EXPRESSION AND CHARACTERIZATION OF A TRUNCATED MURINE Fcγ RECEPTOR

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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, α and β, have been cloned and sequenced (1–3). Transfection of the β gene into melanoma cells results in the appearance, on the cell surface, of the epitope recognized by mAb 2.4G2, an anti-FcγR mAb (4, 5), and the FcγR binding activity. Both the α and β genes encode proteins containing a leader sequence, NH₂-terminal extracellular domains, one putative transmembrane spanning domain, and a cytoplasmic domain. The two genes are 95% homologous in the extracellular domains, which consist of two repeats of 85 amino acids. Moreover, these domains bear significant homology to other members of the Ig gene superfamily, with the most striking similarity found between the β2 domain of mouse Eβ and the most distal NH₂-terminal domain of the FcγR.

To make the secreted form of an FcγR, the coding sequence for the transmembrane and cytoplasmic domains of FcγRβ was deleted from the cDNA, and a termination codon was introduced. This truncated FcγRβ 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 FcγRβ was then amplified by addition of methotrexate in medium. The resulting cell line secrete 2–3 µg/ml/d of truncated FcγRβ. The availability of large quantities of purified receptor has facilitated examination of 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

Cell Culture. Unless otherwise specified, chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). The CHO mutant cell line DG44, which has a deletion of the DHFR gene (6), was provided by Dr. Chasin (Columbia University, New York, NY), and was maintained in DME (Gibco Laboratories, Grand Island, NY) supplemented with 0.016 This work was supported by U.S. Public Health Service grants AI-24322 (J. C. Unkeless) and DK-10080 (J. D. Glass).

Abbreviations used in this paper: DHFR, dihydrofolate reductase; HRP, horse radish peroxidase; TBS, Tris-buffered saline; sulfo-MBS, sulfo-m-maleimidobenzoyl-N-hydroxysuccinimide.

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mM thymidine, 0.1 mM hypoxanthine, 0.3 mM proline, and 10% FCS (Flow Laboratories Inc., McLean, VA). Hypoxanthine was omitted and dialyzed FCS was used in medium for selection of transfectants. The S49.1 cell line was grown in suspension culture in α-MEM (Gibco Laboratories) 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) (7). Secretory products of transfected and amplified CHO cell lines were biosynthetically labeled with \[^{55}S\]methionine (1,100 Ci/mol; Amersham Corp., Arlington Heights, IL) in methionine-free medium supplemented with hormones.

Monoclonal Antibodies. The antidinitrophenyl (DNP) mAbs DHK10.12 (IgG2b) 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 (Weitmann Institute, Rechovot, Israel). The myeloma protein MPC-11 (IgG2b) was a gift of Dr. Eisen (Massachusetts Institute of Technology, Cambridge, MA). The anti-DNP mAbs were purified from conditioned medium by affinity chromatography on a TNP_25BSA Sepharose-4B column, and the bound mAb was eluted with 0.1M DNP, 0.1M Tris-HCl, pH 8.0. The DNP was removed by gel filtration on Sephadex G-25.

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 those of Maniatis et al. (8). Plasmid 3901, a \(\beta_1\) cDNA clone (1), was restricted by Apa I at base 947 within the insert and the resulting 3' overhang was blunted by T4 DNA polymerase. The unique Apa I restriction site is 26 bp 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 Ig-like domains (see Fig. 1). The stop codon was introduced into the reading frame by ligation of the phosphorylated 16-bp universal terminator, GCTTAATTAGC (Pharmacia Fine Chemicals, Piscataway, NJ), to the termini of the linearized plasmid. This results in addition of four accessory amino acid residues, GLIN, before the termination codon is reached. Next, Nco I was used to cut the cDNA at base 338, one base pair before the first methionine codon of the translated sequence. After Hha I digestion to remove concatamerized terminators, the overhangs generated by those two enzymes were blunted by incubation with DNA polymerase Klenow fragment. The 620-bp DNA fragment between Apa I and Nco I restriction sites with the ligated terminator at the 3' end was isolated by preparative agarose gel electrophoresis and ligated into the Sma I restriction site of linearized dephosphorylated pcEXV-3 expression vector (9). The truncated cDNA insert is predicted to encode a protein with 176 amino acid residues. Two plasmids, pFcγRβ-19 (Fig. 1) and pFcγRβ-17, which contain the inserts with sense and antisense orientations, respectively, were obtained after transformation of MM294.

