly, MA) unless otherwise indicated. Calf intestine alkaline phosphatase was from Boehringer Mannheim Biochemicals (Indianapolis, IN). The methods used for construction of expression vector were based on Maniatis *et al.* (1982).

Plasmid 3901, an FcγRIIβ2 cDNA clone (Ravetch *et al.*, 1986), was restricted by ApaI at base 947 within the insert and the resulting 3' overhang was blunted by T4 DNA polymerase. The unique ApaI restriction site is 26 base pairs upstream of the transmembrane encoding region and results in deletion of the transmembrane and cytoplasmic domains, leaving a 172 amino acid protein that contains the two extracellular immunoglobulin-like domains (see Fig. 1). The stop codon was introduced into the

reading frame by ligation of the phosphorylated 16 base pair universal terminator, GCTTAATTAATTAAGC (Pharmacia Inc., Piscataway, NJ), to the termini of the linearized plasmid. This results in addition of 4 accessory amino acid residues, GLIN, before the termination codon is reached. Next, NcoI was used to cut the cDNA at base 338, one base pair before the first methionine codon of the translated sequence. After HhaI digestion to remove concatamerized terminators, the overhangs generated by those two enzymes were blunted by incubation with DNA polymerase Klenow fragment. The 620 base pair DNA fragment between ApaI and NcoI restriction sites with the ligated terminator at the 3' end was isolated by preparative agarose gel electrophoresis and ligated into the SmaI restriction site of linearized and dephosphorylated pcEXV-3 expression vector (Miller *et al.*, 1985). The truncated cDNA insert is predicted to encode a truncated form of FcγRIIβ (tFcγRIIβ) with 176 amino acid residues. Two plasmids, pFcγRβ-19 (Fig. 1) and pFcγRβ-17, which contain the inserts with sense and anti-sense orientations, respectively, were obtained after transformation of MM294.

D. *Transfection and Amplification.* The CHO derived cell line DG44 was seeded at 4 x 10^5 cells per 100 mm dish and cultured overnight. The cells were cotransfected by incubation with a DNA-calcium phosphate precipitate containing pMG1 (0.1 μg per plate), which is the dhfr minigene construct (Mitchell *et al.*, 1986) given to us by Dr. Chasin (Columbia University), the FcγRIIβ cDNA construct (2 μg per plate), and carrier DNA (20 μg per plate) as described by Wigler *et al.* (1979). After two days, the cells were subcultured and the transfected cells were selected in medium without hypoxanthine

containing 10% dialyzed FCS. After 2 weeks, 0.02 μM methotrexate was added to the medium followed by stepwise increments every ten days to 0.05, 0.1, 1.0 and 2.0 μM methotrexate (Kaufman *et al.*, 1985). The cells were then cloned and clones screened for secretion of FcγRIIβ. The cultures were then maintained routinely in the presence of 2.0 μM methotrexate.

E. *Construction of Recombinant Viruses.* To express moFcγRII in the insect cell, Sf9, the plasmid construct, pFcγRIIβ19 was digested with EcoRI to release the insert from the vector (see Fig. 1). The insert was isolated by agarose electrophoresis and inserted into the EcoRI site of pVL1393, a derivative of pVL941 (Luckow and Summers, 1989), resulting in a plasmid pVLFcR, which was used to construct the recombinant virus.

For transfection, 1 μg of purified ECV DNA and 10 μg of pVLFcγR plasmids were coprecipitated in calcium-phosphate buffer as described above. The precipitant was overlaid on 10^6 Sf9 cells in a 25 cm^2 flask for 4 h and then was replaced by fresh medium. Virus infected cells were seen in 3 days. Recombinant viruses were isolated by visual screening for the occlusion-negative plaques formed on an agarose plate, as described by Smith *et al.* (1983a). The isolated viruses were further plaque-purified on an agarose plate to ensure that there was no contamination by wild-type viruses. A recombinant virus, VLFcγR4p3, was selected for production of the recombinant FcγRII.

F. *Construction of FcγR Expression Vector for E. coli and Expression of the*

Truncated FcγRIIβ in E. coli. To express the truncated FcγR in *E. coli*, the EcoRI fragment isolated from pFcγRIIβ-19 was ligated into the pINIII-ompA plasmids (Ghreyed *et al.*, 1984). A HinfI digestion and T4 polymerase treatment of the DNA fragment resulted in the deletion of the sequence coding for the leader peptide. This blunt-end DNA fragment was isolated from agarose gel. Plasmid pINIII-ompA2 and pINIII-ompA3 were digested with HindIII and EcoRI, respectively, followed by treatment with T4 polymerase and calf intestine alkaline phosphatase. Ligation of the isolated DNA fragment with each linearized pINIII-ompA plasmid resulted in fusion of the truncated moFcγRII and *E. coli* ompA leader sequence in frame. Two resulting plasmids, pA2F5 and pA3F6 with the insert in the sense orientation (Fig. 8) were then selected.

Preparation and transformation of competent cells of *E. coli* strains were carried out as described by Miller (1987).

To lacalize the the transformed gene products, the osmotic shock methed was used for cell fractionation (Neu and Heppel, 1965; Koshland and Bostein, 1980) of *E. coli*. Cultured bacterial cells were centrifuged in an Eppendorf centrifuge for 2 min. The supernatant was then removed and seved as conditioned medium fraction. The cell pellet was keeped on ice and resuspended in 0.15 ml cold 20 % sucrose in 10 mM tris-HCl (pH 7.5). The cell were centrifuged again for 5 min in the cold. The supernatant fluid was quickly separated from pellet and saved as sucrose-tris fraction, and the pellet was rapidly resuspended by vigorous agitation in 0.15 ml cold distill water. The mixture was incubated for 10 min on ice and centrifuged for 5 min. The supernatant was saved as pariplasmic fraction, and the pellet was resuspended 0.15 ml water and saved as the

cytoplasmic fraction. All fractions thus prepared were assayed for tFcγRII activity by ELISA.

G. *Immunoassay for MoFcγRIIβ (ELISA).* FcγRIIβ in cell culture medium or purified FcγRIIβ was titrated by a modification of the monoclonal sandwich radioimmunoassay described previously (Unkeless and Healey, 1982). FcγR in assay samples was adsorbed onto flat bottom wells (Immulon-2, Dynatech Laboratories Inc., Alexandria, VA) previously coated overnight with rabbit anti-FcγR IgG (5 μg/ml) in PBS and blocked with 1% BSA. FcγRIIβ was detected by sequential addition of the rat anti-FcγR mAb 2.4G2 (5 μg/ml in PBS), biotinylated goat anti-rat IgG antibody (0.5 μg/ml) (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD) and streptavidin-HRP (0.5 μg/ml) (Kirkegaard and Perry), and developed with o-phenylenediamine as described (Khayat *et al.*, 1987). The assay was calibrated by inclusion of a detergent lysate of S49.1 cells (1% NP-40 in PBS, 10^7 cells/ml).

H. *Cytoplasmic RNA Dot Hybridization.* Preparations of cytoplasmic RNA from tissue culture cells and dot blots were as described by White *et al.* (1982). Prehybridization and hybridization of blots with probes labeled with α-[^{32}P]-CTP (3000 Ci/mmol, Amersham) by nick-translation were carried out as described by Dobner *et al.* (1981). The probes were the FcγRIIβ cDNA insert of pFcγRIIβ-17 and the rat metallothionein 1 cDNA insert of plasmid p2A10 (Andersen *et al.*, 1983), kindly donated by Dr. Bancroft (Mount Sinai School of Medicine, NYC, NY).

I. *Purification of Truncated FcγRIIβ.* Proteins from cell culture supernatant containing 5 or 10% FCS was precipitated between 40-75% ammonium sulfate saturation and collected by centrifugation. The protein was redissolved in a minimal volume of sodium phosphate buffer (10 mM, pH 7.5) and dialyzed against the same buffer. The truncated FcγRIIβ was isolated by passing the concentrated protein solution over a DHK10.12 (IgG2b, kappa) Sepharose-4B (Pharmacia) affinity column (5-10 mg protein/ml bed volume), which was then washed with sodium phosphate buffer (10 mM, pH 7.5) containing 50 mM NaCl until the OD at 280 nm returned to baseline. The bound truncated FcγRIIβ was eluted with sodium acetate buffer (0.1 M, pH 4) containing 0.5 M NaCl, and dialyzed versus PBS. The protein at this stage was judged pure by SDS-PAGE.

J. *SDS-PAGE and Immunoblotting.* SDS-PAGE was performed using Neville buffers (Neville, 1971) on slab gels. For immunoblotting (Towbin *et al.*, 1979), non-reduced protein samples were subjected to SDS-PAGE and transferred to nitrocellulose (Bio-Rad Laboratories, Richmond, CA) with a non-buffer tank electroblotter (Polyblot, American Bionetics, Emeryville, CA) as the manufacturer recommended. After the nonspecific sites were blocked in tris-buffered saline (TBS) containing 3% BSA, the nitrocellulose was incubated with preimmune rabbit antibodies, rabbit anti-FcγR IgG or rat anti-FcγR mAb 6B7C (20 μg/ml in TBS with 1% BSA) on a miniblotter (Miniblotter II, Immunoetics, Cambridge, MA), followed by biotinylated goat anti-rabbit and goat anti-rat IgG (0.5 μg/ml each) and streptavidin-conjugated HRP

(0.5 μg/ml). The bound HRP was visualized with 4-chloro-1-napthol (Zalis and Jaffe, 1987).

K. *Biosynthetic Labeling.* Cells were plated in 60 mm tissue culture dishes at 10^6 cells/dish and incubated overnight. The medium was removed and cell monolayer was washed three times with fresh methionine-free αMEM medium (Gibco). Secretory products of transfected and amplified CHO cell lines were then biosynthetically labeled by incubating cells in methionine-free medium supplemented with hormones and 75 μCi/ml of [^{35}S]-methionine (1100 Ci/mol, Amersham, Arlington Heights, IL) for 6 h. The conditioned media were collected and concentrated with Centricon 10 microconcentrator (Amicon, Beverly, MA). The labeled proteins were separated on acrylamide gel by SDS-PAGE. After electrophoresis, the gel was treated with autoradiography enhancer reagent (Du Pont Co., Wilmington, DE) and analyzed by exposure on X-ray film (Eastman Kodak, Rochester, NY) with an intensifying screen (Du Pont).

Results and Discussion

A. *Isolation of the CHO Cell Line Secreting Truncated FcγRIIβ.* One of the major obstacles in studying Fcγ receptors has been the relatively small quantities of these proteins that can be readily isolated from cultured cells. To overcome this difficulty, we attempted to express a secreted form of moFcγIIβ in mammalian cell lines.

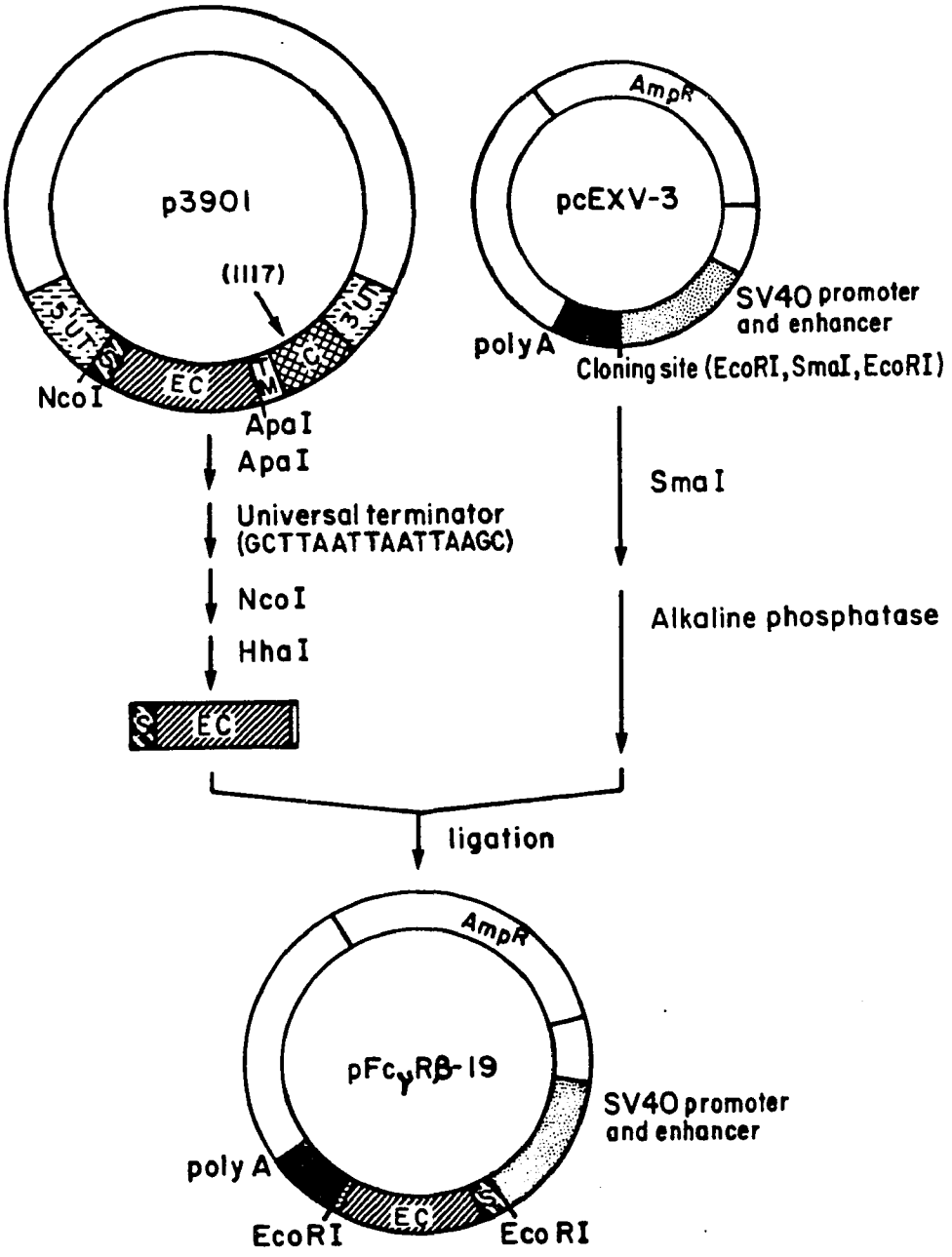

Figure 1. Construction of the truncated FcγRIIβ cDNA and the expression vector.

The plasmid containing the FcγRIIβ2 cDNA insert is illustrated on top left with the predicted coding regions (UT for untranslated region, S for signal sequence, EC for extracellular domains, TM for transmembrane domain,

and C for cytoplasmic domain) and the restriction sites for Apa I and Nco I. Plasmid 3901 is a FcγRIIβ2 cDNA clone which contains the sequence from 5'UT through base 1117 within the C region, as the arrow indicates. The truncated blunt-end cDNA was purified from agarose gel and inserted into the Sma I site of pcEXV-3, which is shown on top right. The plasmid pFcγRβ-19, which has the sense orientation, is shown on the bottom. The two EcoRI sites were preserved in this process and were used to isolate the insert in construction of the baculovirus expression vector.

To convert the FcγRIIβ2, normally an integral membrane protein, to a secreted protein, we deleted the transmembrane and cytoplasmic domains of the receptor, leaving the leader sequence and the extracellular domains. When plasmid 3901, which has an FcγRIIβ2 cDNA insert encoding signal sequence, extracellular and transmembrane domains, and partial cytoplasmic domain, is cleaved with ApaI the transmembrane and cytoplasmic domains are deleted leaving a 172 residue protein with two extracellular immunoglobulin-like domains, as diagramed in Fig. 1 and detailed in Materials and Methods. The truncated FcγRIIβ protein is also missing 8 amino acids just outside the membrane. The truncated FcγRIIβ cDNA was inserted in both orientations into the pcEXV-3 expression vector (Miller *et al.*, 1985) and transfected along with plasmid pCGcos3neo (Southern and Berg, 1982) into the B78H1 melanoma cell line. After G418 selection, transfectants were cloned and supernatants were screened for the presence of moFcγRII.

