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Recombinant soluble Fcγ receptors: production, purification and biological activities

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Abstract

Soluble forms of low affinity receptors for the Fc portion of IgG circulate in body fluids and regulate immune functions. We describe the transfection, production and purification techniques which allow the preparation, at a laboratory scale, of milligram amounts of glycosylated recombinant mouse and human soluble Fcγ receptors. These recombinant products bind IgG and are biologically active on immune responses, like their normal counterparts.

1. Introduction

Receptors for the Fc portion of IgG (FcγR) are a heterogeneous family of membrane proteins that mediate a variety of effector and regulatory functions in hematopoietic cells.

In mice and humans, three types of receptors bind IgG. FcγRI are high affinity receptors for monomeric IgG while FcγRII and FcγRIII bind IgG-containing immune complexes with low affinity. FcγRII are found on lymphocytes, macrophages, platelets, polymorphonuclear cells, mast and Langerhans cells. FcγRIII is the only FcγR expressed by NK cells, and is co-expressed with other FcγR on macrophages, basophils, neutrophils and Langerhans cells (for reviews see refs. 1 and 2).

A significant structural homology, characteristic of the Ig supergene family, defines the FcγR family. In mice, a single gene encodes two transmembrane FcγRII glycoproteins, generated by alternative splicing of the first intracytoplasmic exon. Both receptors are composed of two Ig-like extracellular domains, an hydrophobic transmembrane region and a 94 amino acids (FcγRIIb1) or 47 amino acids long (FcγRIIb2) intracellular tail, respectively. FcγRIIb1 is expressed by lymphocytes, mast and polymorphonuclear cells while FcγRIIb2 is found mostly on macrophages. One gene encodes the IgG-binding α-chain of FcγRIII, which has 95% amino acid sequence homology with FcγRII in its ectodomain. It differs from the latter by its transmembrane and intracellular regions and by the fact that its expression requires its association with a homodimer of γ-chains that are also present in the high affinity FcεRI complex.

In humans, three genes encode FcγRII, where additional protein diversity is provided by alternative splicing of the first intracellular exon

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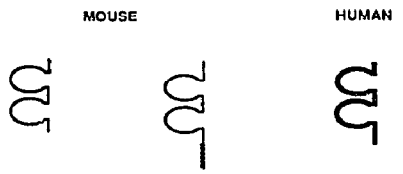
generating two transmembrane isoforms of FcγRIIB [3]. Two genes encode two isoforms of FcγRIII, the NK- and macrophage-specific FcγRIII-A being homologous to its murine counterpart with a transmembrane α-chain associated to a dimer of γ-chains or, in some NK cells to dimers of ζ- or γ/ζ-chains. On neutrophils, FcγRIII-B is a phosphatidyl-inositol anchored molecule. Within the CD nomenclature, FcγRI have been classified as CD64, FcγRII as CD32 [2] and FcγRIII as CD16.

Previously called IgG-Binding Factors (IgG-BF) [4], soluble forms of low affinity FcγR (sFcγR) have been described, both in mice and in humans, in supernatants of cells of the immune system such as activated T and B cells, macrophage and monocyte cell lines, polymorphonuclear cells [1,5] as well as in biological fluids such as serum [6], saliva [7] and colostrum (C. Sautès, unpublished data). At least, two mechanisms were shown to generate these soluble forms, proteolytic cleavage of the membrane receptors or alternative splicing of the FcγRII gene exon(s). The cleavage that occurs in the EC region, close to the cell surface, is made by enzymes not yet characterized, and allows the release of soluble forms corresponding to the two EC domains. This phenomenon was described in the mouse for FcγRIIb1 on activated B and T cells, B and T cell lines [1,4,5] and on fibroblasts expressing a recombinant form of this receptor [8], for FcγRIII on activated NK cells [1], and for FcγRIIb2 and/or FcγRIII on macrophage cell lines [9] as well as on Langerhans cells (In preparation). These soluble forms bind mouse IgG1, IgG2a, IgG2b, but not IgG3, and react with the 2.4G2 mAb [8], like the membrane forms of the receptors. The soluble forms produced by cleavage of FcγRII and FcγRIII (sFcγRII and sFcγRIII, respectively) are structurally very similar, due to the 95% sequence homology in the EC regions of these receptors. They have an apparent molecular mass of 38–40 kDa and after deglycosylation, give rise to a 19 kDa polypeptide [1,8]. In man, soluble forms that comprise only EC region are generated by enzymatic cleavage of membrane-anchored FcγRIIIA on NK cells [10] and FcγRIIIB on neutrophils [11]. They have an apparent molecular mass of 48 kDa, bind human IgG1, IgG3 isotypes and react with the same mAbs than the membrane receptors. Release of human sFcγRII from cell membrane by proteolytic cleavage seems also to occur at least in activated B cells [12] and in Langerhans cells [13].