Transfection of CHO Cell Line and Amplification. The CHO-derived cell line DG44 was seeded at 4 x 10⁵ 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 (10), given to us by Dr. Chasin (Columbia University), the FcγRβ cDNA construct (2 μg per plate), and carrier DNA (20 μg per plate) as described by Wigler et al. (11). After 2 d, the cells were subcultured and the transfected cells were selected in medium without hypoxanthine containing 10% dialyzed FCS. After 2 wk, 0.02 μM methotrexate was added to the medium followed by stepwise increments every 10 d to 0.05, 0.1, 1.0, and 2.0 μM methotrexate (12). The cells were then cloned and clones were screened for secretion of FcγRβ. The cultures were then maintained routinely in the presence of 2.0 μM methotrexate.

Immunoasays for FcγRβ (ELISA). FcγRβ in cell culture medium or purified FcγRβ was titrated by a modification of the monoclonal sandwich radioimmunoassay described previously (13). FcγR in assay samples was adsorbed onto flat-bottomed wells (Immulon-2; Dynatech Laboratories Inc., Alexandria, VA) previously coated overnight with rabbit anti-FcγR IgG (5 μg/ml) in PBS and blocked with 3% BSA. FcγRβ 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 & Perry Laboratories, Inc., Gaithersburg, MD) and streptavidin–horse radish peroxidase (HRP) (0.5 μg/ml) (Kirkegaard & Perry, Inc.), and developed as described (14). The assay was calibrated by inclusion of a detergent lysate of S49.1 cells (1% NP-40 in PBS, 10^7 cells/ml).

A similar protocol was used to analyze the binding of the rat anti-FcγR mAbs 2.4G2 and 6B7C to flat-bottomed plates coated with OVA or a peptide-OVA conjugate.

**Cytoplasmic RNA Dot Hybridization.** Preparations of cytoplasmic RNA from tissue culture cells and dot blots were as described by White and Bancroft (15). Prehybridization and hybridization of blots with probes labeled with α-[32P]CTP (3,000 Ci/mmol; Amersham Corp.) by nick translation were carried out as described by Dobner et al. (16). The probes were the FcγRβ cDNA insert of pFcyRβ-17 and the rat metallothionein 1 cDNA insert of plasmid p2A10 (17), kindly donated by Dr. Bancroft (Mount Sinai School of Medicine, New York, NY).

**Purification of Truncated FcγRβ.** Cell culture supernatant containing 5 or 10% FCS was collected and protein precipitated between 40–75% ammonium sulfate saturation was 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 FcγRβ was isolated by passing the concentrated protein solution over a DHK10.12 (IgG2bK) Sepharose-4B (Pharmacia Fine Chemicals) 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 OD280 returned to baseline. The bound truncated FcγRβ was eluted with sodium acetate buffer (0.1M, pH 4) containing 0.5M NaCl, and dialyzed versus PBS. The protein at this stage was judged pure by SDS-PAGE.

**SDS-PAGE and Immunoblotting.** SDS-PAGE was performed using Neville buffers (18) on slab gels. For immunoblotting (19), nonreduced protein samples were subjected to SDS-PAGE and transferred to nitrocellulose (Bio-Rad Laboratories, Richmond, CA) with a nonbuffer tank electrophoretic apparatus (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 min blotter (Miniblotter II; Immuneins, 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 (20).

**Assay of Binding of Immune Complexes by Truncated FcγRβ.** Crystallized BSA was derivatized with DNP groups by reaction with dinitrobenzene sulfonic acid (Pierce Chemical Co., Rockford, IL) in 2% Na2CO3, and after gel filtration, the extent of substitution was determined as described previously (21). The DNP20BSA (50 μg) was then radiolabeled with 1 μCi of carrier-free Na[125I] (Amersham Corp.) using tubes coated with 1 μg of iodogen (Pierce Chemical Co.) (22). Unincorporated 125I was removed by gel filtration on a G-25M column. The labeled protein had the sp act of 1.25 × 10^6 cpm/μg. Flexible 96-well U-bottomed plates (Micro-2000; Dynatech Laboratories, Inc.) were coated with purified truncated FcγRβ (5 μg/ml in PBS) at 4°C overnight and the wells were washed with 3% nonfat dry milk in PBS at room temperature for 2 h. Immune complexes were formed by incubation of serial twofold dilutions of different anti-DNP mAbs with [125I]DNP20BSA in 0.2× PBS at room temperature for 2 h and the complexes were then transferred to the FcγRβ-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 Ria-Gamma; LKB-Wallac, Turku, Finland).