We developed a sensitive enzyme linked immunosorbent assay (ELISA) by utilizing a rabbit polyclonal anti-FcγR antibody and 2.4G2, an anti-moFcγRII mAb, to

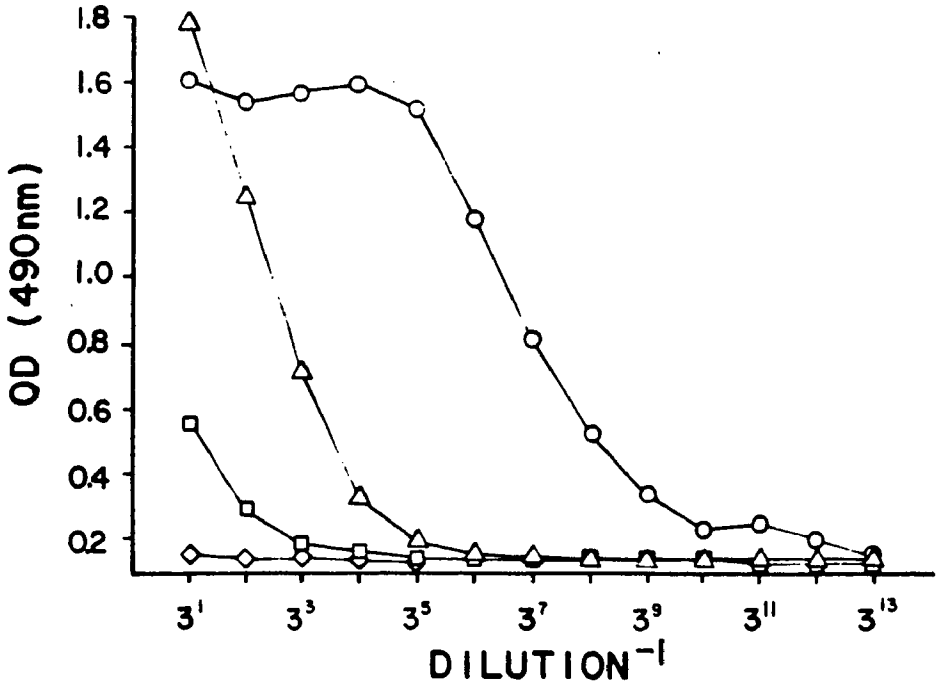

Figure 2. The titration of FcγRII by mAb 2.4G2.
$4x10^5$ cells of B1902, B1904, D1717 and D1959 were seeded in 100 mm tissue culture plates. After 24 h of incubation, the supernatants were taken from the plates and serial 3-fold dilutions assayed. An NP-40 lysate of S49.1 cells at 10^7 cells/ml was used as calibration. (□), B1904; (◇), B1902 and D1717, tFcγRIIβ non-secreting transfectants; (○), D1959; (△), S49.1 lysate.

detect and quantitate moFcγRII. The rabbit anti-FcγR antibody coated onto a microtiter plate is the capture reagent for moFcγRII and mAb 2.4G2 is the detecting reagent. This assay is capable of specifically and quantitatively detecting as little as 0.5 ng of moFcγRII protein in 0.1 ml volume. Of 19 clones transfected with pFcγRIIβ-19, 9 secreted immunoreactive material, but none of the 64 clones transfected with pFcγRIIβ-17 was positive. However, the titer of FcγRIIβ in the supernatant of B1904, one of the better secreting lines, based on comparison with lysates of the FcγR positive S49.1 cell line, was less than 5 ng/ml (Fig. 2), which would make further biochemical studies difficult.

To obtain a higher level of production and secretion of tFcγRII, we transfected pFcγRIIβ-17 and pFcγRIIβ-19 into a DHFR-negative CHO cell line along with a *dhfr* minigene. This co-transfection results in co-integration of *dhfr* minigene and the truncated FcγRIIβ DNA into the chromosomes of transfected cell. The stable transfectants were further selected with increasing concentrations of methotrexate. This leads to amplification of the *dhfr* minigene with co-amplification of the FcγRIIβ constructs that are situated close enough to it. The FcγRIIβ-secreting clones were isolated from mass culture after the culture had become resistant to 2 μM methotrexate. The level of secretion of truncated FcγRIIβ by the D1959 cell line is elevated over 2000-fold relative to the B1904 melanoma transfectant (Fig. 2). As expected, none of the pFcγRIIβ-17 transfected CHO cells secreted immunoreactive material. The slope of the titration of the intact S49.1 cell FcγR is slightly steeper, possibly due to more efficient capture of the intact FcγR by the rabbit anti-FcγR antiserum. We estimate, using the S49.1 lysate to

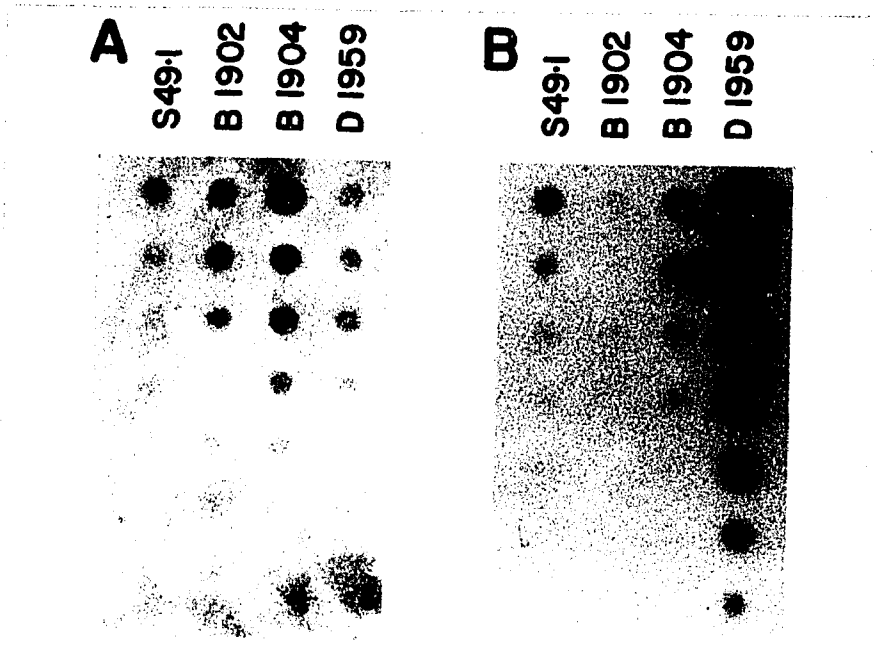

Figure 3. Cytoplasmic RNA dot hybridization.
Cell lysates (10^7 cells/ml) were made from all cell lines except for D1959 in B (10^6 cells/ml) Serial 2-fold dilutions starting from 1:4 were applied onto the nitrocellulose with a blot apparatus (BioRad). A, hybridized with the metallothionein 1 cDNA insert; B, hybridized with the truncated FcγRIIβ cDNA insert. Using only the cDNA inserts as probes was necessary for these experiments because the total plasmid hybridized with mRNA transcribed from plasmid DNA amplified in the D1959 cell line.

calibrate the assay, that the concentration of truncated FcγRIIβ in medium conditioned by confluent D1959 cells is 2-3 μg/ml/d.

The much higher level in synthesis of the truncated FcγRIIβ relative to B1904 and S49.1 should be paralleled by an increase in mRNA level. Cytoplasmic RNA dot hybridization was performed to investigate the levels of FcγRIIβ mRNA in selected cell lines. Hybridization of nick-translation labeled insert DNA fragment of pFcγRIIβ-17 to cytoplasmic RNA showed a greater than 320-fold higher level of expression of FcγRIIβ message by the D1959 cell line relative to B1904 and S49.1 cell lines (Fig. 3B). The levels of hybridization to cytoplasmic RNA found with a rat metallothionein cDNA probe were roughly the same for all the cell lines examined (Fig. 3A).

B. *Identification and Purification of the Truncated FcγRIIβ.* We examined the total [^{35}S]-methionine-labeled secretion products from the original CHO DG44 cell line, D1959, and a companion cell line, D1717, which was transfected with pFcγRIIβ-17 and is also resistant to 2 μM methotrexate. No difference in the profile of labeled secreted proteins was detected between the two FcγR-negative cell lines DG44 and D1717. However, the profile (Fig. 4) of labeled secreted proteins from D1959 has a major additional peak, centered at M_r 31,000, which was absent in the DG44 and D1717 cell line labeled secretion products. By densitometry of the autoradiogram we determined that the 31,000 M_r protein compromises 30% of the total labeled secreted proteins. This is probably an underestimate of the actual amount, since there is only one methionine in the sequence of the truncated FcγRIIβ (Ravetch *et al.*, 1986).

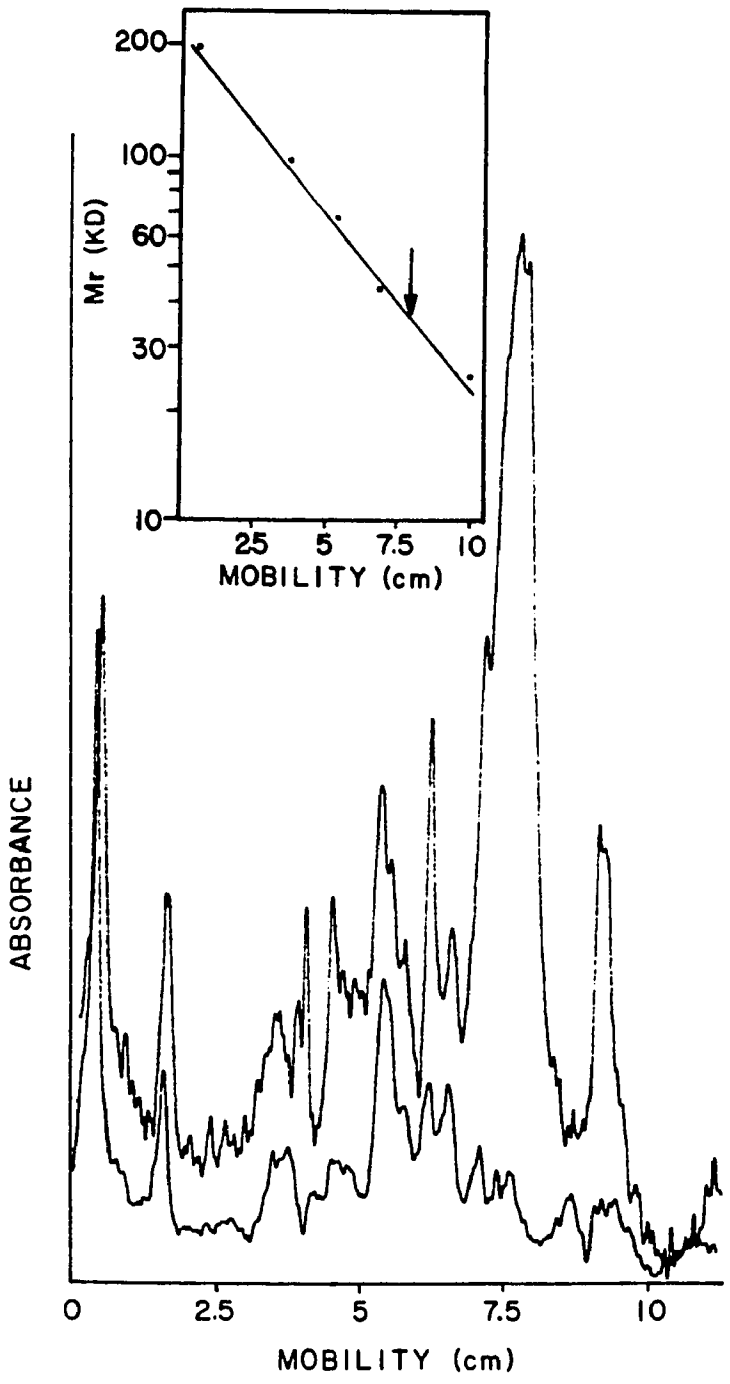

Figure 4. Analysis of [^{35}S]-methionine-labeled secreted products from transfected cell lines.

10^6 cells plated in 60 mm tissue culture dishes for 12 h were rinsed in PBS and labeled for 6 h in 2 ml of methionine-free αMEM medium containing 75 μCi/ml of [^{35}S]-methionine. The supernatants were concentrated and subjected to SDS-PAGE on a 10% gel. After autoradiography, the film was scanned by a densitometer. The profile of densitometry is shown with arbitrary absorbance. Upper curve, D1959; lower curve, D1717. The peak of truncated FcγRIIβ in the profile of D1959 occupies 30% of the total area. In the insert we show the mobility of the truncated FcγRIIβ (indicated by the arrow) and molecular weight standards (BRL, Gaithersburg, MD): myosin (H-chain), 200 kDa; phosphorylase b, 97 kDa; BSA, 68kDa; ovalbumin, 43 kDa; and α-chymotrypsinogen, 25 kDa.

In order to confirm that the major peak shown in the radiolabeled products is the truncated FcγRIIβ, the immunoreactivity of the proteins released from D1959 was examined by immunoblotting after SDS-PAGE. The rabbit anti-FcγR antibodies specifically bound to the same31,000 M_r protein (Fig. 5A) in the secretion products of the D1959 cell line as is seen in the autoradiogram of the [^{35}S]-methionine-labeled secretion products from D1959 cells in Fig. 3. The truncated FcγRIIβ is composed of 176 amino acid residues and should have a peptide core of about 20,000 Daltons. The anomalous electrophoretic mobility of the truncated FcγRIIβ is due to N-linked glycosylation. However, the truncated FcγRII is considerably smaller than intact FcγR from S49.1 or J774 cells, which has a M_r of 60,000 (Mellman and Unkeless, 1980; Margaret *et al.*, 1986; Unkeless *et al.*, 1981; Green *et al.*, 1985). The portion that is deleted of the FcγRIIβ is 127 amino acids (for the β1 gene product) or 81 amino acids (for the β2 gene product), corresponding to the molecular mass of about 14,000 or 9,000 Daltons, respectively, and clearly does not account for the difference in M_r seen on SDS-PAGE. This suggests that CHO cells either synthesize smaller complex carbohydrate moieties, or that as the consequence of over-production of the cloned gene in CHO cells, the glycosylation is faulty.

We purified the protein to homogeneity from the medium conditioned by the D1959 cell line by one cycle of affinity chromatography on IgG2b-Sepharose 4B (Fig. 6, Table I). The first step was a 40-75% saturated ammonium sulfate precipitation of conditioned medium followed by dialysis. This step functions both to concentrate the FcγRIIβ and to lower the ionic strength, which leads to tighter binding of the truncated

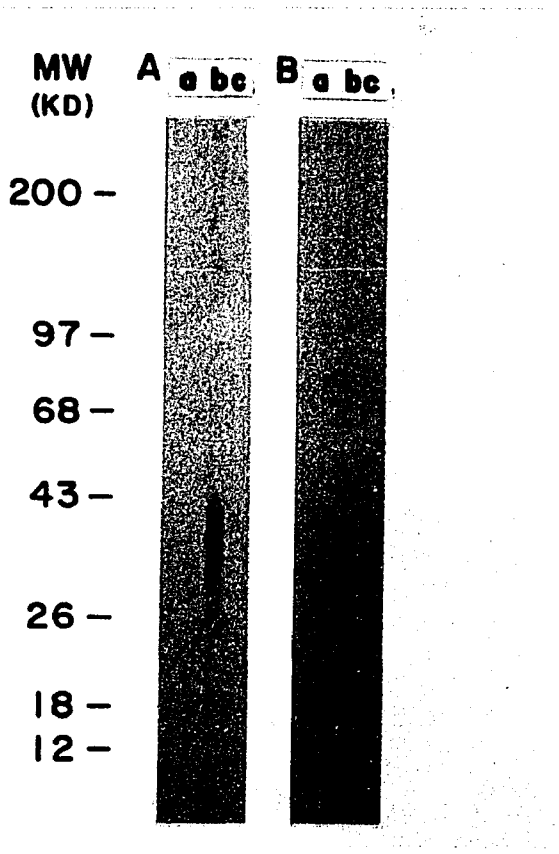

Figure 5. Immunoblotting analysis of the truncated FcγRIIβ.
Proteins were subjected to SDS-PAGE on a 7-17% acrylamide gradient gel, transferred to nitrocellulose paper and stained with: a, mAb 6B7C; b, rabbit anti-FcγR IgG; c, preimmne rabbit IgG. A, concentrated serum-free medium from the D1959 cell line; B, truncated FcγRIIβ purified on DHK10.12-Sepharose 4B.

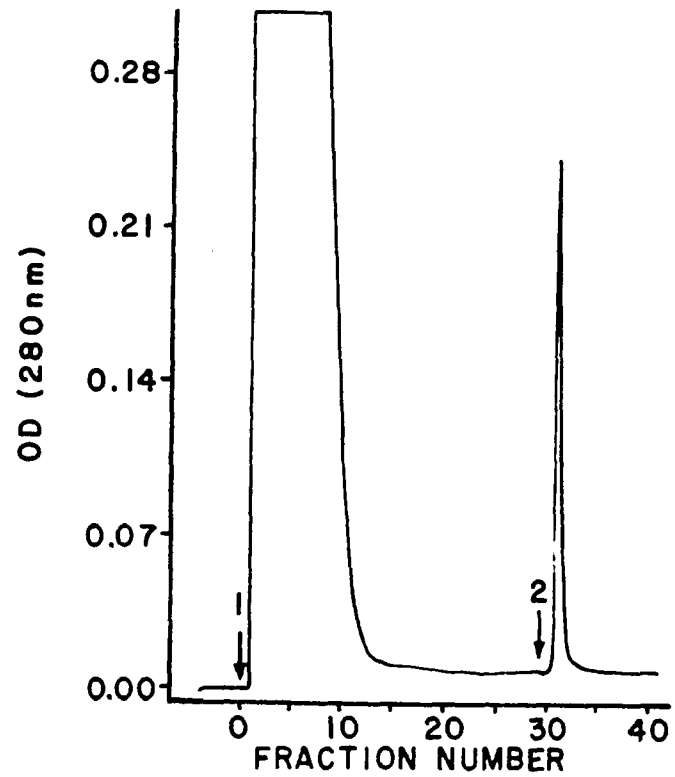

Figure 6. Purification of the truncated FcγRIIβ.
150 ml of D1959 supernatant was concentrated to 2 ml by ammonium sulfate precipitation between 40-70% ammonium sulfate, dialyzed versus 10 mM sodium phosphate buffer, pH 7.5, and loaded (arrow 1) onto the DHK10.12-Sepharose 4B column (3 ml of bed volume). The column was washed with 10 mM sodium phophate buffer, pH 7.5, containing 50 mM NaCl and the truncated FcγRII was eluted (arrow 2) with 0.1 M acetate buffer (pH 4.0), 0.5 M NaCl.