Both in mouse and in man, an alternative splicing of the TM encoding exon was also shown to generate sFcγR. In mouse, splicing of the exon encoding the TM region of FcγRII generates a mRNA encoding a TM-deleted FcγRIIb2. This mRNA is found in cells from a macrophage cell line P388D1 [9], and in Langerhans cells (In preparation). Immunochemical analyses showed that the soluble FcγR is composed of the EC region of FcγRII and of the IC region of FcγRIIb2. This new FcγRII isoform is called FcγRIIb3 [9]. In man, a FcγRIIA mRNA lacking the TM domain-encoding exon has been also observed in erythroleukemia (K562) and histocytic (U937) cells lines, in Langerhans cells [13] and in platelets (A. Astier, personal communication).

Soluble FcγR play important roles in immunoregulation not only because they interact with the Fc portion of IgG and thereby block the Fcγ-mediated functions [1,4,14] but also because they down-regulate immune functions. Semi-purified sFcγR was previously shown to inhibit *in vitro* antibody production by normal [4] and hybridoma [15] B-cells. To better define the functional roles of sFcγR *in vitro* and their potential use *in vivo* in immune disorders, we have prepared in eukaryotic cells and purified mg quantities of recombinant molecules equivalent to the natural isoforms of sFcγR. Three types of recombinant sFcγR were produced: mouse sFcγRII and FcγRIIb3 and human sFcγRIII (sCD16) (Fig. 1). The cDNA encoding sFcγR were expressed in the mouse fibroblastic L cell line [16–18] and more recently in the Baby Hamster Kidney cell line, BHK [14]. In the present paper we will describe the high level expression and production systems in BHK cells, the purification techniques developed to obtain 98% pure material and the biochemical charac-



	sFcγRII	FcγRIIb3	sFcγRIII (sCD16)
ORIGIN	ENZYMATIC CLEAVAGE	TM-EXON SPLICING	ENZYMATIC CLEAVAGE
PRODUCED BY	LYMPHOCYTES MACROPHAGES	MACROPHAGES	NK CELLS NEUTROPHILS
PRESENT IN	SERUM	SERUM	SERUM
APPARENT Mr			
POLYPEPTIDE	19 000	25 000	30 000
GLYCOPROTEIN	38-40 000	40-44 600	48 000

Fig. 1. Characteristics of the natural equivalents of soluble FcγR produced in recombinant form.

teristics and functional properties of the recombinant products. The titration methods developed to follow production and purification of sFcγR, such as the immunodot assays and ELISA have been described elsewhere [14].

2. Production of recombinant soluble FcγR

2.1. Transfection of cDNA

Preparation of sFcγR cDNA

The cDNA encoding truncated sFcγR were produced by introducing stop codons in cDNA encoding membrane forms of the receptors. In mouse, site-directed mutagenesis of FcγRIIb1 cDNA was used to convert the membrane form of FcγRIIb1 into a 174-amino acid-long sFcγRII [16]. In man, a stop-linker was introduced in cDNA encoding FcγRIIIB (NA2 form), resulting in the production of a 194-amino acid-long sFcγRIII containing six accessory amino acid residues (ADPRLV) at its COOH terminus [17]. The cDNA encoding the murine FcγRIIb3 was constructed by PCR amplification of FcγRIIb1 by using specific oligonucleotides.