**Peptide Synthesis and Conjugation.** The peptide ITVQGPSSRSSLRPVL, residues 169–183 of FcγRβ, was synthesized stepwise by solid-phase methods on an automated peptide synthesizer (model 9500; Biosearch, San Rafael, CA). A cysteamine residue was attached to the resin through a thioether linkage before start of automated synthesis (23). After deblocking, this results in a COOH-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 sulphydryl group, the bifunctional reagent sulfo-maleimidobenzoyl-N-hydroxysuccinimide ester (sulfo-MBS) (Pierce Chemical Co.) was used to make the peptide–protein conjugate, as described by Youle and Neville (24). Briefly, 5 mg of 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 OVA was 8.7:1. After incubation at room temperature for 30 min, the acylated OVA was desalted over a Sephadex G-25M column (Pharmacia Fine Chemicals) 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 same buffer at room temperature for 2 h. The molar ratio of peptide to OVA was 5.2:1. The peptide–OVA conjugate, ITVQPKSSRSLPVL-OVA, was then filtered through a small Sephadex G-25M column.

**Disulfide Analysis.** Ellman's reagent (25), 5,5'-dithio-bis(2-nitrobenzoic acid) (Calbiochem-Behring Corp., La Jolla, CA) was used to titrate the number of sulphydryl groups in the truncated FcγRβ as described by Anderson and Wetlaufer (26) 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°C overnight. The alkaline solution was neutralized by the addition of 0.5 ml of 6 N H₃PO₄ 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 sulphydryl groups was calculated from the extinction coefficient of 13,600/M/cm (25). 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) (26).

**Deglycosylation.** Digestion of the truncated FcγRβ with N-glycosidase F (Genzyme, Boston, MA) was based on the procedure described by Tarentino et al. (27). Samples were boiled for 3 min in 0.2 M sodium phosphate buffer (pH 8.6) with 10 mM 1,10-phenanthroline hydrate and varying concentrations of SDS, after which they were incubated with N-glycosidase F for 2 h at 37°C.

### Results and Discussion

**Isolation of the CHO Cell Line Secreting Truncated FcγRβ.** To convert the FcγRβ1, 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 a FcγRβ1 cDNA insert encoding signal sequence, extracellular and transmembrane domains, and partial cytoplasmic domain, is cleaved with Apa I the transmembrane and cytoplasmic domains are deleted, leaving a 172-residue protein with two extracellular Ig-like domains (see Fig. 1 and Materials and Methods). The truncated FcγRβ protein is also missing eight amino acids just outside the membrane. The truncated FcγRβ cDNA was inserted in both orientations into the pcEXV-3 expression vector (9) and transfected along with pCGcos3neo (28) into the B78H1 melanoma cell line. After G418 selection, transfectants were cloned and supernatants were screened by ELISA. Of 19 clones transfected with pFcγRβ19, 9 secreted immunoreactive material, but none of the 64 clones transfected with pFcγRβ17 were positive. However, the titer of FcγRβ in the supernatant of B1904, one of the better secreting lines, based on comparison with lysates of the FcγR* S49.1 cell line, was <5 ng/ml (Fig. 2), which would make further biochemical studies difficult.
To obtain a higher level of secretion, we transfected pFcyRβ-17 and pFcyRβ-19 into a DHFR- CHO cell line along with a dhfr minigene, and selected with increasing concentrations of methotrexate. This leads to amplification of the dhfr minigene and flanking transfected FcyRβ expression plasmid DNA. The FcyRβ-secreting clones were isolated from mass culture after amplification resulting in resistance to 2 µM methotrexate. The level of secretion of truncated FcyRβ by the D1959 cell line is elevated over 2,000-fold relative to the B1904 melanoma transfectant (Fig. 2). As expected, none of the pFcyRβ-17-transfected CHO cells secreted immunoreactive material. Although the slope of the titration of the intact S49.1 cell FcyR is slightly steeper (possibly due to more efficient capture of the intact FcyR by the rabbit anti-FcyR antiserum), we estimate, with a value of $5 \times 10^4$ FcyR per S49.1 cell (results not presented), that the concentration of truncated FcyRβ in medium conditioned by confluent D1959 cells is 2-3 µg/ml/d.

The much higher level in synthesis of the truncated FcyRβ 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 FcyRβ mRNA. Hybridization of nick translation-labeled insert of pFcyRβ-17 cDNA to cytoplasmic RNA showed a greater than 320-fold higher level of expression of FcyRβ 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).