Table I. Purification of the Truncated FcγRIIβ from Cell Culture

Step of purification	Volume (ml)	Titer^{-1} per ml (titer^{-1})	Total activity (mg)[b]	Total protein (titer mg)$^{-1}$	Specific activity	Recovery of[c] activity (%)
Cell culture Supernatant	50	7.0×10^{3}[a]	3.5×10^{5}			
Concentrated supernatant	1.0	3.8×10^{5}	3.8×10^{5}	113	3.4×10^{3}	100
Post affinity supernatant	6	1.7×10^{2}	1.0×10^{3}	60	1.7×10^{1}	
Acid eluant	4.5	7.3×10^{4}	3.3×10^{5}	0.37	8.9×10^{5}	86.8

a. The titer of cell culture supernatant is the average value of several separate assays and was determined, in any one set of assays, by the titer resulting is 50% maximum $OD_{280\ nm}$. The total activity of the concentrated supernatant was always somewhat higher (50-100%) than that of the starting cell culture supernatant.

b. Proteins were quantitated by Lowry's method (Lowry *et al.*, 1951).

c. Comparison of the activity recovered from IgG2b-affinity column.

FcγRIIβ to the IgG matrix. The affinity column removed >99% of immunoreactive material from the initial concentrated conditioned medium. Recovery of truncated FcγRIIβ from the concentrated conditioned medium was >85%. The purified protein showed the same broad electrophoretic mobility on SDS-PAGE (see lanes a and b, Fig. 13A in Chapter Three) and has the same activity with rabbit anti-FcγR antibodies in immunoblotting (Fig. 5B) as the immunoreactive material in the conditioned medium. Based on amino acid composition with 4 tryptophan and 8 tyrosine residues (Ravetch *et al.*, 1986), the truncated FcγRIIβ should have an ϵ_{280nm} = 33,000 $M^{-1}cm^{-1}$ (Mihalyi, 1970). Given a M_r of 31,000 for the truncated FcγRIIβ, the yield of purified protein by measuring OD at 280 nm is 2.5 μg/ml conditioned medium, in good agreement with the ELISA assay titration.

C. *Expression of Recombinant FcγRIIβ in Insect Cells.* The baculovirus AcNPV has been shown to be suitable as an expression vector for production in cultured insect cells of a number of proteins from cloned genes (Miller, 1988; Miller *et al.*, 1986; Luckow and Summers, 1988). We wanted to examine whether high level production of soluble murine FcγRIIβ could be achieved by using this expression system. Since N-linked oligosaccharides made by insect cells are of the high mannose type, and are not sialylated, FcγR synthesized in insect cells might be useful for the studies on the importance of complex forms of oligosaccharides.

Because the truncated FcγRIIβ cDNA insert in plasmid pFcγRIIβ-19 is flanked by two EcoRI restriction sites (see Fig. 1), simple steps were taken to switch the insert from

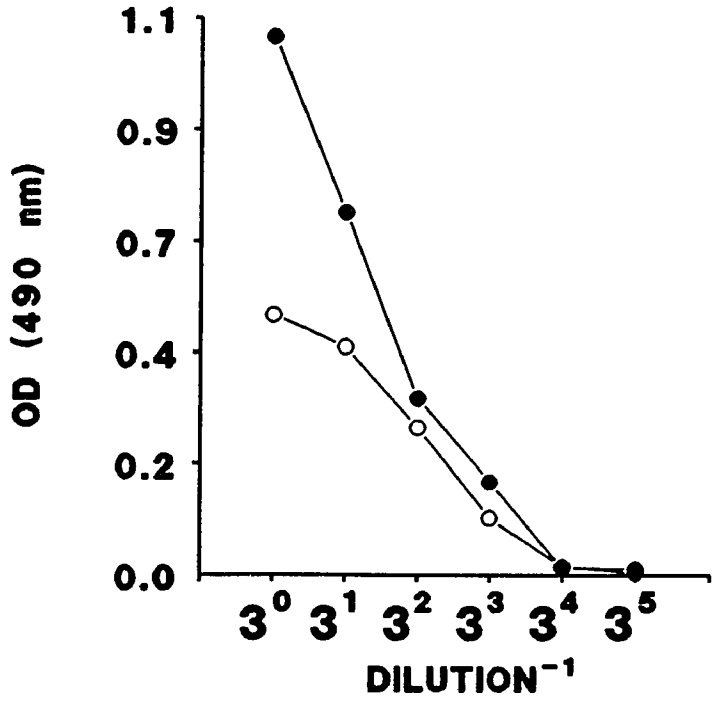

Figure 7. Production of the truncated FcγRIIβ from Sf9 cells infected by the recombinant virus VLFcγR4p3.

Confluent Sf9 cells in tissue culture flask were infected with VL234p3 at a multiplicity of infection of 3 and incubated for 1 h. The medium was exchanged and the cells were incubated for 3 days. The conditioned medium was diluted 100-fold and then serial 3-fold dilutions were assayed by ELISA for tFcγRIIβ. For comparison, the medium conditioned by D1959 was titrated in the same range of dilutions. (●), infected Sf9; (○), D1959.

pFcγRIIβ-19 into the EcoRI site of pVL1393, as detailed in the Material and Methods section. The resulting transfer vector pVLFcR23 in a 100-fold molar excess was mixed with purified intact AcNPV DNA and then co-precipitated with calcium phosphate. The homologous recombination between AcNPV DNA and pVLFcR23 in transfected Sf9 cells resulted in the insertion of truncated FcγRIIβ DNA into the locus of the virus polyhedrin gene. The recombinant viruses thus produced are able to infect cells and to multiply normally. However, they do not form occlusions in infected cells and have a distinctive plaque morphology (Smith et al., 1983a,b). By visual screening for plaques formed by viruses that did not produce occlusions (O^- mutants), putative recombinant viruses were identified, isolated and purified.

As was the case with CHO cells, we expected the truncated FcγRIIβ synthesized in infected cells to be secreted into the cell culture medium. The conditioned media from the cells infected with isolated O^- recombinants were assayed by ELISA. 10 of 16 O^- viruses tested directed production of the soluble FcγRIIβ upon infection. Restriction mapping of purifiedextracellular viral DNA of one of the recombinant viruses revealed an altered pattern of the appropriate EcoRI DNA fragment containing polyhedrin gene (data not shown). A second round of plaque purification was performed to eliminate all wild type virus. The maximum production of the truncated FcγRIIβ in Sf9 cells was reached 3 days after a three MOI (multiplicity of infection) with VLFcγR4p3. The amount of FcγRβ in the supernatant, quantified by ELISA, was comparable to that obtained from the CHO transfectant, D1959 (Fig. 7), estimated at about 10 μg/ml. However, shapes of the titration curves were different between these two species of

tFcγRIIβ. The tFcγR from Sf9 cells apparently bound much better to the microtiter plate coated with rabbit anti-FcγR antibodies. The protein was purified from conditioned medium by ammonium sulfate precipitation, concanavalin A-Sepharose affinity chromatography and IgG-Sepharose affinity chromatography. Like the truncated FcγRIIβ from D1959, the purified protein produced in insect cells also showed broad band on SDS-PAGE (data not shown) with an apparent Mr 30,000-35,000. The yield was about 1 μg/ml.

D. *Expression of tFcγR in E. coli.* Another attractive approach of high level production of proteins is expression of cloned genes in *E. coli* stains. In some cases, foreign proteins expressed in *E. coli* comprised up to 30% of the total proteins produced by each cell (Masui *et al.*, 1984). A number of *E. coli* stains have very high transformation efficiency, which makes manipulation of gene products relatively easy and inexpensive.

The fact that glycosylation is not required for the binding of moFcγRII with IgG (Results and Discussion in next chapter) led us try to express the truncated FcγR in *E. coli*. The expression vector we chose for these studies is the pINIII-ompA set (Ghrayed *et al.*, 1984) of secretion cloning vectors, in which a cloned gene product is produced in large amounts and secreted across the cytoplasmic membrane into the periplasmic space. The oxidizing environment of the periplasm should allow the formation of disulfide bonds, which might be essential for the correct conformation and full biological activity of the protein.

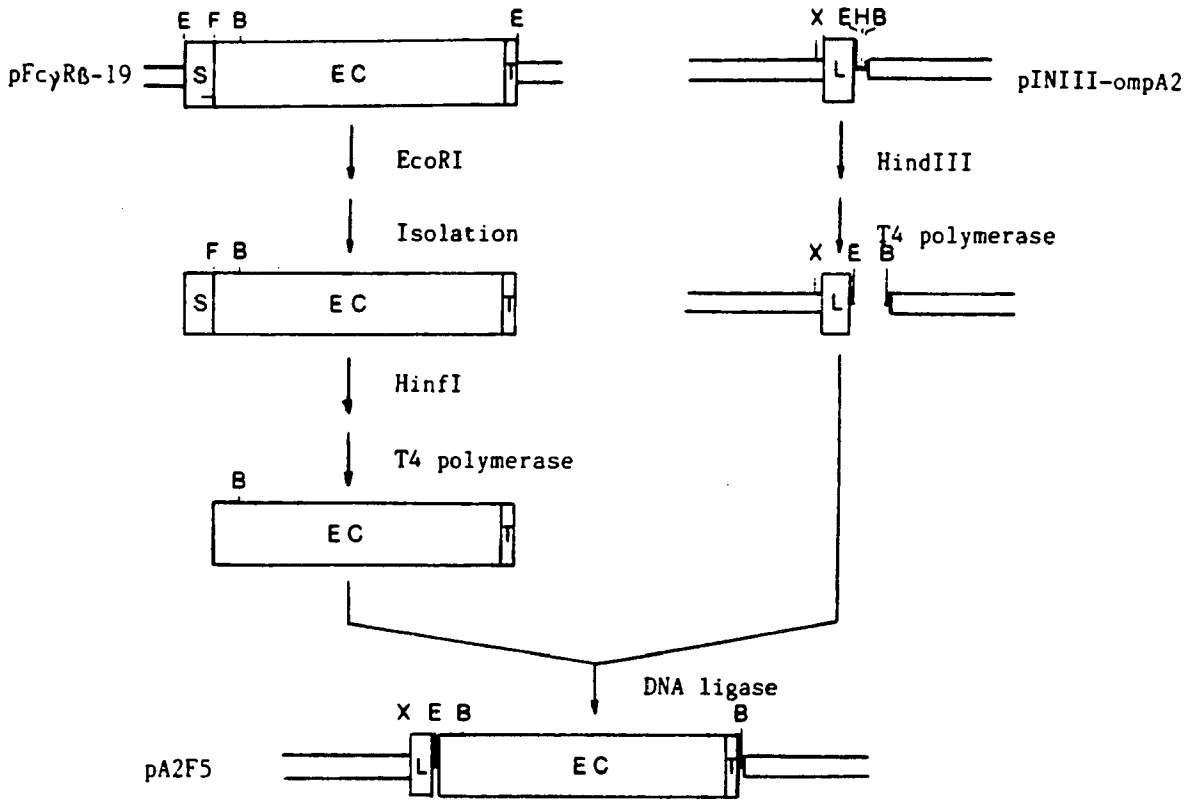

Figure 8. Construction of *E. coli* secretion expression vector for tFcγRIIβ.

The redion containing the tFcγRII insert in pFcγRβ-19 and the region encoding the ompA leader peptide in pINIII-ompA2 are illustrated on the top (S for FcγRIIβ signal peptide encoding sequence, EC for extracellular domains, T for universal terminator ligated to the 3'-end of EC, and L for the ompA leader peptide coding sequence). The resulting plasmid with the insert in the correct orientation is at the bottom. Plasmid pA3F6 was constructed in same way except that the tFcγRIIβ DNA was inserted into the EcoRI site of pINIII-ompA3. The abbreviations for restriction enzymes are: E for EcoRI, B for BamHI, F for HinfI, H for HindIII,and X for XbaI.

To express the truncated FcγRII in *E. coli*, the DNA insert containing the sequences encoding the putative leader peptide, tFcγRII, and the terminator in pFcγRβ-19 was isolated. A HinfI digestion and T4 polymerase treatment resulted in the deletion of the sequence coding for the leader peptide. The resulting blunt-end DNA fragment was isolated and ligated into the hindIII site of pINIII-ompA2 or the EcoRI site of pINIII-ompA3, so as to be fused with the E. coli ompA (outer membrane protein A) leader sequence in frame, as shown schematically in Fig. 8. Two plasmids, pA2F5 and pA3F6 with the insert in the correct orientation were isolated and used for expression experiments.

We transformed of the tFcγR constructs into *E. coli* strains. As expected, only the transformants harboring the plasmids with the insert in the correct orientation expressed the protein with 2.4G2 epitope (Fig. 9A). Unexpectedly, the majority of 2.4G2 activity was detected in the medium and only very little in the periplasmic space (Fig. 4B) and cytoplasm. The secretion of target protein into medium using ompA or pelB (pectate lyase) leader sequences was also observed for active subtilisin E by Takagi et al. (1988) and for an active chimeric antibody fragment by Better et al. (1988). It seems likely that the final location of a secreted protein is dependent not only on the leader sequence but also on the structure of the protein itself.

The expression of a foreign protein with pINIII-ompA should be induced by isopropyl-1-thio-β-D-galactopyranoside (IPTG) because the target gene is under the control of lac UV5 promoter-operator. IPTG is commonly used at a concentration of 2 mM to induce proteins under lac promoter control. However, we observed that the

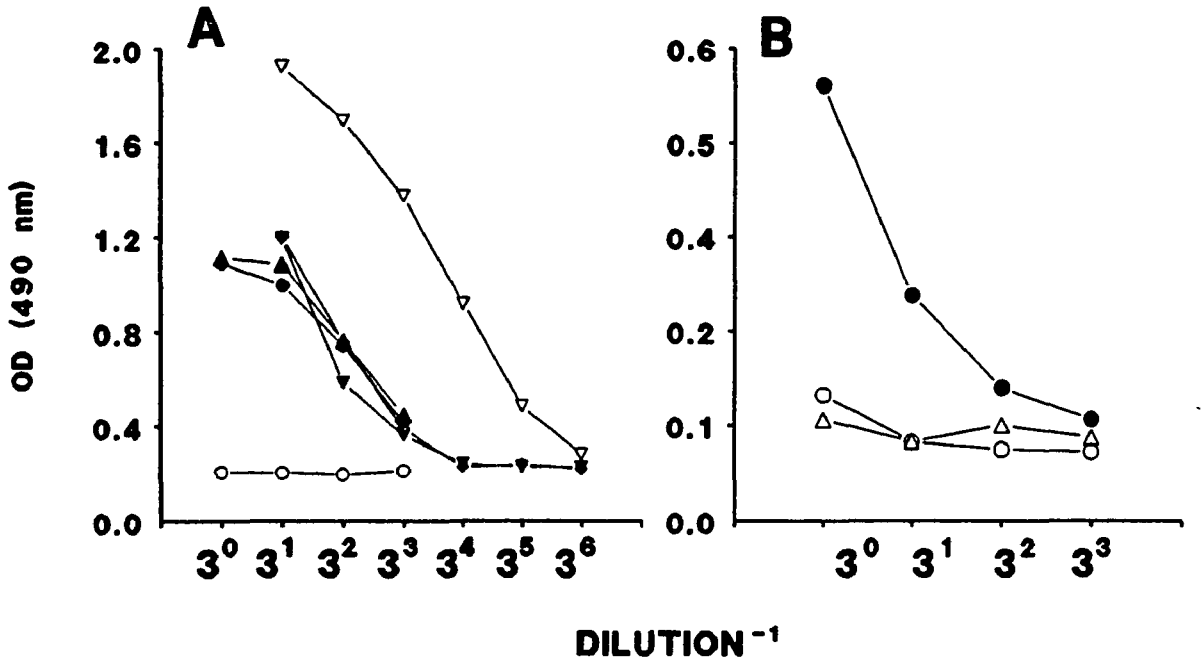

Figure 9. Production and secretion of the truncated FcγRIIβ in *E. coli* strains.

A. JMA2F54, JMA3F61 and JMA3F31 were JM109 transformants harboring plasmids pA2F5, pA3F6 and pA3F3, respectively. The plasmid pA3F3 is a pINIII-ompA3 derivative with the insert DNA in the anti-sense orientation, and pA2F5 and pA3F6 are described in the text. WA2F51 and WA3F62 were W620 strain harboring pA2F5 and pA3F6, respectively. These strains were cultured in 2xTY medium for 20 h. The E. coli cells were removed by centrifugation, the supernatants were diluted to 10 (JM109 strains) or 30 (W620 strains) folds, and then a series of 3-fold dilutions were assayed for tFcγRII by ELISA. The medium conditioned by D1959 was included as calibration. (▲), JMA2F54; (●), JMA3F61; (○), JMA3F31; (▼), WA2F51; (◆), WA3F62; (▽), D1959.