Transfection of BHK cells

To obtain clones secreting high amounts of sFcγR, a co-transfection of two selectable genes into cells from the adherent BHK (Baby Hamster Kidney) cell line was carried out [19]. One million BHK cells grown as monolayer in a 25-cm^3 flask were incubated for 30 min at 37°C in a 5% CO_2 atmosphere with one milliliter of a plasmid mixture with 20 μg pKC3 (containing cDNA encoding sFcγR), 5 μg pSV2 DHFR (containing the dihydrofolate resistance (DHFR) gene), and 5 μg pRMH140 (containing the neomycin resistance gene). Calcium phosphate precipitation was achieved by adding slowly 60 μl $CaCl_2$ (2 *M*) to 0.42 ml plasmid mixture in TE. The mixture was diluted two-fold in 0.48 ml HBS (Hank's Balanced Salt Solution) and incubated for 30 min at 20°C. Five ml culture medium (DMEM/HAM12, 1% sodium pyruvate, 1% glutamine, 10% FCS) were then added to the BHK flask. Five hours later, the supernatant was removed and BHK cells were incubated for 3 min with 5 ml of culture medium, containing 15% glycerol, washed two times and incubated in culture medium at 37°C in a 5% CO_2 atmosphere. Three days later, cells were trypsinized and the transfectants were selected with 0.4 mg/ml G418 and 1 μM methotrexate (MTX). During 14 days, medium was changed every three days and selected clones obtained by day 14 were mixed and exposed to increasing MTX concentrations in the medium (3 and 10 μM). After 15 days of selection in 10 μM MTX, production levels of sFcγR were measured by immunodot or ELISA assays [14]. For the cell lines secreting mouse sFcγRII (6/9C cell line) or FcγRIIb3 (2CII cell line) production in flasks were around 1 $\mu g/10^6$ cells/day. Higher production level was obtained with the JBIX A2 cell line secreting human sFcγRIII: 18 $\mu g/10^6$ cells/day (Table 1).

2.2. Production of sFcγR in bioreactors

In order to scale up the production, the adherent transfected cell lines were grown on hollow fibers in a perfusion cell culture system (Acusyst Jr.; Endotronics, MN, USA), which allows the continuous measurement and control of pH, dissolved oxygen and residual glucose throughout the culture. A volume of 150 ml of culture medium was harvested every week and the production of sFcγR was measured. The

Table 1
Production of sFcγR by transfected cell lines

Species	Mouse	Mouse	Human
Soluble FcγR	sFcγRII	FcγRIIb3	sFcγRIII
Cell line	6/9CII	2CII	JBIX A2
Production in flasks (mg/ml)	1.4	0.3	6
Production in bioreactors			
Amount (mg)	224 ± 54	403 ± 78	308 ± 187
Volume (l)	0.7 ± 0.2	0.6 ± 0.04	1.1 ± 0.3
Duration (days)	43 ± 14	39 ± 4	59 ± 14
Number of bioreactors	2	2	3

1.1-m^3 bioreactors were inoculated with $3 \cdot 10^8$ transfected cells. A continuous flow of culture medium (DMEM/HAM 5%FCS) was applied at 1 ml/h. By day 40 no more FCS was added. As shown in Fig. 2, kinetics of production of sFcγR in bioreactors were different between the 2CII cell line and the JBIX A2 and 6/9CII cell lines. In the former case, high productivity was reached in less than 13 days whereas in the latter ones maximum was reached one week later. Table 1 illustrates the production levels of cell lines 6/9CII secreting mouse sFcγRII, 2CII secreting mouse FcγRIIb3 and JBIX A2 secreting human sFcγRIII. Production varied also between cell lines, from around 220 mg in the case of 6/9C to 400 mg in the case of 2CII and 308 mg in that of JBIX A2. Noteworthy, concentrations of 0.5 mg/ml sFcγR could be reached in harvested culture medium by using these procedures.

3. Purification of soluble FcγR

The high sFcγR concentrations reached in hollow fibers harvests and the low percentage of fetal calf serum used (from 0 to 5%) rendered purification of sFcγR feasible in few steps.