Identification of the Truncated FcyRβ. We examined the total [$^{35}$S]methionine-
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Figure 2. The titration of FcγRβ by mAb 2.4G2. 4 × 10^5 cells of B1902, B1904, D1717, and D1959 were seeded in 100-mm plates. After 24 h, the supernatants were taken from the plates and serial threefold dilutions were assayed. A lysate of S49.1 cells at 10^7 cells/ml was used as calibration. (□) B1904; (○) B1902 and D1717, FcγRβ nonsecreting transfectants; (○), D1959; (△), S49.1 lysate.

Figure 3. Cytoplasmic RNA dot hybridization. Lysates at 10^7 cells/ml were made from all cell lines except for D1959 in B, for which 10^6 cells/ml were used. Serial twofold dilutions starting from 1:4 were applied onto the nitrocellulose. (A) hybridized with the metallothionein 1 cDNA insert; (B) hybridized with the truncated FcγRβ 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.

Labeled secretion products from the original CHO DG44 cell line, D1959, and a companion cell line, D1717, which was transfected with pFcγRβ-17 and is also resistant to 2 μM methotrexate. No difference in the profile of labeled secreted proteins was detected between the two FcγR− 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 comprises 30% of the total secreted proteins. This is probably an underestimate of the actual amount, since there is only one methionine in the sequence of the truncated FcγRβ (1).

To confirm that the major peak shown in the radiolabeled products is truncated
FIGURE 4. Analysis of [³⁵S]methionine-labeled secreted products from transfected cell lines. 10⁶ 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 medium containing 75 µCi/ml of [³⁵S]methionine. The supernatant was concentrated and subjected to SDS-PAGE on 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 FcγRβ in the profile of D1959 but not in that of D1717 occupies 30% of the total area. In the insert we show the mobility of the truncated FcγRβ (arrow) and molecular weight standards: myosin (H chain), 200,000; phosphorylase b, 97,400; BSA, 68,000; OVA, 43,000; and α-chymotrypsinogen, 25,700 (Bethesda Research Laboratories, Gaithersburg, MD).

FcγRβ, the immunoreactivity of the proteins released from D1959 was examined by immunoblotting after SDS-PAGE. The rabbit anti-FcγR antibodies specifically bound to the same 31,000 Mr protein (Fig. 5A) in the secretion products of the D1959 cell line as is seen in the autoradiogram of the [³⁵S]methionine-labeled secretion products from D1959 cells in Fig. 3. FcγR from S49.1 cells is considerably larger, with a Mr of 60,000 (5). The portion that is deleted of the FcγRβ is 127 amino acids (for the β1 transcript) or 81 amino acids (for the β2 transcript) and clearly does not account for the difference in Mr seen on SDS-PAGE. The anomalous electrophoretic mobility of the truncated FcγRβ may be due to carbohydrate.

Characterization of the Truncated FcγRβ. The truncated FcγRβ was purified 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 FcγRβ and to lower the ionic strength, which leads to tighter binding of the FcγRβ to the IgG matrix. The affinity column removed >99% of immunoreactive material from the initial concentrated conditioned medium. Recovery of truncated FcγRβ from the concentrated conditioned medium was >85%. The purified protein shows the same broad electrophoretic mobility on SDS-PAGE (Fig. 7, lane C) and has the same activity with rabbit anti-FcγR antibodies in immunoblotting
FIGURE 5. Immunoblotting analysis of the truncated FcyRβ. Protein was subjected to 7-17% SDS-PAGE, transferred to nitrocellulose, and stained with: (a) mAb 6B7C; (b) rabbit anti-FcyRIgG; (c) preimmune rabbit IgG. (A) Concentrated serum-free medium from the D1959 cell line; (B) truncated FcyRβ purified on DHK10.12-Sepharose 4B. Mol wt × 10⁻⁹ shown at left.

(Fig. 5B) as the immunoreactive material in the conditioned medium. Based on amino acid composition with four tryptophan and eight tyrosine residues, the truncated FcyRβ should have an ε₂₈₀ = 33,000 M⁻¹ cm⁻¹ (30). Given an Mₚ of 31,000 for the truncated FcyRβ, the yield of purified protein by measuring OD₂₈₀ is 2.5 µg/ml conditioned medium, in good agreement with the ELISA assay titration.