B. WA3F62 were cultured in 1 ml of 2xTY for 20 h. Medium and cells were separated by centrifugation. The periplasmic preparations of the

bacteria were made by osmotic shock (Neu and Heppel, 1965; Koshland and botstein, 1980). Cell pellets were resuspended in ice-cold ST solution (20% sucrose and 10 mM tris-HCl, pH 7.5) and cenfrifuged again. The supernatant fluid was quickly removed and saved as sucrose-tris fraction. The pellet was rapidly resuspended by vigorous agitation in cold distill water, incubated on ice for 10 minutes, and centrifuged again. The supernatant was removed and saved as periplasmic fraction. Both sucrose-tris fraction (▵) and periplasmic fraction(○) were adjusted to 1 ml and along with the conditioned medium (●) were diluted 100-fold and then 3-fold dilutions were assayed by ELISA for tFcγRII.

expression of the truncated FcγR was induced at only very low IPTG concentration, 3-5 μM, and 10 μM of IPTG in the medium inhibited the expression significantly. The expression of tFcγR in a rich medium was constitutive, was as described for a human growth hormone-pINIII-ompA construct expressed in *E. coli* (Hsiung *et al.*, 1986), possibly due to the presence in rich medium of enough lactose or other inducer of the *lac* promoter. It is likely that the truncated FcγR is toxic to *E. coli* cells since the strains expressing tFcγR grow much slower than control strains. In addition, growth of tFcγR-expressing bacteria resulted in the selection of more rapidly growing variants, which do not secrete tFcγR. We also transformed the pA2F5 and pA3F6 plasmids into several strains of *E. coli*, including W620, JM109, MM294, and BNN103(lon$^-$), and got similar results.

The 2.4G2 reactive material was isolated by IgG-Sepharose affinity chromatography and showed an apparent Mr 20,000 in SDS-PAGE and immunoblotting

(data not shown), consistent with the size of deglycosylated CHO tFcγRII. However, the preparation by one cycle of affinity chromatography was not pure probably due to the binding of some bacterial proteins to mouse antibody. By manipulating culture conditions, we have achieved expression of the truncated FcγR from *E. coli* at the level of 0.5-1 μg/ml in bacterial medium. Although the yield is not as high as we had hoped, this system can certainly be used for a variety of subsequent studies. Sufficient amount of truncated FcγR can be purified for biochemical analysis. This system can also be used for mutagenesis studies, since, in contrast to the eukaryotic CHO expression system, the expression is good without time-consuming amplification and cloning steps.

E. *Identification and Isolation of tFcγRII-secreting E. coli Colonies.* Specific antibodies can be used to detect and isolate protein-encoding genes from large recombinant DNA libraries expressed in *E. coli* colonies (Kemp and Cowman, 1981; Broome and Gilbert, 1978) or phage plaques (Young and Davis, 1983). Transformed *E. coli* colonies that secrete tFcγR differ from conventional colonies and plaques in that the target protein is not localized within the cells. We have developed a simple protocol to screen the cells secreting tFcγR with mAbs.

Briefly, to screen the colonies secreting tFcγR, a nitrocellulose paper saturated with IPTG is overlaid on the colonies and incubated overnight. The nitrocellulose paper is then blocked with BSA and incubated with medium conditioned by the 2.4G2 hybridoma. Although 2.4G2 does not recognize moFcγRII following reduction and SDS

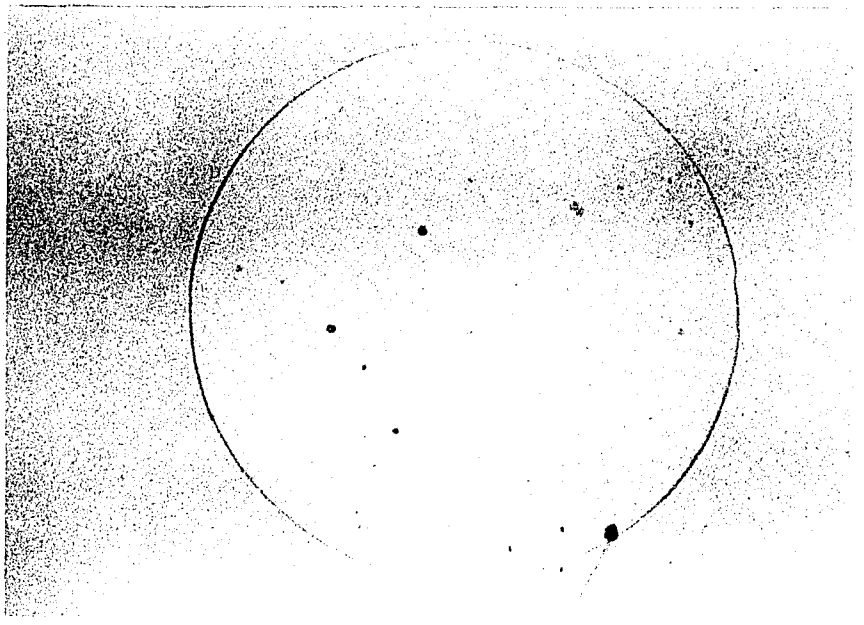

Figure 10. Screening of tFcγRIIβ-secreting colonies with mAb 2.4G2. W620 was transformed with pA3F6 and plated on M9 agar plate supplemented with ampicillin (100 μg/ml). After overnight incubation, a nitrocellulose filter treated with 10 mM IPTG was lifted from the plate and processed as described in text.

denaturation in Western blotting on nitrocellulose paper, mAb 2.4G2 does react with the native tFcγR on the nitrocellulose paper lifted from the bacterial agar plates. The bound 2.4G2 on the blot can be detected and visualized with biotinylated goat anti-rat IgG antibody followed with streptavidin-horse radish peroxidase (HRP), as shown in Fig. 10. This result demonstrated a potential method for structural analysis of FcγR by using the recombinant DNA techonlogy and monoclonal antibodies. For instance, expression of randam mutaions of FcγR molecule could be ralatively easyly achieved with *E. coli* expression secretion vectors. Screening for the reduced or lost activity of the mutated FcγR molecule to anti-FcγR mAbs and mapping the lost epitopes by DNA sequencing could result in useful structural information about native FcγR. Peterson and Seed (1987, 1988) succeeded in determining the ligand binding site on CD2 and the HIV binfing site on CD4 with this kind of mapping.

Summary

We have isolated a recombinant secreted FcγRIIβ molecule by deletion of the transmembrane and cytoplasmic domain encoding sequences from a FcγRIIβ2 cDNA clone, and insertion of the truncated cDNA into a eukaryotic expression vector, pcEXV-3. To express and amplify the production of the truncated FcγRIIβ molecule, we transfected the truncated cDNA plasmid into a dihydrofolate reductase-minus CHO cell line along with a *dhfr* minigene, and amplified the gene products with methotrexate. The resulting cell line secretes 2-3 μg/ml/24 h of truncated FcγRIIβ. The secreted product

showed an apparent M_r of 30,000-35,000 on SDS-PAGE and the immunoreactivity with anti-FcγR antibodies on immunoblots. The truncated FcγRIIβ was purified to homogeneity by affinity chromatography on IgG-Sepharose. The availability of the truncated FcγRIIβ in mg quantities should facilitate further analysis of structure and function of these receptors.

We also constructed a recombinant baculovirus, VLFcγR4p3, that contains the cDNA sequence for the tFcγRII. The Sf9 insect cells infected by the recombinant virus are capable of producing and secreting the truncated FcγRII at a high level. This provides an alternative system to produce large quantity of tFcγRII.

This eukaryotic protein was also expressed in *E. coli* by using the pINIII-ompA vectors, which directed the secretion of tFcγRII. A screening protocol for the colonies secreting tFcγRII was tested by using anti-FcγRII mAb 2.4G2. This procedure might be valuable for screening mutant FcγRs generated by site-specific or random mutagenesis.

CHAPTER THREE

CHARACTERIZATION OF THE TRUNCATED MOUSE Fcγ RECEPTOR

Introduction

Cloning of the cDNAs of murine (Ravetch *et al.*, 1986; Lewis *et al.*, 1986; Hibbs *et al.*, 1986; Hogarph *et al.*, 1987; Sears *et al.*, 1990) and human FcγRs (Allen and Seed, 1989; Stuart *et al.*, 1987; Stengelin *et al.*, 1988; Hibbs *et al.*, 1988; Seki, 1989; Stuart *et al.*, 1989; Brooks *et al.*, 1989; Simmons and Seed, 1988; Scallon *et al.*, 1989; Ueda *et al.*, 1989, Ravetch and Perussia, 1989; Edberg *et al.*, 1989; Selvaraj *et al.*, 1989; Peltz *et al.*, 1989) has shed light on the structural features of this group of proteins. It is evident that the common binding capacity of FcγRs results from the structural similarity in the extracellular domains. The sequences of the leukocyte FcγRs in this region are highly homologous to each other with 30-90% amino acid identity, including conserved cysteine residues. Moreover, these domains bear significant homology to other members of the immunoglobulin superfamily, with the most striking similarity found between the β2 domain of mouse class II MHC protein Eβ and the most distal N-terminal domain of the moFcγRII. Four or more potential N-linked glycosylation sites in each receptor were also predicted from sequencing data. The structural elements important to the biochemical and physiological properties of FcγRs need to be elucidated.

Having succeeded in obtaining high level expression and secretion of the truncated form of moFcγRII (tFcγRII) in cultured cells. we further studied its immunological

properties. The recombinant receptor has similar binding activity and specificity as intact FcγRII, providing us a simplified system to investigate the nature of interaction between FcγR and IgG molecules. The availability of large quantities of purified receptor has facilitated the examination of biochemical properties and the ternary structure of the protein, and allowed us to confirm the membrane orientation and to map an epitope recognized by an anti-FcγR mAb, 6B7C.

Materials and Methods

A. *Monoclonal Antibodies.* The anti-dinitrophenyl (anti-DNP) mAbs DHK10.12 (IgG1b) and DHK109.3 (IgG1) were a generous gift of Dr. Schlessinger (Washington University, St. Louis, MO). U12.5 (IgG2b), U7.27 (IgG2a) and U7.6 (IgG1) were the kind gift of Dr. Eschar (Weitzmann Institute, Israel). The myeloma protein MPC-11 (IgG2b) was a gift of Dr. Eisen (MIT, Cambridge, MA). The anti-DNP mAbs were purified from conditioned medium by affinity chromatography on a trinitrophenyl$_{25}$BSA Sepharose-4B column, and the bound mAb was eluted with 0.1 M dinitrophenol, 0.1 M Tris-HCl, pH 8.0. The free dinitrophenol was removed by gel filtration on Sephadex G-25 (Pharmacia) and the bound dinitrophenol removed by passing the antibody solution through a Dowex-1 column.

B. *Assay of Binding of Immune Complexes by Truncated FcγRIIβ.* Crystallized bovine serum albumin (BSA) was derivatized with dinitrophenyl groups by reaction with

dinitrobenzene sulfonic acid (Pierce, Rockford, IL) in 2% Na_2CO_3 and after gel filtration the extent of substitution was determined as described previously (Unkeless, 1977). The $DNP_{25}BSA$ (50 μg) was then radiolabeled with 1 mCi of carrier free Na[^{125}I] (Amersham) using tubes coated with 1 μg of iodogen (Pierce) (Fraker and Speck, 1978). Unincorporated ^{125}I was removed by gel filtration on a G-25M column. The labeled protein had the specific activity of 1.25 x 10^5 cpm/μl. Flexible 96 well U-bottom plates (Micro-2000, Dynatech) were coated with purified truncated FcγRIIβ (5 μg/ml in PBS) at 4°C overnight and the wells quenched by 3% non-fat dry milk in PBS at room temperature for 2 h. Immune complexes were formed by incubation of serial two-fold dilutions of different anti-DNP mAbs with [^{125}I]-$DNP_{25}BSA$ in 0.2x PBS at room temperature for 2 h and the complexes were then transferred to the FcγRIIβ-coated wells. After further incubation at room temperature for 2 h, the plates were rapidly rinsed in 10 mM phosphate buffer (pH 7.5). The wells were cut out and radioactivity was measured by gamma counter (1217 RiaGamma, LKB-Wallac, Finland).

C. *Disulfide Analysis.* Ellman's reagent (Ellman, 1959), 5,5'-dithio-bis(2-nitrobenzoic acid) (Calbiochem, Behring Diagnostics, La Jolla, CA) was used to titrate the number of sulfhydryl groups in the truncated FcγRIIβ as described by Anderson and Wetlaufer (1975) for peptides. Protein dissolved in 0.1 M acetic acid and 0.2% SDS (0.25 ml) was mixed with an equal volume of 6 N NaOH and boiled for 5 min or incubated at 37° overnight. The alkaline solution was neutralized by the addition of 0.5 ml of 6 N H_3PO_4 containing 2 mM EDTA. 100 μl of the Ellman's reagent (1 mg/ml in

20 mM sodium acetate buffer, pH 5.5, with 1 mM EDTA) was then added into the neutralized solution, and the absorbance at 412 nm, due to the release of 2-nitro-5-thiobenzoic acid, was measured. The concentration of free sulfhydryl groups was calculated from the extinction coefficient of 13,600/M/cm (Ellman, 1959). Crystallized BSA was used to determine the yield of free -SH groups per disulfide bond following alkaline cleavage. The yield of -SH/S-S experimentally determined for BSA (1.31, see Table II) agrees well with the value determined for model peptides, e.g. 1.34 for oxidized glutathione (Anderson and Wetlaufer, 1975).

D. *Enzymetic Deglycosylation.* Endoglycosidase F/N-glycosidase F (endo F) and neuraminidase were purchased from Boehringer Mannheim Biochemicals. Purified tFcγRIIβ was treated with either endo F or neuraminidase, or both. The incubations of tFcγRIIβ with these enzymes were performed at 37°C in 50 mM sodium acetate buffer, pH 5.5, with 10 mM $CaCl_2$ and 10 mM EDTA for 18 h (Moyle *et al.*, 1975; Freeze and Wolgast, 1986).

E. *Inhibition of Glycosylation, Biosynthetic Labeling and Immunoprecipitation.* Antibodies and crystallized BSA were immobilized on CNBr-activated Sepharose 4B (Pharmacia) under the conditions recommended by the manufacturer. Soluble FcγR secreted by the CHO cell line was biosynthetically labeled as described in the previous chapter. D1959 cells were seeded in 60 mm tissue culture dish at 10^5 cells/plate and incubated overnight. The cell monolayers were then washed and incubated in fresh

methionine-free αMEM (Flow) supplemented with 10% dialyzed FCS, [^{35}S]-methionine, and different concentrations of tunicamycin for 5 h. The conditioned media were collected and incubated at room temperature with mAb 2.4G2- or U7.6-coupled Sepharose beads or with BSA-coupled beads as the background control. The Sepharose beads were washed by centrifugation in PBS. The bound proteins were dissociated from the Sepharose matrix by boiling in electrophoresis sample buffer and analyzed on SDS-PAGE.

F. *Crosslinking of tFcγRIIβ with Heterobifunctional Crosslinker.* The cleavable, photoreactive, heterobifunctional crosslinker, sulfosuccinimidyl-2-(*p*-azidosalicylamido)-ethyl-1,3'-dithiopropinate (SASD) was from Pierce. SASD and protein conjugates were prepared according to the manufacturers recommended procedure. 550 nmol of SASD in 0.2 ml of PBS, pH 7.4, was first iodinated with 1 mCi of carrier-free Na^{125}I (13.5 mCi/μg, Amersham) in an Iodo-Gen (Pierce) coated test tube at room temperature for 30 sec. The reaction mixture (0.1 ml) was then transferred into the tube containing 5 nmol of protein in 0.2 ml of 0.1 M sodium borate buffer, pH 8.4 and the reaction was allowed to proceed for 30 min at room temperature. Free SASD and Na^{125}I were removed by Excellulose GF-5 desalting column (Pierce) equilibrated with PBS. The eluted protein fraction had specific activity of 0.5-1 x 10^5 cpm/μl and protein concentration of 6 μM. The crosslinking was induced by irradiating the conjugated proteins with long wave UV light (366 nm) from a handheld long wavelength UV source (UVP Inc., San Gabriel, CA) and analyzed by reducing and non-reducing SDS-PAGE.

G. *Non-denaturing PAGE.* The procedure for determining the molecular weight of tFcγRIIβ in a nondenaturing polyacrylamide gel system was a modification of Davis (1964) and Bryan (1977). Marker proteins and tFcγRIIβ were electrophoresed on a set of gels with polyacrylamide concentration of 6%, 7%, 8%, 9% and 10% and stained with Coomassie brilliant blue. The electrophoretic mobility, R_f of each marker protein species relative to the tracking dye was measured and 100Log(R_f x 100) was plotted against the gel concentrations. The slope of such a plot is the Retardation Coefficient (K_r). The logarithm of the negative K_r is then plotted against the logarithm of the molecular weight of each protein resulting in a linear plot. The molecular weight of tFcγRIIβ was determined on the plot from its K_r. The marker proteins were α-lactalbumin (14.2 kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa), BSA monomer (66 kDa), BSA dimer (132 kDa).

H. *Sucrose Density Gradient Ultracentrifugation.* Centrifugation was performed by using an SW41 swinging bucket rotor (Beckman) at 37,000 rpm for 20 h at 18°C. Linear gradients (11 ml, 5-30% sucrose) were prepared in PBS. Purified tFcγRIIβ, marker proteins, lactalbumin and BSA, or the mixture of them were applied to each gradient in a total volume of 100 μl. After centrifugation, each gradient was fractionated into 0.2 ml fractions. Protein was determined by the Coomassie brilliant blue G (Bradford, 1976; Spector, 1978).

I. *Isoelectric Focusing (IEF).* IEF was performed on a horizontal electrophoresis

apparatus (BioRad) with a Servalyt IEF kit and Servalyt 100 μ precasted gels (Serva Feinbiochemica, Heidelberg, Germany) following the procedures recommended by the manufacturers.