3.1. Mouse sFcγRII

Mouse sFcγRII was purified in two or three steps, depending on the sFcγRII concentration in the starting material. For sFcγRII concentrations between 0.05 and 0.2 mg/ml, *i.e.* for a 2 to 3 mg/ml total protein concentration, two ion-exchange steps followed by affinity chromatography were required for purification [14]. For sFcγRII concentrations between 0.2 and 0.5 mg/ml, *i.e.* for a 2.8 to 4 mg/ml total protein concentration, one ion-exchange chromatography step followed by affinity chromatography led to 99-100% pure material. The following procedures describe the purification scheme for 120 ml of culture medium from 6/9CII cell line grown on hollow fibers and containing 0.4 mg/ml sFcγRII for a 2.8 mg/ml total protein concentration.

Chromatography equipment

Steps were performed by using a gradient programmer (GP250, Pharmacia, St-Quentin-en-Yvelines, France), a fraction collector (FRAC 300, Pharmacia), a UV absorbance recorder (Uvicord SII 2238, Pharmacia) and, for equilibration and ion-exchange steps, a conductivity meter (2195, Pharmacia).

Purification follow-up

Protein concentrations were estimated by measuring absorbance at 280 nm · 0.7. Detection of sFcγR in column fractions was performed by a semi-quantitative immunodot assay [14]. The sFcγR concentrations of the different pools were measured by sandwich type ELISA.

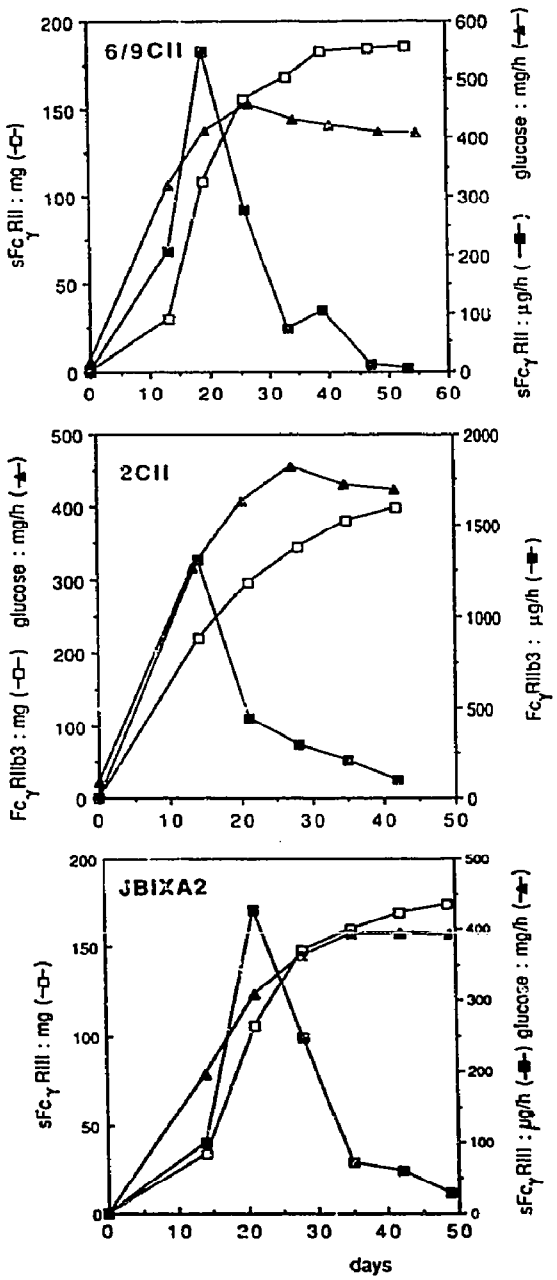


Fig. 2. Growth and productivity, on hollow fibers, of eukaryotic cell lines secreting sFcγR;(▲) glucose consumption of the bioreactors, (■) sFcγR productivity, (□) total sFcγR production of the 6/9CII cell line secreting mouse sFcγRII, 2CII cell line secreting mouse FcγRIIb3 and JBIX A2 cell line secreting human sFcγRIII.

