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, NH_2-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 $\beta 2$ domain of mouse Eβ and the most distal NH_2-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)[1]-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 secretes 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

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

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 [^{35}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 (Weitzmann 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 $TNP_{25}BSA$ Sepharose-4B column, and the bound mAb was eluted with 0.1 M DNP, 0.1 M 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 β_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, GCTTAATTAATTAAGC (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 × 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 (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.

Immunoassays for FcγRβ (ELISA). FcγRβ in cell culture medium or purified FcγRβ was titered by a modification of the monoclonal sandwich radioimmune assay 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-[^{32}P]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 pFcγRβ-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 (IgG2bκ) 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 OD_{280} returned to baseline. The bound truncated FcγRβ 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.

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 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; Immunetics, 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% Na_2CO_3, and after gel filtration, the extent of substitution was determined as described previously (21). The $DNP_{20}BSA$ (50 μg) was then radiolabeled with 1 mCi of carrier-free Na[^{125}I] (Amersham Corp.) using tubes coated with 1 μg of iodogen (Pierce Chemical Co.) (22). Unincorporated ^{125}I was removed by gel filtration on a G-25M column. The labeled protein had the sp act of 1.25×10^7 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 quenched by 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 [^{125}I]$DNP_{20}BSA$ 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 ITVQGPKSSRSLPVL, 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 sulfhydryl group, the bifunctional reagent sulfo-*m*-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, ITVQGPKSSRSLPVL-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 sulfhydryl 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 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 (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 pFcγRβ-19, 9 secreted immunoreactive material, but none of the 64 clones transfected with pFcγRβ-17 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.

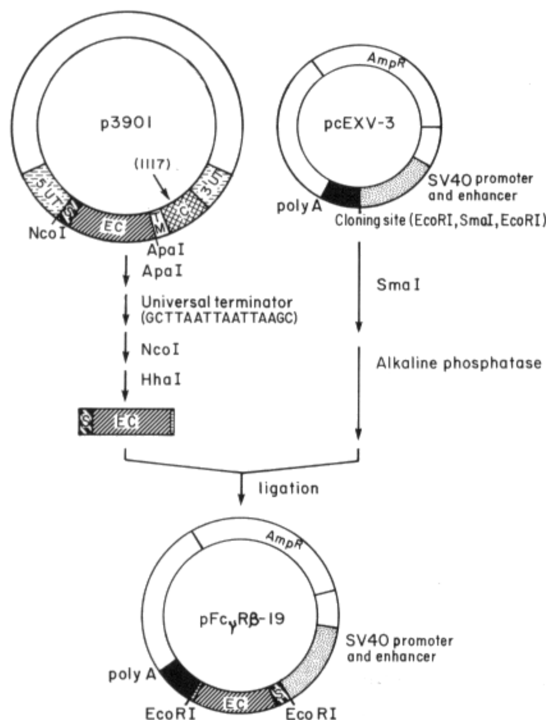

FIGURE 1. Construction of the truncated FcγRβ cDNA and the expression vector. The plasmid containing the FcγRβ1 cDNA insert is illustrated on top left with the predicted coding regions (UT for untranslated region, S for signal sequence, EC for extracellular domains, TM for transmembrane domain, and C for cytoplasmic domain) and the restriction sites for Apa I and Nco I. Plasmid 3901 is a FcγRβ1 cDNA clone that 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.

To obtain a higher level of secretion, we transfected pFcγRβ-17 and pFcγRβ-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 FcγRβ expression plasmid DNA. The FcγRβ-secreting clones were isolated from mass culture after amplification resulting in resistance to 2 μM methotrexate. The level of secretion of truncated FcγRβ by the D1959 cell line is elevated over 2,000-fold relative to the B1904 melanoma transfectant (Fig. 2). As expected, none of the pFcγRβ-17–transfected CHO cells secreted immunoreactive material. Although 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, with a value of 5×10^4 FcγR per S49.1 cell (results not presented), that the concentration of truncated FcγRβ in medium conditioned by confluent D1959 cells is 2–3 μg/ml/d.

The much higher level in synthesis of the truncated FcγRβ 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 FcγRβ mRNA. Hybridization of nick translation–labeled insert of pFcγRβ-17 cDNA to cytoplasmic RNA showed a greater than 320-fold higher level of expression of FcγRβ message by the D1959 cell line relative to B1904 and S49.1 cell lines (Fig. 3*B*). 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. 3*A*).