J. *Peptide Synthesis and Conjugation.* The peptide ITVQGPKSSRSLPVL, residues 169-183 of FcγRIIβ was synthesized stepwise by solid phase methods on a Biosearch 9500 automated peptide synthesizer. A cysteamine residue was attached to the resin through a thioether linkage before start of automated synthesis (Glass and Buku, 1987). After deblocking, this results in a carboxyl-terminal sulfhydryl group for derivatization. The peptide was purified by reverse phase chromatography on a Vydac C-18 HPLC column. Amino acid analysis after hydrolysis was consistent with the sequence.

Since the peptide contained a free sulfhydryl group, the bifunctional reagent sulfo-m-maleimidobenzoyl-N-hydroxysuccinimide ester (sulfo-MBS) (Pierce) was used to make the peptide-protein conjugate, as described by Youle and Neville (Youle and Neville, 1980). Briefly, 5 mg of ovalbumin (OVA) was dissolved in 0.5 ml of sodium phosphate buffer (10 mM, pH 7.5) and mixed with 0.5 mg of sulfo-MBS in 10 μl of dimethylformamide by vortexing. The molar ratio of sulfo-MBS to ovalbumin was 8.7:1. After incubation at room temperature for 30 min, the acylated-OVA was desalted over a Sephadex G-25M column (Pharmacia) in 10 mM phosphate buffer (pH 7.5) with 15 mM NaCl. The protein was then incubated with 1 mg of peptide in 2 ml of the same buffer at room temperature for 2 h. The molar ratio of peptide to OVA was 5.2:1. The

peptide-OVA conjugate, ITVQGPKSSRSLPVL-OVA, was then filtered through a small Sephadex G-25M column.

K. *Immunoassay for the Synthetic Peptide.* A modification of the monoclonal sandwich radioimmunoassay detailed in previous chapter was used to analyze the binding of the rat anti-FcγR mAbs 2.4G2 and 6B7C to the synthetic peptide-ovalbumin conjugate. Serial dilutions of ovalbumin and peptide-conjugated ovalbumin in sodium carbonate buffer, pH 8.5, were absorbed onto 96-well microtitration plate by incubation overnight at 4°C. After the wells were blocked with 3% BSA in PBS, the rat anti-FcγR mAb 2.4G2 or 6B7C (5 μg/ml in PBS) were added and the plate was incubated at room temperature for 2 h. Binding of the peptide with mAbs was analyzed by sequential addition of biotinylated goat anti-rat IgG antibody (0.5 μg/ml) and streptavidin-HRP (0.5 μg/ml), followed by developing with o-phenylenediamine and H_2O_2.

L. *Crystallization.* For crystallization, large batches of purified tFcγRII, 20-30 mg, were treated with either endo F or neuraminidase or both as was detailed in the section D. The enzyme-digested tFcγRII was repurified by affinity chromatography on IgG-Sepharose 4B column as was described in the section I of Materials and Methods in the previous chapter. Purified protein was then dialyzed against 10 mM cacodylate buffer, pH 8.0 and concentrated to 7-15 mg/ml by use of Centricon 10 microconcentrators. The crystallization of tFcγRII was carried out by the vapor diffusion method in 10 μl hanging drops (Reid *et al.*, 1973; Wlodawer and Hodgson, 1975)

equilibrated with 8-15% polyethylenglycol (PEG) 3350 (J.T.Baker Inc., Phillipsburg, NJ) in 20-50 mM cacodylate buffer, pH 7.4-7.6, with 0-50 mM EDTA and 0-50 mM $CaCl_2$. Briefly, about 1 ml of equilibration solutions with varying combinations of PEG 3350, $CaCl_2$, EDTA and cacodylate were prepared in the wells of 24-well tissue culture plates (Linbro, Flow Laboratories). A 5 μl aliquot of the concentrated protein solution was mixed, on a siliconized microscope cover glass slide, with an equal volume of equilibration solution from a well. The glass slide was then inverted and placed over on the well. The border between the glass and the well was sealed with high vacuum grease, resulting in a closed chamber. The crystals were grown at room temperature.

Results and Discussion

A. *The Biological Activity of the Truncated FcγRIIβ.* Murine FcγRII was formerly described as a low affinity, trypsin-resistant receptor with specificity for aggregated IgG1, IgG2a and IgG2b. We wanted to know whether the truncated FcγRIIβ binds IgG. The purification of truncated FcγRIIβ by affinity chromatography on IgG2b-Sepharose demonstrated the retention of specificity by the recombinant truncated receptor. To determine the isotype specificity of the truncated FcγRIIβ, we examined the binding of [^{125}I]-DNP_{25}BSA-anti-DNP mAb complexes to truncated FcγRIIβ absorbed to microtiter plates. The assay was done at low ionic strength, since this had been reported to enhance the binding of low affinity FcγR (Jones *et al.*, 1985). Non-specific binding of labeled DNP_{25}BSA to the plate was very low. No binding was seen for the control mAb,

MPC-11, a myeloma protein (Fig. 11D). The immune complexes formed from IgG1 (U7.6 and DHK 109.3), IgG2a (U7.6) and IgG2b (DHK10.12) anti-DNP mAbs bound to the truncated FcγRIIβ (Fig. 11A, B and C). The binding of IgG1 antibody-antigen complexes to the truncated FcγRIIβ was significantly stronger than that for IgG2a and IgG2b complexes. However, no binding was observed for the IgG2b anti-DNP-mAb U12.5, which may be due to a low avidity of U12.5 for DNP. From these results, we conclude that the affinity of the truncated FcγRIIβ for murine IgG isotypes as immune complexes is IgG1 > IgG2b=IgG2a. This conclusion, however, assumes that these anti-DNP mAbs all form equal size complexes with DNP-BSA. It can not be excluded that the IgG1 mAb U7.6 might have a higher affinity to DNP-BSA or form larger complexes than other mAbs used in these assays do.

With the same assay for binding of immune complexes, we examined the effect of pH on truncated FcγRIIβ activity by using the IgG1 anti-DNP mAb U7.6 and [^{125}I]-DNP_{25}BSA. There was more binding of the labeled DNP_{25}BSA in immune complexes at more acid pH (Fig. 12). This differs from a previous report by Mellman and Unkeless (1980) in which the binding of labeled intact FcγR, isolated from macrophages, to rabbit immune complexes was examined, and found to decrease sharply at pH 5.0. The failure of FcγR internalized with immune complexes to recycle to the cell surface, but rather to be digested in the lysosomal compartment (Mellman *et al.*, 1983; Mellman and Plutner, 1984), is more easily explained by the failure of the receptor-ligand complex to dissociate at acidic pH. The intact FcγR in the absence of detergent might exist in a protein micelle, due to aggregation of the hydrophobic transmembrane

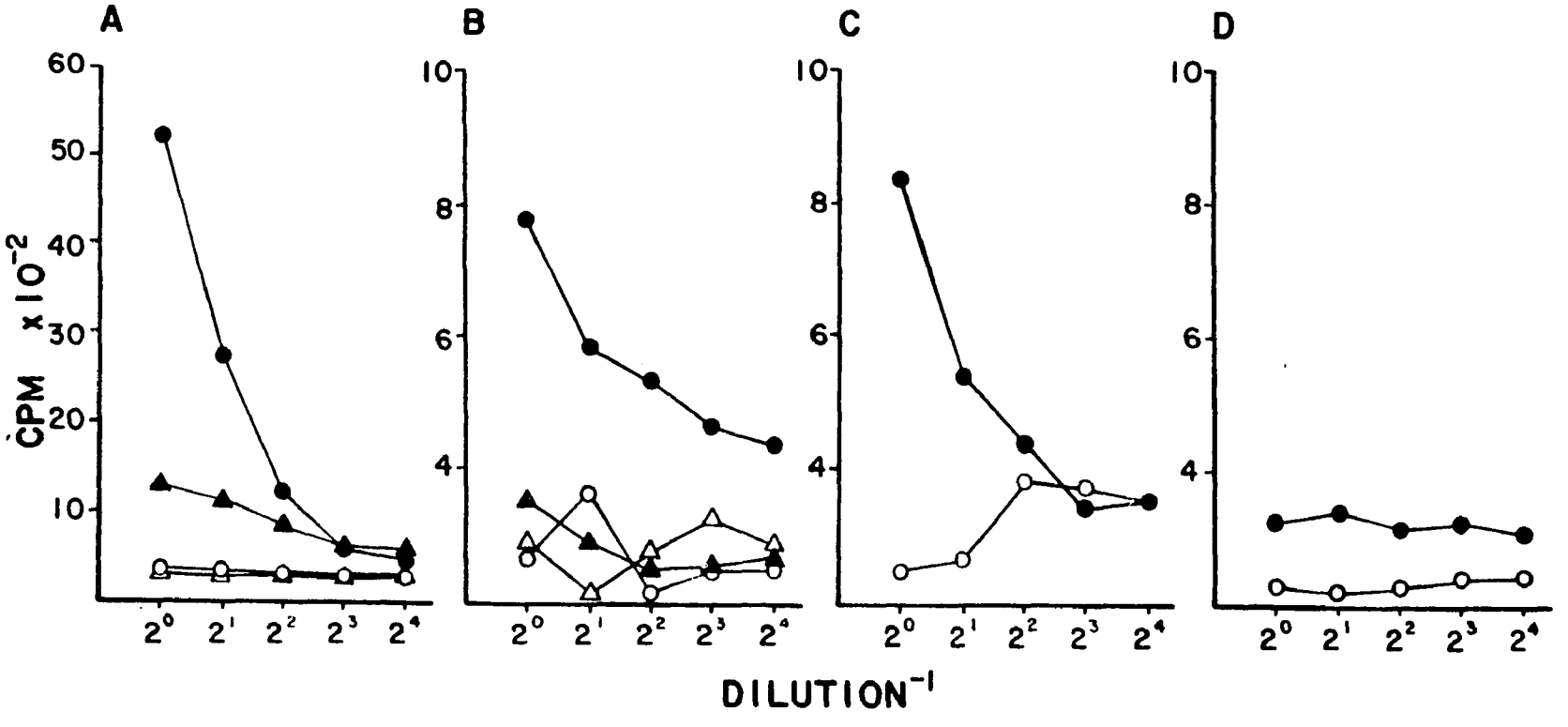

Figure 11. Binding of different subclasses of IgG immune complexes by purified truncated FcγRII.
Immune complexes were formed by incubation of 50 μl aliquots of serial dilutions of anti-DNP mAbs, beginning at 10 μg/ml of IgG, with 50 μl of [^{125}I]-DNP$_{25}$BSA (1 μg/ml, 1.2x10^6 cpm/ng). The immune complexes were then transferred into control wells or wells coated with truncated FcγRII. After 2 h at room temperature, the wells were rinsed to remove unbound complexes, and the wells were cut out for assay of bound [^{125}I]-DNP$_{25}$BSA. Open symbols: truncated FcγRII coated wells; filled symbols: control wells. A, IgG1, (○) U7.6, (△) DHK 109.3; B, IgG2b, (○) DHK 10.12, (△) U12.5; C, IgG2a, U7.27; D, IgG2b, MPC-11.

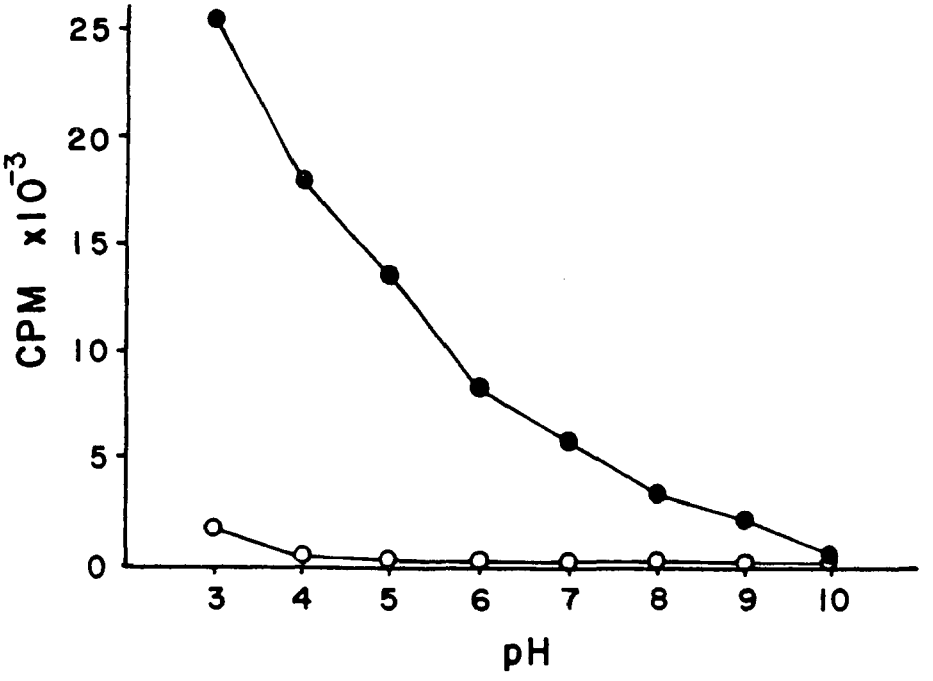

Figure 12. Binding by truncated FcγRII of IgG1 U7.6-DNP$_{25}$BSA immune complexes as a function of pH.

Citrate-phosphate buffer was used between pH 3-5, sodium phosphate buffer between pH 6-8, and glycine buffer between pH 9-10 (Gomori, 1955). All buffers were adjusted to 10 mM and contained 0.1 M NaCl. The immune complex was prepared as described in Fig. 11 with a final concentration of 5 μg/ml U7.6 and 0.5 μg/ml [^{125}I]-DNP$_{25}$BSA. The immune complex was diluted 10 fold with the different buffers before transfer into the truncated FcγRII coated wells and control wells. (●), truncated FcγRII coated wells; (○), control wells.

domains. It is possible that this micelle at low pH dissociates, resulting in a loss of cooperative binding necessary for binding to the immune complexes. This consideration did not apply in the present experiments in which the truncated FcγRIIβ was adsorbed onto the plastic surface.

B. *Determination of the Number of Disulfhydryl Groups in the Truncated FcγRIIβ.* From the cDNA sequencing data, it was predicted that the external domain of the moFcγRII has four cysteine residues, two located in each immunoglobulin-like domain (Ravetch *et al.*, 1986; Lewis *et al.*, 1986; Hogarph *et al.*, 1987). The thiol groups of these cysteine residues could form two disulfide bridges. We performed titrations using Ellman's reagent, 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) to determine the number of free sulfhydryl groups and found that truncated FcγRIIβ has no free sulfhydryl groups (Table II). The procedure is based on the reaction of the thiol with DTNB to give the mixed disulfide and 2-nitro-5-thiobenzoic acid (TNB), which is quantified by the absorbance of its dianion form (TNB^{2-}) at 412 nm (Riddles et al., 1979). Alkaline hydrolysis of BSA resulted in 22.9 free -SH groups per mol, a ratio of 1.3 titratable -SH per disulfide bond. This value is in agreement with the proposed mechanism of the chemical reaction of thiol with DTNB (Donovan and White, 1971; Anderson and Wetlaufer, 1975) and the literature reports (Anderson and Wetlaufer, 1975) for the yield of free sulfhydryls released from model disulfide-containing peptides. Alkaline hydrolysis of the truncated FcγRIIβ gave 2.51-SH per mol. When corrected for the yield of -SH/S-S, a value of 1.91 S-S bonds/mol of truncated FcγRIIβ was obtained. Reverse

Table II. Titration of tFcγRIIβ Sulfhydryl Groups with Ellman's Reagent

Protein	OD_{280nm}/M	-SH/protein	Number of S-S bonds	-SH/S-S
Albumin	0	0[a]	17.5[b]	-
Albumin, alkali treated	3.25×10^5	22.9	-	1.31
Truncated FcγRII	0	0	1.92[c]	-
Truncated FcγRII, alkali treated	0.35×10^5	2.51	-	NA

a. There is one free sulfide group in bovine albumin (Brown, 1977).

b. Since titration with Ellman's reagent of albumin without hydroxide cleavage was negative, we assume the albumin preparation was dimerized.

c. Calculated from this experiment by dividing experimental -SH/S-S for albumin into the -SH/protein obtained for the truncated FcγRII.

phase HPLC profiles of tryptic digests of the truncated FcγRIIβ with and without reduction were also compatible with the presence of intrachain disulfide bonds (data not shown). Like many other members of the immunoglobulin superfamily, the cysteines form intramolecular linkages in the FcγRIIβ and, therefore, the two repeats of the extracellular domain form two C2-set Ig-like loops (Williams and and Barclay, 1988).

The disulfide linkages in the tFcγRIIβ are important for the binding activity. In attempting of labeling tFcγRII with a fluorescein probe, we synthesized crabescein, a disulfhydryl-derivative of fluorescein (Packard *et al.*, 1986) and incorporated the probe into purified tFcγR by mild reduction followed by reoxidizing with crabescein. However, although FcγR could be derivatized with crabescein, the derivatized tFcγR no longer bound to the IgG-Sepharose column (results not shown). In the case of IgG, the interaction between two heavy chains below the hinge region is relatively weak (Davies and Metzger, 1983), and therefore, incorporation of a molecule the size of crabescein has little effect on the molecule. In FcγR, the intrachain disulfide bond might play a crucial role for the strong interaction in a Ig-like domain and addition of crabescein might significantly disturb the disulfide bond, subsequently affecting the overall structure of the domain or even the whole molecule. The crucial role of disulfide bonds in stabilizing the structure and activity of tFcγR needs to be further examined.