Equilibration step

A 120-ml volume of culture medium was applied at 180 ml/h on a column (75 × 2.6 cm I.D., C26/100, Pharmacia) containing 400 ml Sephadex G25 medium (Pharmacia) equilibrated in 0.1 *M* sodium citrate buffer (pH 5). Gel was washed at the same flow-rate with the same buffer. Fractions containing proteins (absorbance at 280 nm above 0.1) and eluting before the conductivity peaks were pooled (160 ml at 2.1 mg/ml).

Cation-exchange

A 160-ml volume of material equilibrated in 0.1 *M* sodium citrate buffer (pH 5) was applied at 150 ml/h on a column (3.8 cm × 2.5 cm, 25 G200, Sepracor, Villeneuve-La-Garenne, France) containing 20 ml S-Sepharose (S-Sepharose fast flow, bead diameter 45–165 μm, Pharmacia) equilibrated in 0.1 *M* sodium citrate buffer (pH 5). Gel was washed with the same buffer at the same flow-rate. Proteins were eluted at 30 ml/h with 0.1 *M* sodium citrate buffer (pH 5) containing 0.5 *M* NaCl. The pool (25 ml) contained 2.4 mg/ml proteins.

Equilibration step

A 25-ml volume of S-Sepharose eluate was applied at 120 ml/h on a column (62 × 1.6 cm I.D., C16/100, Pharmacia) containing 150 ml Sephadex G25 medium (Pharmacia) equilibrated in 20 m*M* Tris buffer (pH 7.6). Gel was washed at the same flow-rate with the same buffer. Fractions containing proteins (absorbance at 280 nm above 0.1) and eluting before the conductivity peak were pooled (50 ml at 1.2 mg/ml).

Affinity chromatography

A 50-ml volume of material equilibrated in Tris 20 m*M* pH 7.6 buffer was applied at 15 ml/h on a column (15 × 1.6 cm I.D., 16/G200, Sepracor) containing 30 ml of insolubilized IgG prepared by coupling CNBr-activated Sepharose 4B (45–165 μm, Pharmacia) with rabbit IgG (Nordic, Tilburg, Netherlands) at 10 mg IgG per ml gel, according to manufacturer recommendations. The column was washed with the same buffer at the same flow-rate and sFcγRII was eluted with 0.1 *M* sodium acetate buffer (pH 4). Fractions with an absorbance at 280 nm above 0.1 were pooled (15 ml at 0.73 mg/ml).

Equilibration step

The sFcγRII was equilibrated in PBS (pH 7.3) by dialysis using boiled dialysis bags (Visking 8 × 32). Chromatography on Sephadex G25 (fine, Pharmacia) in the same buffer can also be performed for equilibration. Hundred percent recoveries are obtained by using a 1/4 (v/v) sample/gel ratio and a 5 cm/h flow-rate. After equilibration, sFcγRII was spun at 10 000 *g* for 15 min, and frozen at −80°C in aliquots.

Experimental conditions

All steps were performed at 4°C and buffers were made with MilliQ water (Millipore, St. Quentin en Yvelines, France) filtered through 0.22-μm filters. Tubings as well as cation-exchange columns were washed with 0.2 *M* NaOH, washed with H_2O, and kept in 20% ethanol. If endotoxin-free sFcγRII preparations were needed, sterile water was used to prepare buffers and the affinity chromatography column made with endotoxin-free IgG, was used only once [14].

Results

As shown in Table 2, this purification scheme allows the purification of sFcγRII with a 27% total yield and a purification factor of 8 Nevertheless, substantial amounts of sFcγRII (33%) were not absorbed onto the affinity chromatography column. Reapplication of the effluent onto a new IgG column did not lead to further adsorption of this material. Ion-exchange step led to complete sFcγRII adsorption, but with a