The external domain of the FcyR has four cysteine residues, two located in each Ig domain (1, 2). We performed titrations using Ellman’s reagent, 5,5'-dithio-bis-(2-nitrobenzoic acid) to determine the number of free cysteine residues and found that truncated FcyRβ has no free sulfhydryl groups (Table II). 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 literature reports (26) for the yield of free sulfhydryls from model disulfide-containing
Purification of the Truncated FcγRβ from Cell Culture

| Step of purification       | Volume | Titer per ml | Total activity (titer) | Total protein | Specific activity titer/mg | Recovery of activity % |
|----------------------------|--------|--------------|------------------------|---------------|----------------------------|------------------------|
| Cell culture supernatant   | 50     | 7.0 x 10⁴    | 3.5 x 10⁵              |               |                            |                        |
| Concentrated supernatant   | 1.0    | 3.8 x 10⁵    | 3.8 x 10⁵              | 113           | 3.4 x 10⁴                  | 100                    |
| Post affinity supernatant  | 6      | 1.7 x 10⁵    | 1.0 x 10⁵              | 60            | 1.7 x 10⁴                  |                        |
| Acid eluant                | 4.5    | 7.3 x 10⁴    | 3.3 x 10⁵              | 0.37          | 8.9 x 10⁴                  | 86.8                   |

* Titer is expressed as the reciprocal of the dilution.
† Proteins were quantitated by Lowry's method (29).
‡ Comparison of the activity recovered from IgG2b affinity column.
§ 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 in 50% maximum OD₅₆₀. The total activity of the concentrated supernatant was always somewhat higher (50-100%) than that of the starting cell culture supernatant.

Titration of Truncated FcγRβ Sulfhydryl Groups with Ellman’s Reagent

| Protein                     | OD₄₁₀/M | -SH/protein | Number of S-S bonds | -SH/S-S |
|-----------------------------|---------|-------------|----------------------|---------|
| Albumin                     | 0       | 0*          | 17.5                 | —       |
| Albumin, alkali treated     | 3.25 x 10⁵ | 22.9       | 1.51                 |         |
| Truncated FcγRβ             | 0       | 0           | 1.92                 | —       |
| Truncated FcγRβ, alkali treated | 0.35 x 10⁶ | 2.51       | —                    | NA      |

* There is one free sulfide group in bovine albumin (31).
† Since titration with Ellman’s reagent of albumin without hydroxide cleavage was negative, we assume the albumin preparation was dimerized.
‡ Calculated from this experiment by dividing experimental -SH/S-S for albumin into the -SH/protein obtained for the truncated FcγRβ.

Peptides. Alkaline hydrolysis of the truncated FcγRβ 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 FcγRβ was obtained. Reverse-phase HPLC profiles of tryptic digests of the truncated FcγRβ with and without reduction were also compatible with...
the presence of intrachain disulfide bonds (data not shown). Like many other members of the Ig gene superfamily, the cysteines form intramolecular linkages in the FcγRβ and, therefore, the two repeats of the extracellular domain form two loops, although these loops are much shorter (42–45 amino acids) than in other members of the family.

The predicted amino acid sequence of mFcγR (both α and β genes) has four potential sites for N-linked glycosylation (1, 2). Limited endoglycosidase F digestion of FcγR isolated from J774 after a short pulse with [35S]methionine resulted in five intermediates, consistent with four glycosylation sites (32). The truncated FcγRβ purified by affinity chromatography on IgG2b-Sepharose 4B shows the same broad electrophoretic mobility as the intact FcγR, suggesting it is also glycosylated. Digestion of the truncated FcγRβ with N-glycosidase F confirmed the presence of carbohydrate (Fig. 7), and resulted in a deglycosylated core of 19,000 M₉, which is in agreement with the predicted length of the peptide backbone of the truncated FcγRβ, 176 amino acids. The protein could be deglycosylated only after partial denaturation, either by SDS or by boiling; intact truncated FcγR was very poorly digested by N-glycosidase F. The 12,000 M₉ difference between the glycosylated and deglycosylated truncated FcγRβ is somewhat less than that found for J774 FcγR (60,000 versus 37,000 M₉).