C. *Glycosylation and Microheterogeneity of tFcγRIIβ.* The predicted amino acid sequence of moFcγRII (both α and β genes) has four potential sites for N-linked glycosylation (Ravetch *et al.*, 1986; Lewis *et al.*, 1986; Hogarth *et al.*, 1987). Limited

endoglycosidase F digestion of FcγR isolated from J774 after a short pulse with [^{35}S]-methionine resulted in 5 intermediates, consistent with 4 glycosylation sites (Green *et al.*, 1985). The truncated FcγRIIβ purified by affinity chromatography on IgG2b-Sepharose 4B shows the M_r of 30,000-35,000 and the same broad electrophoretic mobility as the intact FcγR, suggesting it is also glycosylated. Digestion of the truncated FcγRIIβ with endoglycosidase F/N-glycosidase F confirmed the presence of carbohydrates (Fig. 13A), and resulted in a fully deglycosylated core of 20,000 M_r, which is in agreement with the predicted length of the peptide backbone of the truncated FcγRIIβ, 176 amino acids.

Deglycosylation of native truncated FcγRIIβ with endoglycosidase F/N-glycosidase F (endo F) was only partially achieved (Fig. 13A). Fractionation of endoglycosidase F treated tFcγRII by ion exchange chromatography on a Mono S column (Pharmacia) eluted with a 0-0.5 M Na_2SO_4 gradient revealed that only about 20-30% of the protein was converted to the fully deglycosylated form (Fig. 14). This ratio was not changed by increasing the amount of enzyme used and extending the time of digestion (data not shown). It is possible that not all of oligosaccharide moities on tFcγRIIβ are exposed on the surface and that the oligosaccharides buried in the truncated FcγRIIβ are protected from endoglycosidase digestion. Thus, denaturing the protein is necessary for complete deglycosylation with endo F. Furthermore, it seems likely that the extent of glycosylation varies from one molecule to another. The species that can be completely deglycosylated might lack the buried sugar moieties because of an insufficient glycosylation system for the over-produced tFcγRIIβ in CHO cells. In addition to the difference on molecular size,

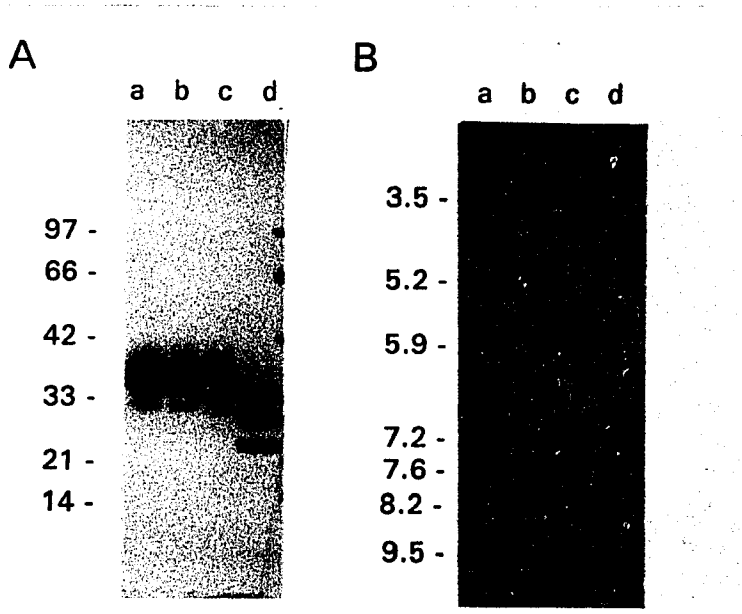

Figure 13. Digestion of truncated FcγRIIβ with endo F or neuraminidase.

Purified tFcγRIIβ were treated with either endo F or neuraminidase as detailed in text. Enzyme-treated and untreated tFcγRIIβ were subjected to SDS-PAGE on a 7-17% gradient gel (A) or IEF on a pH 3-10 precasted gel (B). Lanes a and b, affinity-purified tFcγRIIβ; lane c, neuraminidase-treated tFcγRIIβ; lane c, endo F-treated FcγRIIβ. At left of each panel, the markers of molecular weight (A) and pH (B) are indicated.

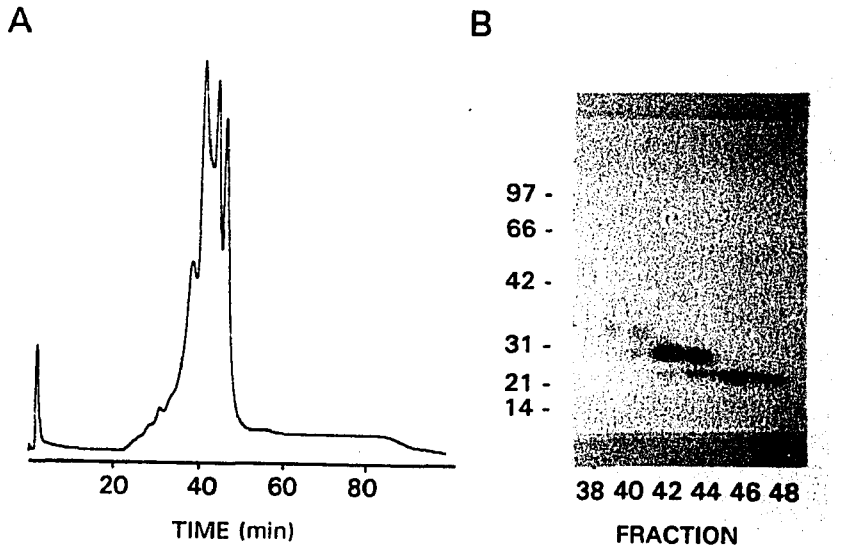

Figure 14. Fractionation of deglycosylated tFcγRIIβ on Mono S column.

A. Endo F treated and re-purified tFcγRII was dialyzed in 20 mM sodium phosphate buffer, pH 7.4 and concentrated. The protein was loaded on the Mono S culumn equilibrated with 20 mM sodium phosphate. Elution was performed with a Na_2SO_4 gradient of 0-0.5 M, 0.4 ml/min, and the fractions were collected at 1 min intervals.

B. The peak fractions were subjected to SDS-PAGE on a 7-17% gradient minigel. The fraction number shown on the bottom of each lane and M_r x 10^{-3} at left.

microheterogeneity of the truncated FcγRII can also be revealed by the differences in isoelectric point of different species. The sialic acids, N-acyl derivatives ofneuraminic acid, are widely distributed in eukaryotic cells and frequently found on membrane and secreted glycoproteins as the outermost residues in the N-linked oligosaccharides. This is true for both intact FcγR on cell surface (Gorczyca *et al.*, 1989) and the truncated FcγR produced by CHO cells. Isoelectric focusing (IEF) of purified tFcγRII on polyacrylamide gel separated tFcγRII into at least seven bands with pI range from 6.0 to 7.5 (Fig. 13B). Digestion of tFcγRII with neuraminidase (sialidase) to remove the sialic acids resulted in a decrease of charge heterogeneity and an increase in the pI of the protein. Two major bands with pI 7.8 and 8.0, respectively, were seen in the desialylated tFcγRII. Since sialic acids always occupy distal positions to the peptide backbone, treatment with neuraminidase had no significant effect on the size heterogeneity (lane c in Fig. 13A). Most carbohydrate moieties were still unchanged. These observations is consistent with the published data by Anderson (1982). In contrast, digestion of tFcγRII with endoglycosidase F significantly reduced the size of the truncated FcγRII and its dispersion in the gel (Fig. 13A) but had a smalleffect on the range of PI, although the pI's were increased (Fig. 13B).

D. *Activity and Immunological Epitopes of Deglycosylated tFcγRIIβ.* One of the major considerations that led us initially to express the soluble form of FcγRIIβ using mammalian cell lines was that the oligosaccharide moieties on the glycoprotein might be important for its binding activity. To examine the immunoreactivity of aglycosylated

tFcγRII protein, we used tunicamycin, a potent antibiotic that blocks the first stage in formation of the oligosaccharide-lipid donor. In the presence of tunicamycin, cells can still synthesize proteins but cannot add N-linked sugar to the peptide backbones. The truncated FcγRIIβ thus produced is unglycosylated. We immunoprecipitated tFcγRIIβ from medium conditioned by cells growing in the presence of tunicamycin with antibody-coated Sepharose 4B. The molecular weight of tFcγRIIβ secreted from decreased to 20,000 M_r (Fig. 15), consistent with the mass of the unglycosylated peptide chain. The aglycosylated tFcγRIIβ was immunoprecipitated with both mAb 2.4G2 and the anti-DNP mAb U7.6 (Fig. 15, Lane j-l). In the unglycosylated FcγRIIβ, the antigenic epitope recognized by mAb 2.4G2 is preserved, as is the binding site for IgG. Therefore, the carbohydrate moieties are not important for binding activity of Fcγ receptors.

In contrast, carbohydrate is important to maintain conformation of IgG that enables it to bind to huFcγRI and murine FcγRII and mediate cell functions. Leatherbarrow *et al.* (1985) showed that aglycosylated murine IgG2a did not bind to human monocytes, and Kurlander and Gartrell (1983) showed that immune complexes of aglycosylated murine IgG2b inefficiently mediated ADCC. Carbohydrate-deficient IgG2b prepared by treating hybridoma cells with tunicamycin lost the ability to bind to moFcγRII on macrophages and to induce ADCC (Nose and Wigzell, 1983). Leatherbarrow *et al.* (1985) also demonstrated that the binding affinity of aglycosylated mouse IgG2a mAb to huFcγRI on monocytes is 40-fold reduced relative to native IgG2a. This lowered affinity of the aglycosylated IgG2a is probably due to conformational change in the hinge region of IgG2a, rather than direct involvement of carbohydrate in

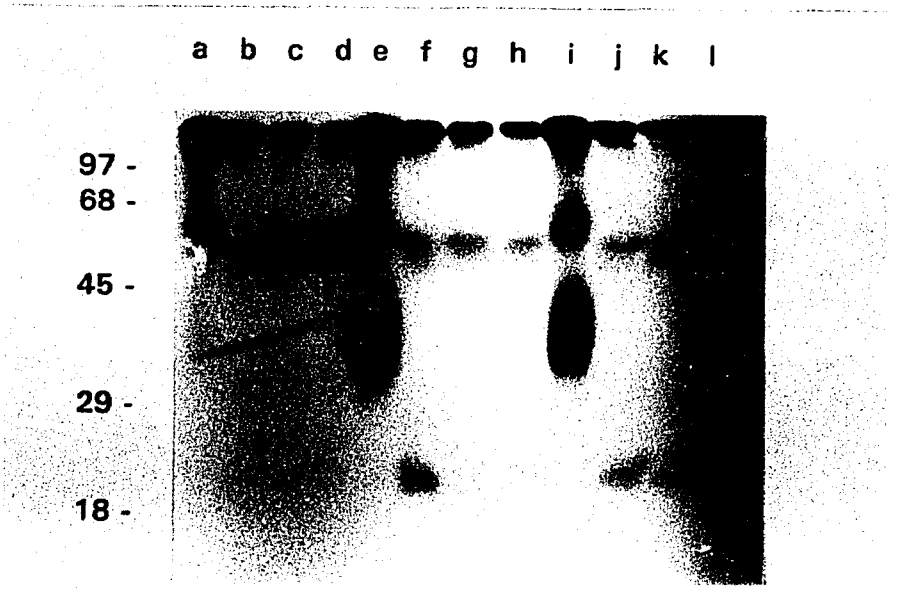

Figure 15. Immunoprecipitation of tFcγRIIβ and aglycosylated tFcγRIIβ.

D1959 cells were seeded at 10^6 cells per 60 mm plate and incubated overnight. The medium was removed and cells were washed twice with PBS. The cells were then incubated for 5 h in methionine-free medium supplemented with [^{35}S]-Methionine (125 μCi/ml) and 0.025 mM (lanes b, f and j), 0.125 mM (lanes c, g and k), or 0.625 mM tunicamycin (lanes d, h and l). The control cells (lanes a, e and i) were same labeled without tunicamycin. The conditioned media were then collected and incubated with BSA- or antibody-conjugated Sepharose 4B for 3 h with nutation. The beads were collected and washed four times with NaCl, 50 mM, sodium phosphate, 50 mM, pH 7.0, resuspended in sample buffer and boiled for 3 min. The proteins were analyzed by SDS-PAGE (13.5% acrylamide) followed by fluorography. Lanes a-d, conditioned media were incubated with BSA coated beads; lanes e-h, with rat anti-FcγRII mAb 2.4G2; lanes j-l, with mouse anti-DNP mAb U7.6.

binding.

The independence of glycosylation for binding activity of the truncated FcγRIIβ was also confirmed by repurification of the deglycosylated protein. Partially and completely deglycosylated species were all recovered from IgG-Sepharose 4B affinity column (Fig. 13A). However, these results do not exclude the possibility that glycosylation is important, and that there are conformational differences between glycosylated and unglycosylated species. A more obvious role the oligosaccharides might play may relate to the expression of FcγR on the cell surface. In CHO cells, tunicamycin, at the concentration as low as 0.025 mM, inhibited the production or secretion of tFcγRIIβ. As is shown in Fig. 15, the amount of immunoprecipitable material in the conditioned media containing tunicamycin was decreased profoundly (Lane e and i). In the presence of 0.0625 mM tunicamycin, almost no aglycosylated tFcγRIIβ was precipitated (lane g and h). This decrease was also seen on SDS-PAGE when total labeled proteins in the supernatant were loaded (data not shown). In contrast, the production of a number of other secretory proteins was not affected by 0.0625 mM tunicamycin (data not shown). It is known that N-linked oligosaccharides are required for efficient secretion of some proteins. Fcγ receptors might belong to this category of proteins. Alternatively, carbohydrates might play a role in enhancing the stability of maturing FcγRII by protecting it from digestion by tissue proteases.

E. *Determination of the Size of the Native Truncated FcγRIIβ.* SDS-PAGE is commonly used to estimate molecular weights and subunit composition of proteins.

However, the method of electrophoresis, the nature of the proteins, and the source of SDS can markedly affect the results (Swaney, et al., 1974; Bachrach and Hess, 1973). As Tung and Knight (1972) discussed, the results of SDS-PAGE for estimation of molecular weights should be confirmed by other methods, such as non-denaturing PAGE, gel-exclusion chromatography, and velocity sedimentation. These methods are especially valuable to determine the size of non-denatured proteins.

The size of the truncated FcγRII determined by gel filtration on Superose 6 column (Pharmacia) was unexpectedly much smaller than that determined by SDS-PAGE. The protein eluted between the marker proteins soybean trypsin inhibitor (21,000 Daltons) and cytochrome C (12,270 Daltons), with a M_r of 14,000 (data not shown). The inconsistency between the SDS-PAGE and the anomalous gel filtration results is probably due to interaction of the tFcγRII with the column matrix resulting in retardation of the protein on the column.

To determine the size of native tFcγRIIβ, we used a non-denaturing PAGE system (Bryan, 1977; Davis, 1964) and analyzed the results with Ferguson plots (Ferguson, 1964; Hedrick and Smith, 1968), as was detailed in the Materials and Methods section. The results of the analysis is shown in Fig. 16. Five major species of tFcγRII, were resolved in the gel)Fig. 16A), due to the charge heterogeneity. This agrees with the isoelectric focusing results. The molecular sizes of these different species estimated on the Ferguson plot were similar, about 55,000 M_r (Fig.16B). The higher molecular weight seen in the non-denaturing gel might result from the charged sugar residues on the peptides interacting with gel matrix or from the tertiary structure of the glycoprotein. It

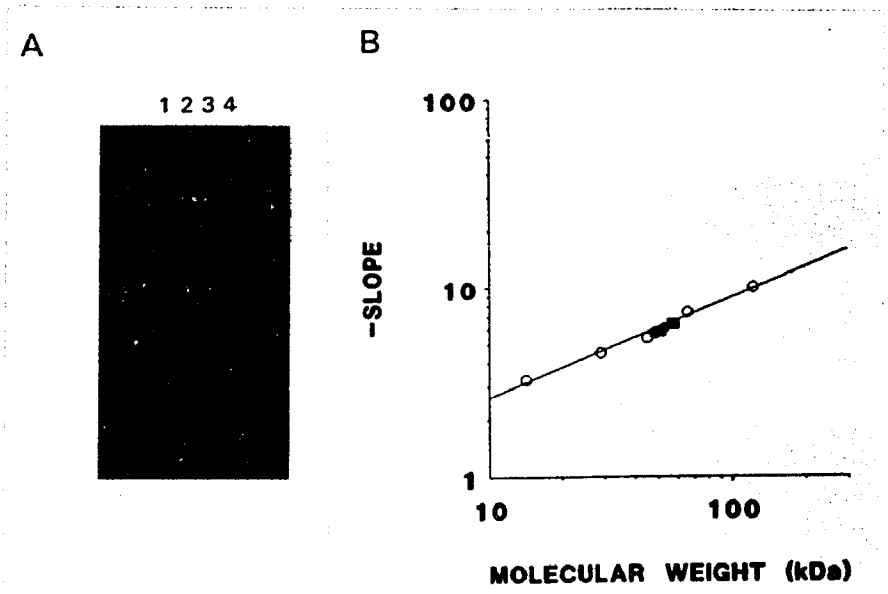

Figure 16. Non-denaturing PAGE.