Identification of the Truncated FcγRβ. We examined the total [^{35}S]methionine-

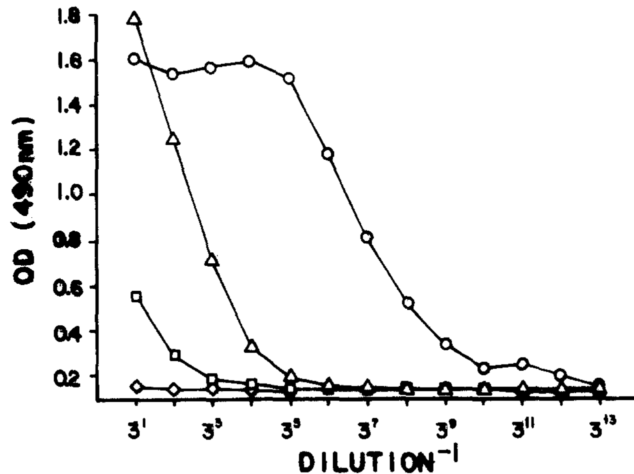

FIGURE 2. The titration of FcγRβ by mAb 2.4G2. 4 × 10^5 cells of B1902, B1904, D1717, and D1959 were seeded in 100-mm plates. After 24 h, the supernatants were taken from the plates and serial threefold dilutions were assayed. A lysate of S49.1 cells at 10^7 cells/ml was used as calibration. (□) B1904; (◇) B1902 and D1717, FcγRβ nonsecreting transfectants; (○), D1959; (△), S49.1 lysate.

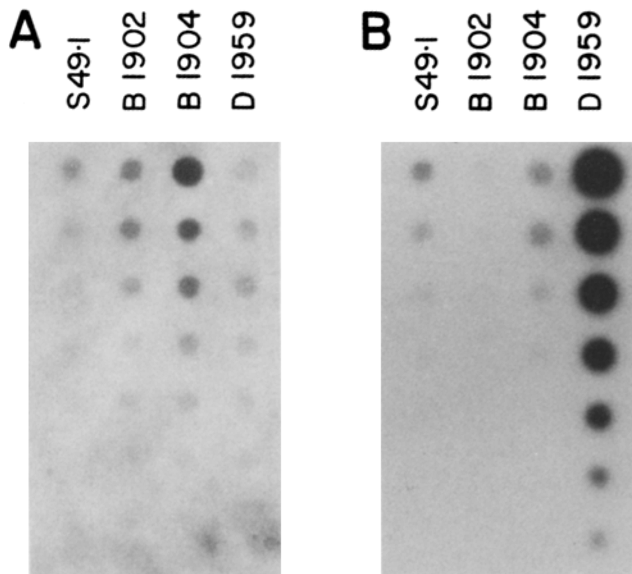

FIGURE 3. Cytoplasmic RNA dot hybridization. Lysates at 10^7 cells/ml were made from all cell lines except for D1959 in *B*, for which 10^6 cells/ml were used. Serial twofold dilutions starting from 1:4 were applied onto the nitrocellulose. (*A*) hybridized with the metallothionein 1 cDNA insert; (*B*)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

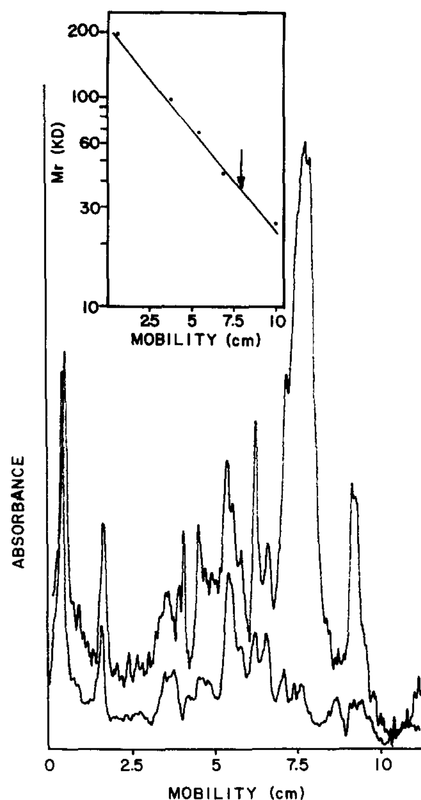

FIGURE 4. Analysis of [35S]methionine-labeled secreted products from transfected cell lines. 10^6 cells plated in 60-mm tissue culture dishes for 12 h were rinsed in PBS and labeled for 6 h in 2 ml of methionine-free medium containing 75 μCi/ml of [35S]methionine. The supernatant was concentrated and 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 M_r protein (Fig. 5*A*) 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 M_r 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 M_r 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