The Biological Activity of the Truncated FcγRβ. The purification of truncated FcγRβ by affinity chromatography on IgG2b-Sepharose demonstrated the retention of specificity of the recombinant truncated receptor. To determine the isotype specificity of the truncated FcγRβ, we examined the binding of [35S]-

DNP₂₀BSA–anti-DNP mAb complexes to truncated FcγRβ adsorbed to microtiter plates. Labeled immune complexes formed from IgG1 anti-DNP mAbs (U7.6 and DHK 109.3), IgG2a (U7.6), and IgG2b (DHK10.12) bound to the truncated FcγRβ (Fig. 8). The avidity of the truncated FcγRβ for IgG1 antibody–antigen complexes 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. No binding was seen for the MPC-11 control (Fig. 8D). From these results, we conclude that the affinity of the truncated FcγRβ for murine IgG isotypes as immune complexes is IgG1>IgG2b=IgG2a.

Using the same assay for binding of immune complexes, we examined the effect of pH on truncated FcγRβ activity using the IgG1 anti-DNP mAb U7.6. There was more binding of the labeled DNP₂₀BSA in immune complexes at more acid pH (Fig. 9). This differs from a previous report by Mellman and Unkeless (5) in which the binding of labeled intact FcγR, isolated from macro-

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\[ \text{FIGURE 7. N-glycosidase F digestion of truncated FcγRβ. 25 µg of truncated FcγRβ was digested with 0.25 U N-glycosidase F, and then subjected to SDS-PAGE on a 12.5% gel. (Lane A) Molecular weight standards; (lane B) 25 µg of native truncated FcγRβ digested with 1.5 U N-glycosidase F; (lane C) purified truncated FcγRβ without digestion; (lanes D–G) truncated FcγRβ boiled for 5 min with either 0.5% (D), 0.1% (E), 0.02% (F), or no (G) SDS before digestion.} \]
Figure 8. Binding of different subclasses of IgG immune complexes by 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 [125I]DNP-αBSA (1 μg/ml, 1.2 × 10^4 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 [125I]DNP-αBSA. (Filled symbols) truncated FcγRII-coated wells; (open symbols) control wells. (A) IgG1, (C) U7.6, (Δ) DHK 109.3; (B) IgG2b, (C) DHK 10.12, (Δ) U12.5; (C) IgG2a, U7.27; (D) IgG2b, MPC-11.
A TRUNCATED Fcγ RECEPTOR

Binding by truncated FcγRβ of IgG1 U7.6 immune complexes as a function of pH. Citrate-phosphate buffer was used for pH 3–5, sodium phosphate buffer for pH 6–8, and glycine buffer for pH 9–10 (33). All buffers were adjusted to 10 mM and contained 0.1 M NaCl. The immune complex was prepared as described in Fig. 8 with a final concentration of 5 μg/ml U7.6 and 0.5 μg/ml [125I]-DNP-20BSA. The immune complex was diluted 10-fold with the different buffers before transfer into the truncated FcγRβ-coated wells and control wells. (○) truncated FcγRβ-coated wells; (●) control wells.

The failure of FcγR internalized with immune complexes to recycle to the cell surface, but rather to be digested in the lysosomal compartment (34, 35), is more easily explained by the failure of the receptor–ligand complex to dissociate at acid pH. The intact FcγR in the absence of detergent exists in a protein micelle, due to aggregation of the hydrophobic transmembrane 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 FcγRβ was adsorbed onto the plastic surface.

Mapping of the Epitope Recognized by mAb 6B7C. 6B7C is an anti-moFcγR mAb that effectively reacts with FcγR from all cells after SDS-PAGE and transfer to nitrocellulose. However, it reacts variably with FcγR on viable FcγR+ 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 (36). When we attempted to detect the secreted FcγRβ using mAb 6B7C, we were surprised to find that this antibody did not recognize the truncated FcγRβ on immunoblots (Fig. 5). Furthermore, although in the ELISA assay, mAb 6B7C bound to the FcγRβ from detergent lysates of S49.1 cells as well as mAb 2.4G2 did, it failed to react with the truncated FcγRβ (Fig. 10A).