Purified tFcγRII protein and marker proteins were run of a set of non-denaturing gels with 6%, 7%, 8%, 9% and 10% polyacrylamide. The gels was stained with Coomassie brilliant blue R. The data were analyzed by Ferguson plots as detailed in text. A. the 8% nondenaturing polyacylamide gel. Lane 1, tFcγRII; lane 2, BSA; lane 3, ovalbumin; lane 4, bovine milk α-lactalbumin. The tFcγRII species with the slowest mobility is band 1. B. The retardation coefficiet for marker protein species was plotted against the M_r. The molecular weight of each resolved species of tFcγRIIβ was caculated from the regression. (■), band 1, (◆), band 2; (▲), band 3; (+), band 4; and (●), band 5.

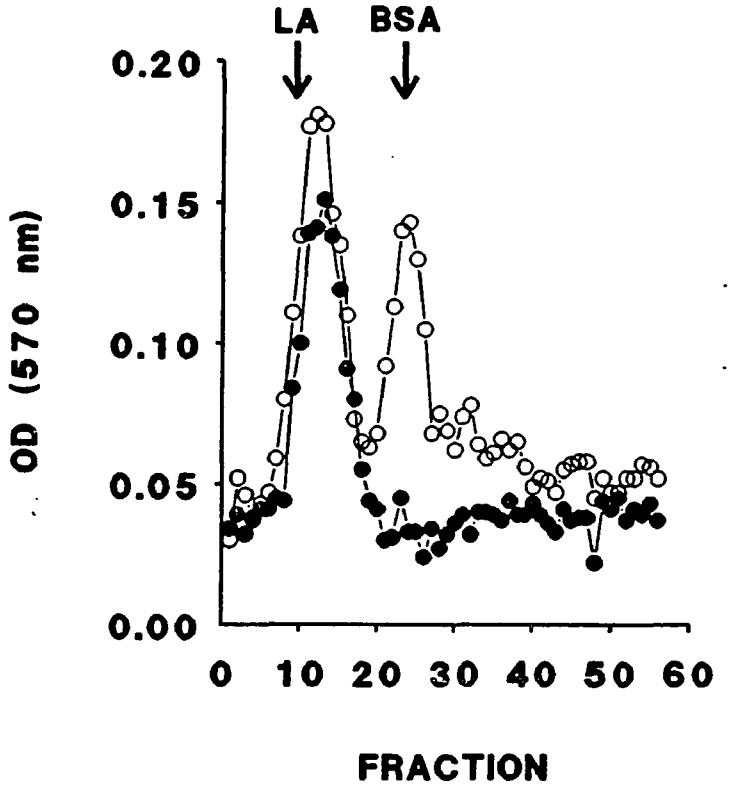

Figure 17. Sucrose density ultracentrifugation.
Deglycosylated tFcγRII alone or the tFcγRII mixed with BSA (0.1 ml in volume) were sedimented in 5-30% sucrose density gradients (11 ml). Parallel gradients were run with α-lactalbumin, and BSA. The gradients were collected in 0.2 ml aliquots. The protein concentration of all fractions was measured by Coomassie brilliant blue G in 96-well microtiter plates 570 nm. (●), tFcγRII alone; (○), tFcγRII and BSA. The marker positions shown with arrows are AL, α-lactalbumin (14.2 kDa); BSA monomer (66 kDa).

is also possible that there is interaction between tFcγRII molecules. The data from the non-denaturing electrophoresis are not sufficient to conclude that the tFcγRII is or is not a dimer.

In an effort to clarify whether tFcγRII molecules in solution exist as monomers, dimers, or oligomers, the deglycosylated tFcγRIIβ was subjected to sucrose density gradient ultracentrifugation. As shown in Fig. 17, deglycosylated tFcγRII sedimented slightly faster than the marker protein α-lactalbumin (M_r 14,200) and much slower than BSA monomer (M_r 66,000). The results with glycosylated tFcγRIIβ were similar (data not shown). From these results we concluded that the truncated FcγRIIβ in solution is a monomer.

F. *Crosslinking of tFcγRIIβ.* The extracellular domains of murine and human FcγRs are homologous to other members of immunoglobulin superfamily (Williams and Barclay, 1988). Representative members in this large and complex group include proteins involved in adhesion or binding, cell-cell recognition, and antigen presentation, and in many cases these receptors trigger intracellular events following interaction with ligands or crosslinking. A number of proteins of this family exist on cell surface as homo- or heterodimers or oligomers. The interactions between Ig-related molecules are likely a common feature. To examine the possibility of FcγR-FcγR interactions, we carried out a series of experiments with the heterobifunctional crosslinker, sulfosuccinimidyl 2-(*p*-azidosalicylamido)ethyl-1,3'-dithiopropionate(SASD). SASD is a heterobifunctional reagent containing a chemically reactive N-hydroxysuccinimide (NHS) ester and a

photoaffinity group, azido-salicylate, that is activated by UV light. The resulting nitrene reacts non-selectively with any nearby chemical group to from a covalent bond. This crosslinker can be iodinated with ^{125}I, and since there is a disulfide moiety between the two crosslinking functional groups, the crosslinked proteins can be cleaved with reducing reagents, resulting in iodination of the crosslinked proteins.

The iodinated SASD was covalently linked to the primary amine group of tFcγRIIβ through the NHS-ester. The derivatized protein was then subjected to long wavelength UV irradiation. If there is association between the protein molecules, they will be crosslinked by SASD. We analyzed the crosslinked products on SDS-PAGE under reducing and non-reducing conditions. With increasing UV irradiation, a large proportion of tFcγRII was crosslinked forming dimer, trimer and high molecular weight complexes (Fig. 18 and Fig. 19) that were so big that they did not enter the running gel. To quantify the amount of tFcγRII as monomer or complexes, we cut the gel and counted the radioactivity associated with each fraction on a gamma counter. As is shown in Fig. 20A and C, the fractions corresponding to the dimer and trimer reached to maximum in 10-20 sec period of irradiation and about 50% of total labeled protein was converted to large complexes after 10-20 min of irradiation. To control for collisional crosslinking, parallel experiments were performed with BSA. There were no large complexes formed with BSA crosslinked with SASD, although the mobility of BSA in SDS-PAGE was broadened by intra-molecular crosslinking (Fig. 19). The large complexes we observed are mediated by the crosslinker, because reduction to cleave the disulfide bridge resulted in dissociation of the complexes to monomers (Fig. 18B). The effect of crosslinking was

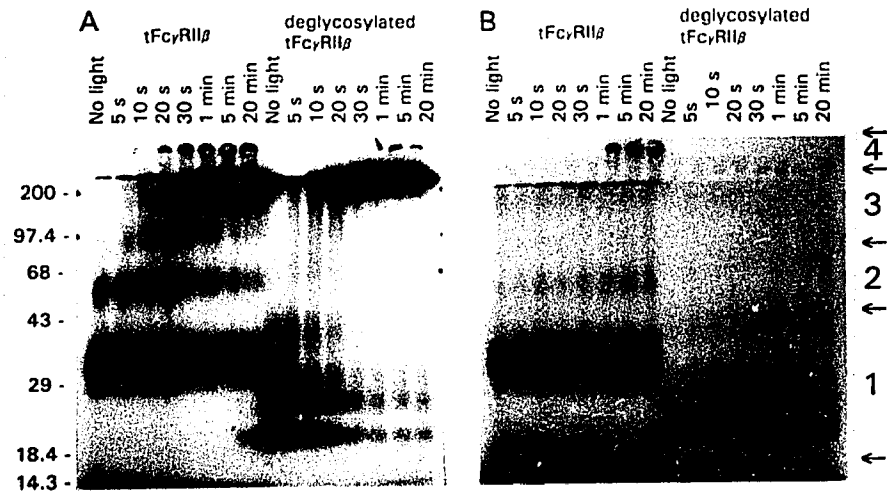

Figure 18. Crosslinking of tFcγRIIβ and deglycosylated tFcγRIIβ with SASD.

SASD-derivatized proteins were irradiated for different time intervals as indicated on the top of each lane. Crosslinked proteins were then subjected to non-reducing (A) and reducing (B) SDS-PAGE on 10% gels. To quantify the radioactivity, each lane of the gels were cut in four pieces, as indicated at right, and measured for radioactivity. M_r x 10^{-3} shown at left.

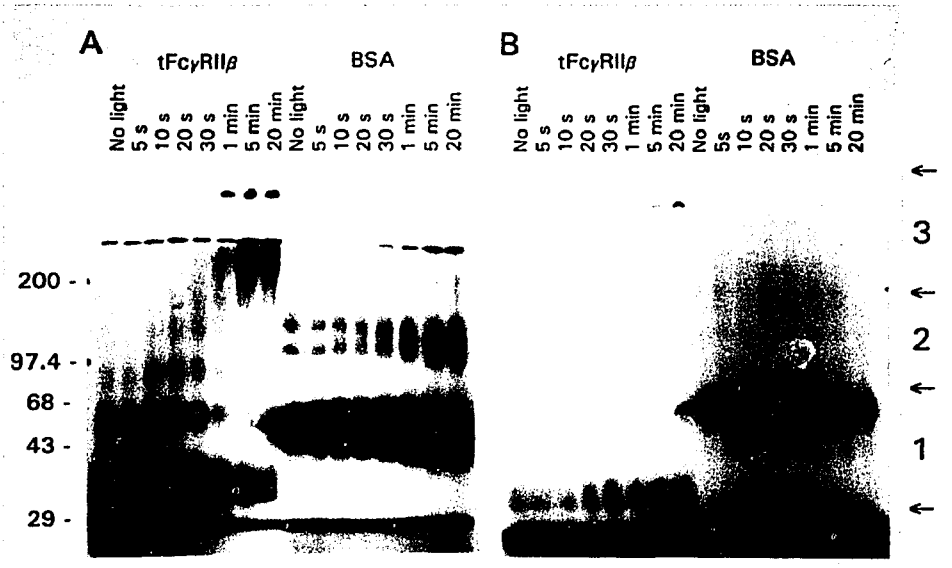

Figure 19. Comparison of SASD-mediated crosslinking of tFcγRIIβ and that of BSA.

SASD-derivatized tFcγRII and BSA were irradiated as described in Fig. 18. Crosslinked proteins were then subjected to non-reducing (A) and reducing (B) SDS-PAGE on 7% gels. To quantify the radioactivity of BSA, each lane of the gels were cut in three pieces, as indicated at right, and measured for radioactivity. M_r x 10^{-3} shown at left.

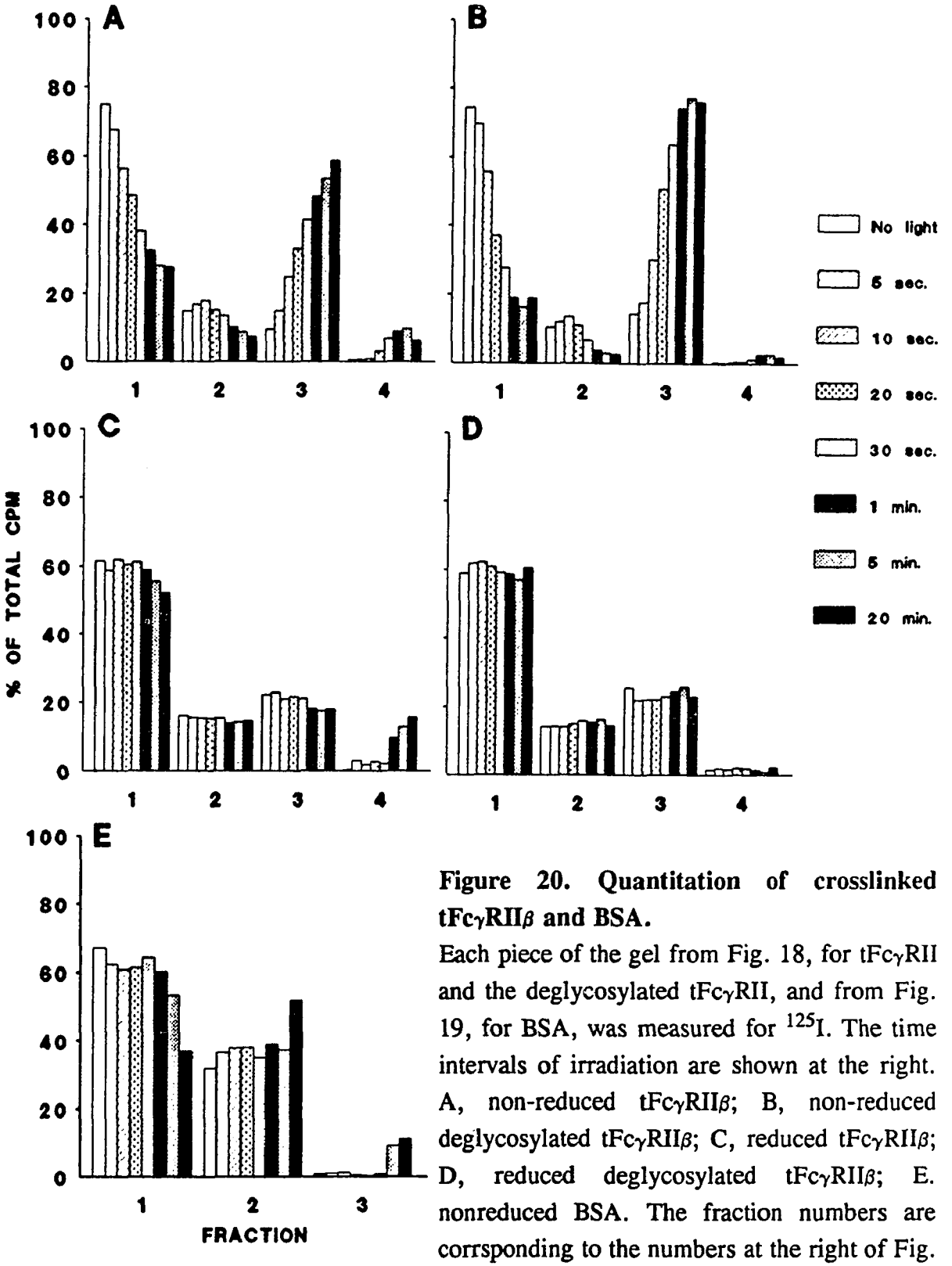

Figure 20. Quantitation of crosslinked tFcγRIIβ and BSA.

Each piece of the gel from Fig. 18, for tFcγRII and the deglycosylated tFcγRII, and from Fig. 19, for BSA, was measured for ^{125}I. The time intervals of irradiation are shown at the right. A, non-reduced tFcγRIIβ; B, non-reduced deglycosylated tFcγRIIβ; C, reduced tFcγRIIβ; D, reduced deglycosylated tFcγRIIβ; E. nonreduced BSA. The fraction numbers are corrsponding to the numbers at the right of Fig. 18 and Fig. 19, respectively.

even more dramatic on deglycosylated tFcγRIIβ as can be seen in Fig. 18 and Fig. 20. The amount of large complexes reached a maximum in just 5 min and occupied over 70% of total labeled protein. These results demonstrated the association between tFcγRIIβ molecules. The presence of carbohydrates might minimize the interaction.

To study the nature of the interaction between tFcγRIIβ molecules we performed the crosslinking at varying concentrations of protein. The SASD-derivatized deglycosylated protein was diluted up to 20-fold, from 6 μM to 0.3 μM, and then subjected to extensive irradiation. We found that the extent of crosslinking did not differ between high and low concentration of tFcγRIIβ in this range (Fig. 21). In addition, the time courses of crosslinking performed at 3 μM, 0.3 μM and 0.15 μM tFcγRIIβ were almost identical (Fig. 21). These results suggested that the Ka for association between tFcγRII molecules is $>3 \times 10^7$ M^{-1}, the lowest concentration of tFcγRIIβ tested. However, the size of tFcγRIIβ determined by velocity sedimentation on sucrose density gradient clearly demonstrates the monomer nature of tFcγRII in solution. In order to confirm that high concentration of sucrose in the gradient did not affect FcγR-FcγR interaction, the SASD crosslinking was carried out in the presence of up to 10% of sucrose, which had no effect on the extent of crosslinking (data not presented). An explanation for this discrepancy could be that there is a rapid and dynamic association between tFcγRIIβ molecules. Although the affinity of one tFcγRIIβ molecule to another is fairly high, probably higher than 10^{-7} M, the dissociation rate and dissociation rates are fast. In the steady state, tFcγRIIβ has more probability to exist as a monomer. $T_{1/2}$ of the activated nitrene is on the order of 10^{-4} sec, which is fast enough to crosslink

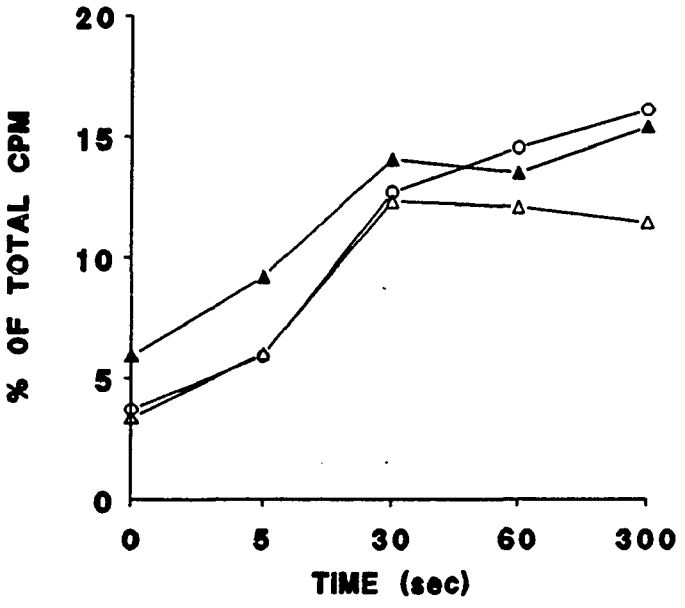

Figure 21. Concentration and time course.