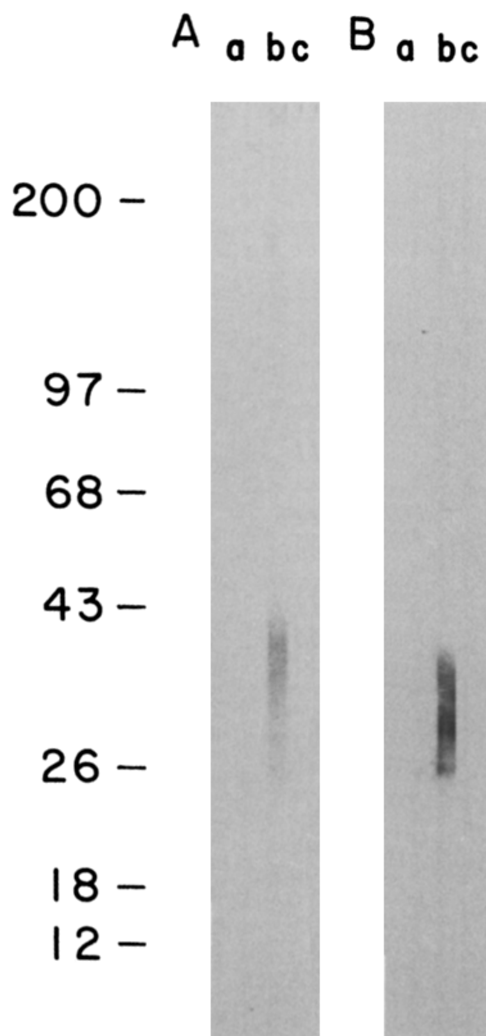

FIGURE 5. Immunoblotting analysis of the truncated FcγRβ. Protein was subjected to 7–17% SDS-PAGE, transferred to nitrocellulose, and stained with: (*a*) mAb 6B7C; (*b*) rabbit anti-FcγR IgG; (*c*) preimmune rabbit IgG. (*A*) Concentrated serum-free medium from the D1959 cell line; (*B*) truncated FcγRβ purified on DHK10.12-Sepharose 4B. Mol wt × 10^{-3} shown at left.

(Fig. 5*B*) as the immunoreactive material in the conditioned medium. Based on amino acid composition with four tryptophan and eight tyrosine residues, the truncated FcγRβ should have an ϵ_{280} = 33,000 M^{-1} cm^{-1} (30). Given an M_r of 31,000 for the truncated FcγRβ, the yield of purified protein by measuring OD_{280} is 2.5 μg/ml conditioned medium, in good agreement with the ELISA assay titration.

The external domain of the FcγR 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 FcγRβ 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

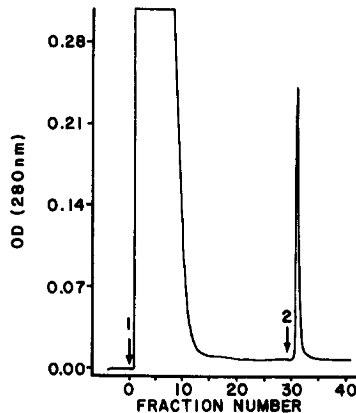

FIGURE 6. Purification of the truncated FcγRβ. 150 ml of D1959 supernatant was concentrated to 2 ml by ammonium sulfate precipitation, dialyzed versus 10 mM sodium phosphate buffer, pH 7.5, and loaded (*arrow 1*) onto the DHK10.12-Sepharose 4B column (3 ml of bed volume). The column was washed with 50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

TABLE I

Purification of the Truncated FcγRβ from Cell Culture

Step of purification	Volume	Titer per ml*	Total activity (titer)	Total protein‡	Specific activity	Recovery of activity§
	ml			*mg*	*titer/mg*	*%*
Cell culture supernatant	50	7.0×10^{3} ‖	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

* 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 OD_{490}. The total activity of the concentrated supernatant was always somewhat higher (50–100%) than that of the starting cell culture supernatant.