Since the truncated FcγRβ is missing the 8 amino acids nearest the membrane of the external domain, the epitope may be in the deleted peptide. Alternatively, it is also possible that the FcγRβ made in CHO cells is different by virtue of altered glycosylation, or that the truncated FcγRβ, due to altered conformation, has lost the 6B7C epitope. To test these possibilities, we synthesized the peptide ITVQGPSSRSLPV (amino acids 169–183 of the FcγRβ) and tested this peptide, coupled to OVA, as a ligand. As can be seen in Fig. 10B, 6B7C bound to ITVQGPSSRSLPV-OVA-coated wells in a dose-dependent fashion, but there was no binding of either 6B7C to OVA-coated wells, or 2.4G2 to either OVA- or ITVQGPSSRSLPV-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 FcγRβ). Furthermore, since the predicted FcγRa and FcγRβ protein sequences are quite different in this region, mAb 6B7C is a FcγRβ-specific probe, which we have confirmed in preliminary experiments (Schreiber,
We have isolated a recombinant secreted FcγRβ molecule by deletion of the transmembrane and cytoplasmic domains encoding sequence from a FcγRβ1 cDNA clone, and insertion of the truncated cDNA into a eukaryotic expression vector, pcEXV-3. To express and amplify the production of the truncated FcγRβ 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 FcγRβ, which can be readily purified by affinity chromatography on IgG-Sepharose. The truncated FcγRβ has a $M_r$ of 31–33,000 on SDS-PAGE and is glycosylated. N-glycosidase F cleavage reduces the $M_r$ to 19,000, 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$_{20}$BSA and anti-DNP mAbs 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.

We were surprised to observe that the truncated FcγRβ 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 FcγRβ, which is just outside the plasma membrane.

The availability of the truncated FcγRβ in microgram quantities should facilitate further analysis of structure and function of these receptors.

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References

1. Ravetch, J. V., A. D. Luster, R. Weinschank, J. Kochan, A. Pavkovec, D. A. Portnoy, J. Hulmes, Y-C. E. Pan, and J. C. Unkeless. 1986. Structure heterogeneity and functional domains of murine immunoglobulin G Fc receptor. Science (Wash. DC). 234:718.

2. Lewis, V. A., T. Koch, H. Plutner, and I. Mellman. 1986. A complementary DNA clone for a macrophage-lymphocyte Fc receptor. Nature (Lond.). 324:372.

3. Hibbs, M. L., I. D. Walker, L. Kirzbaum, G. A. Pietersz, N. J. Deacon, G. W. Chambers, I. F. McKenzie, and P. M. Hogarth. 1986. The murine Fc receptor for immunoglobulin: purification, partial amino acid sequence, and isolation of cDNA clones. Proc. Natl. Acad. Sci. USA. 83:6980.

4. Unkeless, J. C. 1979. Characterization of a monoclonal antibody directed against mouse macrophage and lymphocyte Fc receptors. J. Exp. Med. 150:580.

5. Mellman, I. S., and J. C. Unkeless. 1980. Purification of a functional mouse Fc receptor through the use of a monoclonal antibody. J. Exp. Med. 152:1048.

6. Urlaub, G., P. J. Mitchell, E. Kas, L. A. Chasin, V. L. Funanage, T. T. Myoda, and J. Hamlin. 1986. Effect of gamma rays at the dihydrofolate reductase locus: deletions and inversions. Somatic Cell. Mol. Genet. 12:555.

7. Sells, M. A., J. Chernoff, A. Cerda, C. Bowers, D. A. Shafritz, N. Kase, J. K. Christman, and G. Acs. Long-term culture and passage of human fetal liver cells that synthesize albumin. In Vitro. 21:216.

8. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 545 pp.

9. Miller, J., T. R. Malek, W. J. Leonard, W. C. Greene, E. M. Shevach, and R. N. Germain. 1985. Nucleotide sequence and expression of a mouse interleukin 2 receptor. J. Immunol. 134:4212.

10. Mitchell, P. J., A. M. Carothers, J. H. Han, J. D. Harding, E. Kas, L. Venolia, and L. A. Chasin. 1986. Multiple transcription start sites, DNase I-hypersensitive sites, and an opposite-strand exon in the 5' region of the CHO dhfr gene. Mol. Cell. Biol. 6:425.

11. Wigler, M., A. Pellicer, S. Silverstein, R. Axel, G. Urlaub, and L. Chasin. 1979. DNA-mediated transfer of the adenine phosphoribosyl transferase locus into mammalian cells. Proc. Natl. Acad. Sci. USA. 76:1373.

12. Kaufman, R. J., L. C. Wesley, A. J. Spiliotes, A. D. Gossels, S. A. Latt, G. R. Larsen, and R. M. Kay. 1985. Coamplification and coexpression of human tissue-type plasminogen activator and murine dihydrofolate reductase sequences in Chinese hamster ovary cells. Mol. Cell. Biol. 5:1750.

13. Unkeless, J. C., and G. A. Healey. 1982. Quantitation of proteins and internal antigen pools by a monoclonal sandwich radioimmune assay. J. Immunol. Methods. 56:1.