The deglycosylated tFcγRII was derivatized with SASD and diluted to 3 μM (○), 0.6 μM (△) and 0.3 μM (▲) before irradiation. The irradiation and analysis were the same as in Fig. 18. Plotted is the % of total radioactivity in the third (high molecular weight) fraction of the polyacrylamide gel. The extent and time course of generation of high M_r adducts did not vary with concentration.

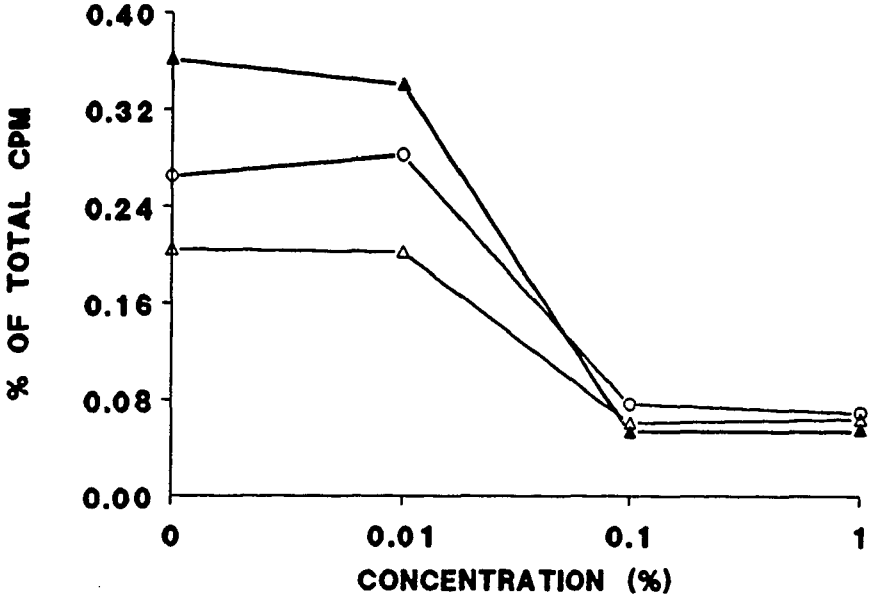

Figure 22. SDS in crosslinking.

SASD-derivatized deglycosylated tFcγRIIβ was incubated in varying concentrations of SDS at room temperature (○), 50°C (△) or 100°C (▲) for 5 min prior to irradiation for 5 min. The crosslinked protein was then analyzed as described in Fig. 18. Only the percentage of total radioactivity in fraction 3 (high M_r adducts) of each lane is plotted.

transiently associated FcγR-FcγR aggregates.

If the crosslinking results from the fast dynamic association between tFcγRIIβ molecules, it should be dependent on the tertiary structure of the protein. To test this possibility, we crosslinked the deglycosylated tFcγRIIβ in the presence of 0-10% SDS. Fig. 22 shows that the formation of large complexes was completely inhibited when the concentration of SDS was higher than 0.1%, a concentration of SDS that is commonly used to denature proteins. A SDS concentration of 0.01% is too low to denature protein completely, therefore, the crosslinking was only slightly inhibited. The treatment of tFcγRIIβ with SDS prior to irradiation was also carried out at higher temperatures to enhance the effect of SDS. There was less high molecular weight complexes when the protein in 0.01% SDS had been incubated at 50°C. Interestingly, when SASD-derivatized protein was boiled, more large complexes were formed, probably due to heat-activated crosslinking. These results suggest strongly that the crosslinking of tFcγRII by SASD is dependent on a native tertiary structure of tFcγRII.

The nature of the interaction between the tFcγRII molecules is not known. The formation of very large complexes suggests that more than one interactive sites might exist on one tFcγRII molecule. Since the truncated FcγRII is composed of two repeats of Ig-like domain, it is possible that FcγR-FcγR interaction is actually the interaction between the Ig-like domains of different molecules. Alternatively, a tFcγRII molecule might have asymmetric sites for other molecules and the association between any two molecules be unidirectional. This kind of "head to tail" interaction would result in rapid formation of very large complexes.

G. *Mapping of the Epitope Recognized by mAb 6B7C.* Monoclonal antibody 6B7C is directed against moFcγRII of macrophage origin (Pure *et al.*, 1988). This antibody effectively reacts with moFcγRII from all cells following SDS-PAGE and transfer to nitrocellulose. However, it reacts variably with FcγR on viable FcγR-positive cells. For example, mAb 6B7C binds to the surface of LPS-activated B cells and J774 cells, but not to primary macrophages or B cells (Pure *et al.*, 1988). When we attempted to detect the secreted FcγRIIβ using mAb 6B7C, we were surprised to observed that this antibody did not recognize the truncated FcγRIIβ on immunoblots (Fig. 5B). Furthermore, although in the ELISA assay, mAb 6B7C bound to the FcγRIIβ from the detergent lysates of S49.1 cells as well as mAb 2.4G2 did, it failed to react with the truncated FcγRIIβ (Fig. 23A).

In construction of the expression plasmid, a stretch of sequence coding for 8 amino acid residues (174-181) located at the C-terminal end of external domain proximal to the transmembrane domain was deleted due to the restriction site used. Since the truncated FcγRIIβ is missing the 8 amino acids, the epitope in intact moFcγRII recognized by mAb 6B7C may be just in the deleted peptide. Alternatively, it is also possible that the FcγRIIβ made in CHO cells is different by virtue of altered glycosylation, or that the truncated FcγRIIβ, due to altered conformation, has lost the 6B7C epitope. To test these possibilities, we synthesized the peptide ITVQGPKSSRSLPVL (amino acids 169-183 of the FcγRIIβ) and tested this peptide, coupled to OVA, as a ligand. As can be seen in Fig. 23B, 6B7C bound to ITVQGPKSSRSLPVL-OVA coated wells in a dose-dependent fashion, but there was no

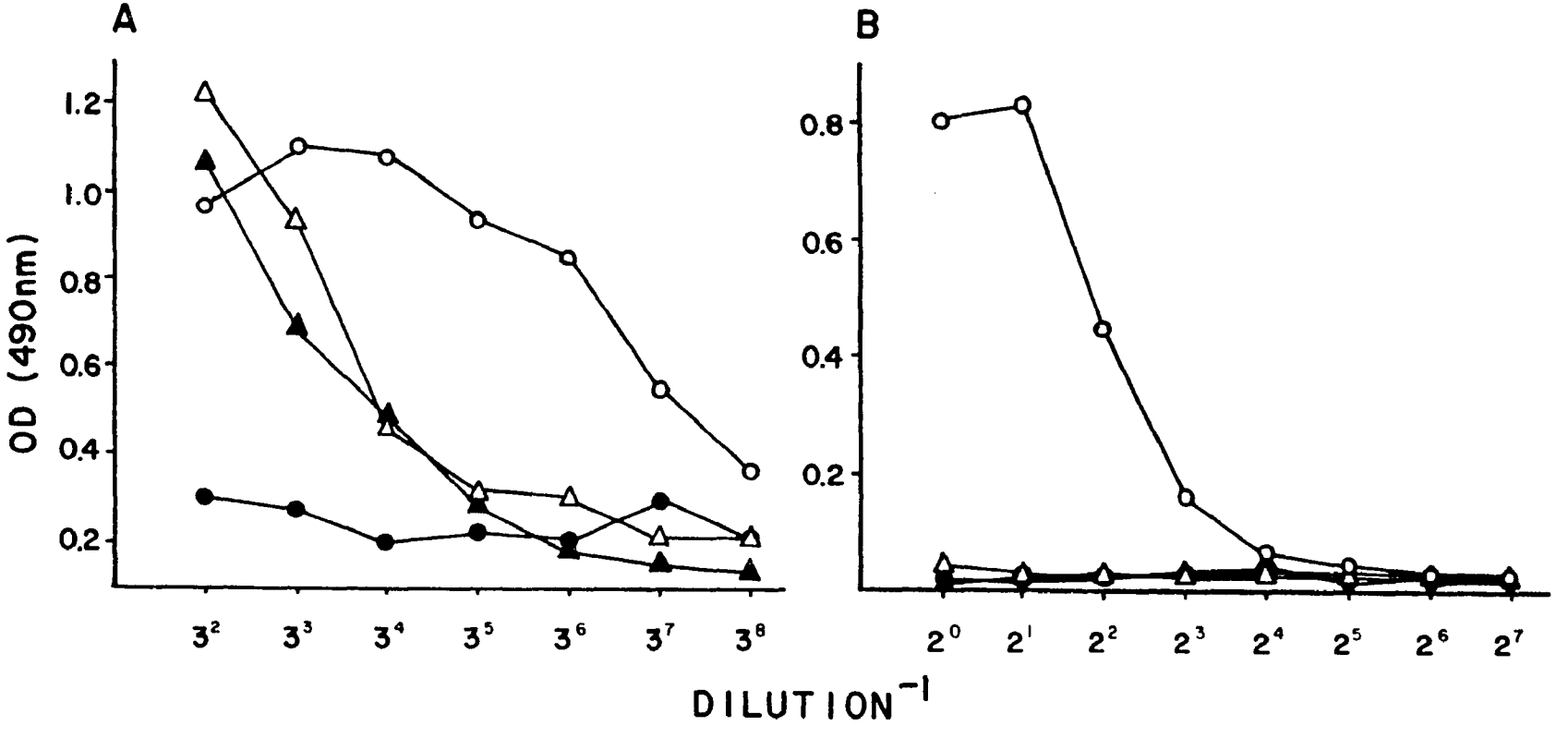

Figure 23. Identification of the epitope of FcγRIIβ recognized by mAb 6B7C.

A. Reactivity of mAbs 2.4G2 (○ and △) and 6B7C (● and ▲) with conditioned medium from the D1959 cell line (○ and ●) and an NP-40 lysate (10^7 cells/ml) of S49.1 cells (△ and ▲). B. Reactivity of mAbs 2.4G2 (● and ▲) and 6B7C (○ and △) with ITVQGPKSSRSLPVL-OVA (○ and ●) and OVA (△ and ▲). Serial dilutions of ovalbumin or ITVQGPKSSRSLPVL-OVA conjugate coating the wells began at 400 μg/ml.

binding of either 6B7C to OVA coated wells, or 2.4G2 to either OVA or ITVQGPKSSRSLPVL-OVA coated wells. These results indicate that the epitope recognized by mAb 6B7C is certainly within amino acids 169-183 and that crucial residues for the 6B7C epitope lie after residue 173 (the terminus of the truncated FcγRIIβ). Furthermore, since the predicted FcγRα and FcγRIIβ protein sequences are quite different in this region, mAb 6B7C is a FcγRIIβ specific probe, which we have confirmed in preliminary experiments (R. Schreiber and J. Unkeless, unpublished results). The location of the 6B7C epitope, just at the plasma membrane, suggests that the epitope may be masked, perhaps by other proteins or cell surface constituents, in the cells bearing FcγRIIβ that fail to bind mAb 6B7C.

H. *Crystallization of the Truncated FcγRIIβ.* The ultimate solution of three dimensional structure of a protein is dependent on crystallographic data and the success of purification and crystallization of the protein is the key to X-ray diffraction studies. Since milligram quantities of purified tFcγRII protein were available, we decided to crystallize the truncated FcγRIIβ in collaboration with Dr. Davies (NIH, Bathesda, MD). Initial attempts to crystallize tFcγRII either yielded an oil from which thin needle-like crystals were formed, or no crystals at all. We reasoned that the difficulties in crystallizing tFcγRII was due to the microheterogeneity of glycosylation and sialylation. Since carbohydrates are not required for biological activity of the receptor, we attempted to crystallize deglycosylated tFcγRII instead.

Several deglycosylation methods were tested and we chose to use endoglycosidase

F treatment. The protein was deglycosylated with endoglycosidase F under non-denaturing condition followed by re-purification on IgG-Sepharose 4B column and concentration to about 6-15 mg/ml. The re-purified tFcγRIIβ was composed of two types of species, 20,000 M_r and 23,000-25,000 M_r on SDS-PAGE (data not shown), corresponding to completely deglycosylated peptide core and partially deglycosylated forms, respectively. Crystallization of tFcγRII was performed by the vapor diffusion method (Reid *et al.*, 1973; Wlodawer and Hodgson, 1975), which allows us to screen a variety of crystallization conditions in a systematic way with small amounts of sample and which is relatively easy to control. We tested a wide range of crystallization conditions to find one that yields crystals suitable for X-ray diffraction analysis, and succeeded in crystallizing the deglycosylated tFcγRII. The best results were obtained in growing crystals in 15-18% PEG 3550 at pH 7.4-7.6 with 5-20 mM $CaCl_2$ and 10-20 mM EDTA. Some single crystals of tFcγRII were approximately 1.0x0.2x0.2 mm (Fig. 24), approaching the size required for high resolution X-ray diffraction analysis. Most importantly, these crystals diffracted to about 3 Å resolution as examined by Dr. Davies.

Crystallization of macromolecules is still a "trial and error" procedure (McPherson, 1985) and there is no universal protocol for all proteins. The physical properties of the protein itself might be the most important determinants for crystallization. In the case of tFcγR, deglycosylation of the tFcγRII seems to be necessary since we were never successful in growing crystals of glycosylated tFcγRII. The desialylated tFcγRII behaved like native tFcγRII in crystallization trials as tested by both ourselves and the group at NIH (data not shown). We were successful in crystallizing the

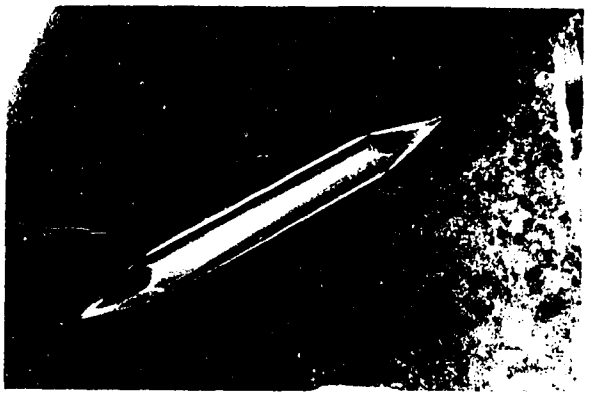

Figure 24. Crystallization of deglycosylated tFc$_\gamma$RII$_\beta$.
The single crystal of deglycosylated tFc$_\gamma$RII shown here grew from the solutions containing 18% PEG 3350 in 20 mM cacodylate buffer, pH 7.4, with 12.5 mM CaCl and 5 mM EDTA.

desialylated tFcγRII, but the crystals were small and unsuitable for X-ray analysis. Lustbader *et al.* (1989) reported crystallization of both chemically deglycosylated and neuraminidase treated human chorionic gonadotropin (hCG). They found that chemically deglycosylated hCG remained half of the carbohydrates but still crystallized and that crystals of neuraminidase-treated hCG diffracted better than those of deglycosylated hCG. Our results support their view that the presence of carbohydrates does not necessarily preclude the crystallization of at least some of glycoproteins. However, in view of their results, treating the truncated FcγRIIβ with both endoglycosidase F and neuraminidase might improve the process of crystallization and the quality of the crystals. We have prepared a batch of endo F/neuraminidase-treated tFcγRIIβ. The crystallization is under way.

Summary

We have characterized the truncated FcγRII purified from the medium conditioned by D1959 cells. The truncated FcγRIIβ has a M_r of 30-35,000 on SDS-PAGE and is glycosylated and sialylated. Endoglycosidase F cleavage reduces the M_r to 20,000, consistent with the size of the truncated product, 176 amino acid residues. There are two disulfide bonds in the protein. Binding of immune complexes formed between $DNP_{25}BSA$ and anti-DNP monoclonal antibodies reveals better binding of IgG1 aggregates than that of IgG2b and IgG2a aggregates. The binding of the immune complexes was somewhat better at more acidic pH, in contrast to previous experiments with binding of purified

FcγR to immune complex coated beads. The glycosylation of tFcγRII is not required for the binding activity since both aglycosylated and deglycosylated tFcγRII bind with IgG.

We provided evidence that although tFcγRII exists as monomer in solution, there is interaction between tFcγRII molecules. Up on crosslinking with photoaffinity crosslinker, the truncated FcγRII monomers form very large complexes. The formation of these complexes is independent of concentration of tFcγRIIβ in the range of 3 μM to 0.3 μM. Complex formation dose not occur after denaturation of the protein with SDS.

We were surprised to observe that the truncated FcγRIIβ did not react with the anti-FcγR mAb 6B7C. Previous work had shown that 6B7C reacts with FcγR on immunoblots, fails to bind to the surface of resting B cells and peritoneal macrophages, but does bind to macrophage cell lines and LPS-stimulated B cells. We show, by binding of mAb 6B7C to a peptide conjugate, that the 6B7C epitope lies within residues 169-183 of the intact FcγRIIβ, which is just outside the plasma membrane.

With the available of large quantity of purified tFcγRII protein, we have been carrying out the studies on crystallization of the protein. The partially deglycosylated protein was crystallized considerablly better than glycosylated one. Single crystals were grown to approximate 1.0 x 0.2 x 0.2 mm in size and diffracted to 3 Å resolution.

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