Table 2
Purification of mouse sFcγRII and human sFcγRIII

Steps	Volumes (ml)	Proteins (mg)[a]	Yield per step	Total yield (%)	sFcγR (mg)[b]	Yield per step	Total yield (%)	Purification factor per step	Purification factor (total)
Mouse									
	120	336	–	–	49	–			
G25									
	60	336	100	100	49	100	100	1	1
S-Sepharose									
Effluent	160	168	–	–	0	–	–	–	
Eluate	25	60	18	18	31.5	64	64	3.5	3.5
G25	50	60	100	18	31.5	100	64	1	3.5
IgG-Immunoadsorbent									
Effluent	50	44	–	–	10.4	–	–	–	
Eluate	15	11	18	3.3	13.2	42	27	2.3	8
Human									
	150	435	–	–	62	–	–	–	
G25									
	240	232	100	100	62	100	100	1	1
S-Sepharose									
Effluent	240	240	–	–	0.3	–	–	–	–
Eluate	55	200	45	45	30	49	49	1.1	1.1
G25	85	199	100	45	30	100	49	1	1.1
IgG-immunoadsorbent									
Effluent	8.5	111	–	–	5.2	–	–	–	–
Eluate	22.5	13.5	6.7	3	11.6	38.6	19	5.7	6.3

[a]As estimated by absorbance at 280 × 0.7.
[b]As estimated by ELISA.

64% recovery in the eluate. Modifications of pH or increase of salt concentration did not lead to better recoveries using the S-Sepharose matrix.

3.2. Mouse FcγRIIb3

A similar two-step purification scheme has been used to purify FcγRIIb3 from 50 ml of culture medium of the 2CII cell line. Both steps lead to a purification factor of 5.5 and a 22% recovery in FcγRIIb3.

3.3. Human sFcγRIII

Human sFcγRIII was purified in two steps–cation-exchange and affinity chromatography–from culture medium of JBIXA2 cell line grown on hollow fibers. The following procedures describe the purification scheme of 150 ml of culture medium containing 0.5 mg/ml sFcγRII for a 2.9 mg/ml total protein concentration.

Chromatography equipment and purification follow-up

These procedures were the same as those used for mouse sFcγRII.

Equilibration

A 150-ml volume of culture medium was applied at 180 ml/h on a column (80 × 2.6 cm I.D., C26/100, Pharmacia) containing 450 ml Sephadex G25 medium (Pharmacia) equilibrated in 0.1 *M* sodium citrate buffer (pH 5). Gel was washed at the same flow-rate with the same buffer. Fractions containing proteins (absorbance at 280 nm above 0.1) and eluting before the conductivity peak were pooled (240 ml at 1.8 mg/ml total proteins).

Cation-exchange

A 240-ml volume of material equilibrated in 0.1 *M* sodium citrate buffer (pH 5) was applied at 150 ml/h on a column (8 × 2.5 cm I.D., 25 G200, Sepracor) containing 40 ml S-Sepharose (S-Sepharose fast flow, bead diameter 45–165 μm, Pharmacia) equilibrated in 0.1 *M* sodium citrate buffer (pH 5). Gel was washed with the same buffer at the same flow-rate. Proteins were eluted at 30 ml/h with 0.1 *M* sodium citrate buffer (pH 5) containing 0.75 *M* NaCl. The pool (55 ml) contained 3.6 mg/ml proteins.

Equilibration step

A 55-ml volume of S-Sepharose eluate was applied at 120 ml/h on a column (110 × 1.6 cm I.D., C16/100, Pharmacia) containing 220 ml Sephadex G25 medium (Pharmacia) equilibrated in 20 m*M* Tris buffer pH 7.6. Gel was washed at the same flow-rate with the same buffer. Fractions containing proteins (absorbance at 280 nm above 0.1) and eluting before the conductivity peak were pooled (85 ml at 2.35 mg/ml).

Affinity chromatography

About one third of the material (30 ml) equilibrated in Tris 20 m*M* buffer (pH 7.6) was applied at 15 ml/h on a column (15 × 1.6 cm I.D., 16/G200, Supracor) containing 30 ml of IgG coupled-Sepharose 4B (prepared as described earlier) and equilibrated in the same buffer. The column was washed with the same buffer at the same flow-rate and sFcγRIII was eluted with 0.1 *M* sodium acetate buffer (pH 4). Fractions with an absorbance at 280 nm above 0.1 were pooled (10 ml at 0.45 mg/ml). The overall procedure was repeated with each of the two other thirds of material.

Equilibration and experimental conditions

These were the same as those described for mouse sFcγRII.