TABLE II

Titration of Truncated FcγRβ Sulfhydryl Groups with Ellman's Reagent

Protein	OD_{412}/M	-SH/protein	Number of S-S bonds	-SH/S-S
Albumin	0	0*	17.5‡	—
Albumin, alkali treated	3.25×10^{5}	22.9	—	1.31
Truncated FcγRβ	0	0	1.92§	—
Truncated FcγRβ, alkali treated	0.35×10^{5}	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

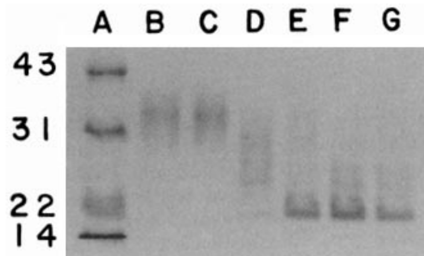

FIGURE 7. *N*-glycosidase F digestion of truncated FcγRβ. 25 μg of truncated FcγRβ was digested with 0.25 U *N*-glycosidase F, and then subjected to SDS-PAGE on a 12.5% gel. (Lane *A*) Molecular weight standards; (lane *B*) 25 μg of native truncated FcγRβ digested with 1.5 U *N*-glycosidase F; (lane *C*) purified truncated FcγRβ without digestion; (lanes *D–G*) truncated FcγRβ boiled for 3 min with either 0.5% (*D*), 0.1% (*E*), 0.02% (*F*), or no (*G*) SDS before digestion.

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 moFcγR (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 [^{35}S]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 M_r, 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 M_r difference between the glycosylated and deglycosylated truncated FcγRβ is somewhat less than that found for J774 FcγR (60,000 versus 37,000 M_r).

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 [^{125}I]-DNP_{20}BSA–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. 8*D*). 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 DNP_{20}BSA 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-