14. Khayat, D., C. Geffrier, S. Yoon, E. Scigliano, C. Soubrane, M. Weil, J. C. Unkeless, and C. Jacquillat. 1987. Soluble circulating Fcγ receptor in human serum: a new ELISA assay for specific and quantitative detection. J. Immunol. Methods. 100:235.
15. White, B. A., and F. C. Bancroft. 1982. Cytoplasmic dot hybridization: simple analysis of relative mRNA levels in multiple small cell or tissue samples. J. Biol. Chem. 257:8569.

16. Dobner, P. R., E. S. Kawasaki, L.-Y. Yu, and F. C. Bancroft. 1981. Thyroid or glucocorticoid hormone induces pre-growth-hormone mRNA and its probable nuclear precursor in rat pituitary cells. Proc. Natl. Acad. Sci. USA. 78:2230.

17. Andersen, R. D., B. W. Birren, T. Ganz, J. E. Piletz, and H. R. Herschman. 1983. Molecular cloning of the rat metallothionein 1 (MT-1) mRNA sequence. DNA (NY). 2:15.

18. Neville, D. M., Jr. 1971. Molecular weight determination of protein-dodecyl sulfate complexes by gel electrophoresis in a discontinuous buffer system. J. Biol. Chem. 246:6328.

19. Towbin, H., T. Staehehin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA. 76:4350.

20. Zalis, M., and C. L. Jaffe. 1987. Routine dot-blot assay of multiple serum samples using a simple apparatus. J. Immunol. Methods. 101:261.

21. Unkeless, J. C. 1977. The presence of two Fc receptors on mouse macrophages: evidence from a variant cell line and differential trypsin sensitivity. J. Exp. Med. 145:931.

22. Fraker, P. J., and J. C. Speck, Jr. 1978. Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. Biochem. Biophys. Res. Commun. 80:849.

23. Glass, J. D., and A. Buku. 1987. Thioether linkages between peptide synthetic intermediates and solid supports. In Peptides 1986: Proceedings of the 19th European Peptide Symposium. D. Theodoropoulos, editor. Walter de Gruyter, Berlin. 163–165.

24. Youle, R. J., and D. M. Neville, Jr. 1980. Anti-Thy 1.2 monoclonal antibody linked to ricin is a potent cell-type-specific toxin. Proc. Natl. Acad. Sci. USA. 77:5483.

25. Ellman, G. L. 1959. Tissue sulfhydryl groups. Arch. Biochem. Biophys. 82:70.

26. Anderson, W. L., and D. B. Wetlauffer. 1975. A new method for disulfide analysis of peptides. Anal. Biochem. 67:493.

27. Tarentino, A. L., C. M. Gomez, and T. H. Plummer, Jr. 1985. Deglycosylation of asparagine-linked glycans by peptide: N-glycosidase F. Biochemistry. 24:4665.

28. Southern, J. P., and P. Berg. 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. 1:327.

29. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265.

30. Mihalyi, E. 1970. Numerical values of the absorbances of the aromatic amino acids in acid, neutral and alkaline solutions. In Hand Book of Biochemistry: Selected Data for Molecular Biology. 2nd ed. H. A. Sober, editor. The Chemical Rubber Co., Cleveland. B75–B77.

31. Brown, J. R. 1977. Structure and evolution of serum albumin. In Albumin: Structure, Biosynthesis, Function. T. Peters and I. Sjoholm, editors. Pergamon Press, Oxford. 1–10.

32. Green, S. A., H. Plutner, and I. Mellman. 1985. Biosynthesis and intracellular transport of the mouse macrophage Fc receptor. J. Biol. Chem. 260:9867.

33. Gomori, G. 1955. Preparation of buffers for use in enzyme studies. Methods Enzymol. 1:138.

34. Mellman, I. S., H. Plutner, R. M. Steinman, J. C. Unkeless, and Z. A. Cohn.
Internalization and degradation of macrophage Fc receptors during receptor-mediated phagocytosis. 1983. *J. Cell Biol.* 96:887.

35. Mellman, I. and H. Plutner. 1984. Internalization and degradation of macrophage Fc receptors bound to polyvalent immune complexes. *J. Cell Biol.* 98:1170.

36. Pure, E., M. D. Witmer, J. B. Lum, I. Mellman, and J. C. Unkeless. 1988. Properties of a second epitope of the murine Fc receptor for aggregated IgG. *J. Immunol.* 139:4152.