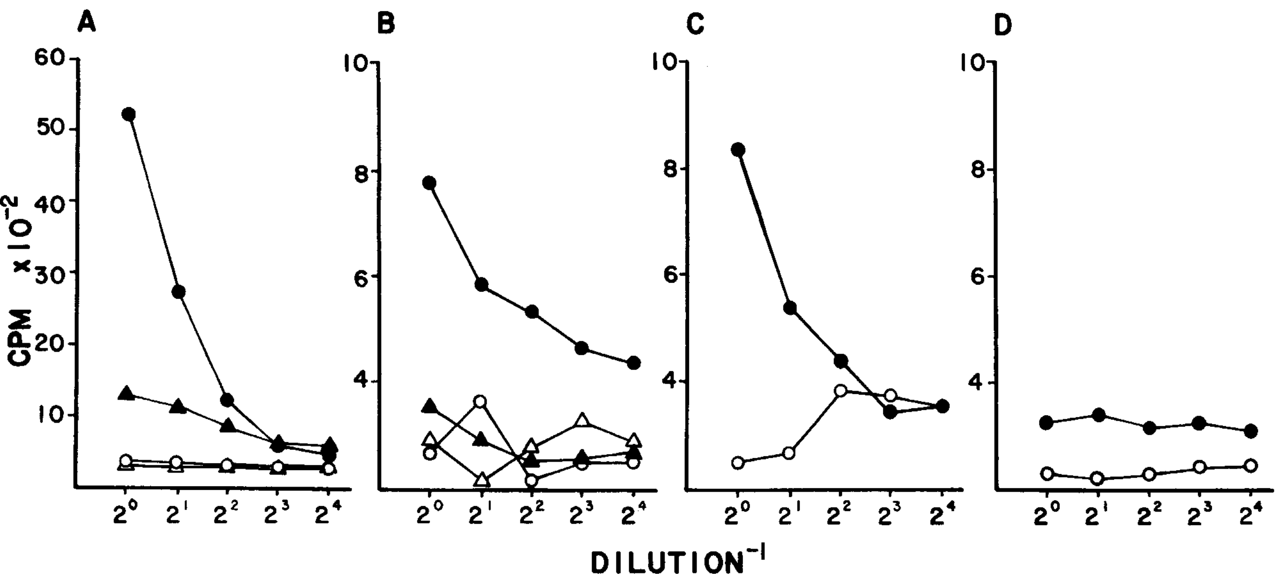

FIGURE 8. Binding of different subclasses of IgG immune complexes by truncated FcγRβ. Immune complexes were formed by incubation of 50-μl aliquots of serial dilutions of anti-DNP mAbs, beginning at 10 μg/ml of IgG, with 50 μl of [^{125}I]DNP$_{20}$BSA (1 μg/ml, 1.2 × 10^4 cpm/ng). The immune complexes were then transferred into control wells or wells coated with truncated FcγRβ. 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$_{20}$BSA. (*Filled symbols*) truncated FcγRβ-coated wells; (*open 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.

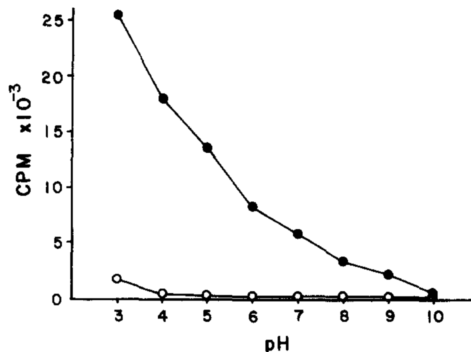

FIGURE 9. Binding by truncated FcγRβ of IgG1 U7.6 immune complexes as a function of pH. Citrate–phosphate buffer was used for pH 3–5, sodium phosphate buffer for pH 6–8, and glycine buffer for pH 9–10 (33). All buffers were adjusted to 10 mM and contained 0.1 M NaCl. The immune complex was prepared as described in Fig. 8 with a final concentration of 5 μg/ml U7.6 and 0.5 μg/ml [^{125}I]-DNP$_{20}$BSA. 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.

phages, 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 (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. 10*A*).

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 ITVQGPKSSRSLPVL (amino acids 169–183 of the FcγRβ) and tested this peptide, coupled to OVA, as a ligand. As can be seen in Fig. 10*B*, 6B7C bound to ITVQGPKSSRSLPVL-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 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 FcγRβ). Furthermore, since the predicted FcγRα 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,

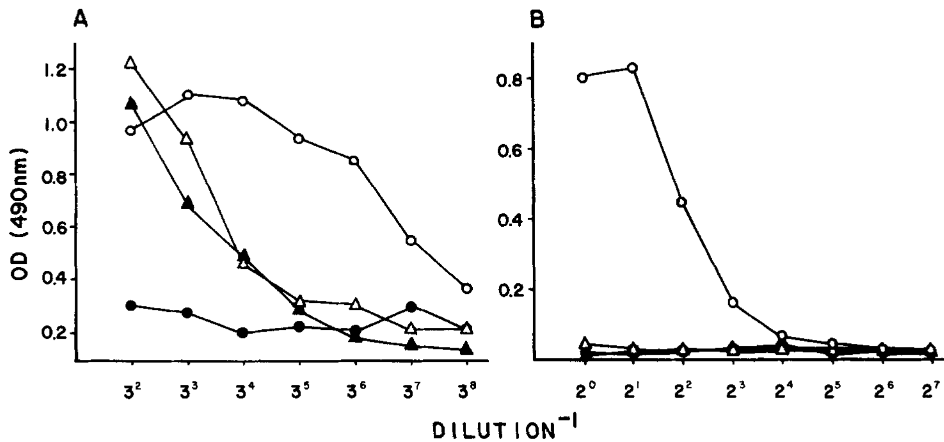

FIGURE 10. Identification of the epitope of FcγRβ recognized by mAb 6B7C. (*A*) Reactivity of mAbs 6B7C (● and ▲) and 2.4G2 (○ and △) with conditioned medium from the D1959 cell line (● and ○) and a NP-40 lysate (10^7 cells/ml) of S49.1 cells (▲ and △). (*B*) Reactivity of mAbs 2.4G2 (● and ▲) and 6B7C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

R., 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 FcγRβ that fail to bind mAb 6B7C.

Summary

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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