Results

As shown in Table 2, this purification scheme leads to the isolation of sFcγRIII with a 19% yield and a purification factor of 6.3. In contrast with mouse sFcγRII, the S-Sepharose step did not achieve any further purification of sFcγRIII. Modifications of pH or salt elution conditions did not improve the purification factor on this column. In any case, this step was maintained to avoid the direct application of culture medium on immunoadsorbents. The affinity chromatography procedure was efficient, with adsorption of 78% sFcγRIII on the insolubilized IgG-columns,

a 38.6% sFcγRIII yield and a purification factor of 5.7.

4. Characterization of recombinant sFcγR

The sizes of the sFcγR expressed in BHK cells were compared by discontinuous SDS-PAGE in 12% polyacrylamide gels stained by Coomassie blue. Before deglycosylation, sFcγR migrated as heterogeneous glycoproteins of apparent M_r between 32 and 34 000 in the case of mouse sFcγRII (Fig. 3A), 40 to 46 000 in the case of mouse FcγRIIb3 (Fig. 3A) and 47 to 56 000 in that of human sFcγRIII (Fig. 3B). After deglycosylation, the major polypeptides had an apparent M_r of 19 000 in the case of sFcγRII Fig. 3A), of 26 000 (Fig. 3A) in the case of FcγRIIb3 and 30–32 000 in the case of sFcγRIII (Fig. 3B). The sizes of the mouse sFcγR major polypeptides were identical to those of their natural equivalents [8,9]. Faint bands corresponding to components of higher M_r were present after deglycosylation of mouse sFcγR. They reflect most probably incomplete deglycosylation of sFcγR since they were absent when higher doses of glycosidases were used [14,16]. Nevertheless, when expressed in L cells, sFcγRII and sFcγRIII glycoproteins were of higher apparent size M_r (38–40 000 and 48 000 respectively) [16–18] than when expressed in BHK cells, suggesting a glycosylation difference between the recombinant products made in these different cell types.

The p*I* of the various sFcγR were also examined on 5% polyacrylamide slab gels using pH 3–10 ampholine gradients [20]. As shown in Fig. 4, the three sFcγR differed in their apparent respective p*I*, sFcγRII being located between pH 7 and 9, FcγRIIb3 between pH 4.8 and 6.6

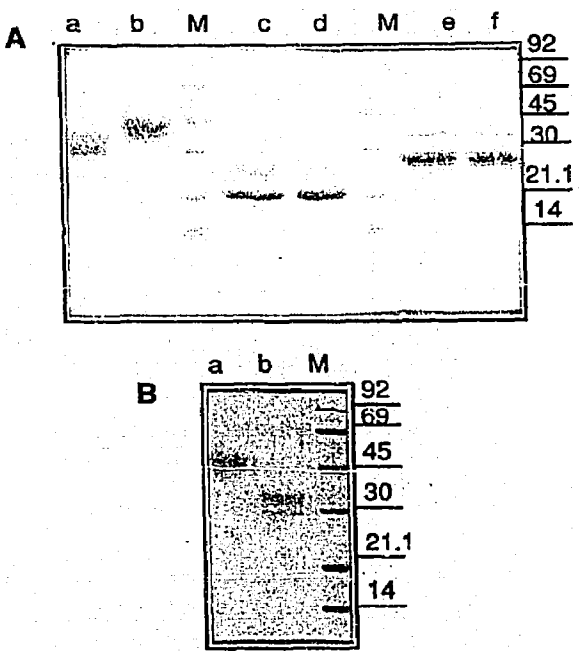


Fig. 3. Size analysis, of recombinant mouse (A) and human (B) sFcγR, as determined by discontinuous SDS-PAGE and Coomasie blue staining. (A) 5 μg of sFcγRII deglycosylated or not (a) with PNGase (c) or N-glycanase (d), and 5μg of FcγRIIb3 deglycosylated or not (b) with PNGase (e) or N-glycanase (f) were boiled in the presence of SDS and MEO and applied to a 12% polyacrylamide gel. (B) 5 μg of sFcγRIII were deglycosylated (b) or not (a) with N-glycanase, boiled in the presence of SDS and MEO and applied to a 12% polyacrylamide gel. M: molecular mass markers.

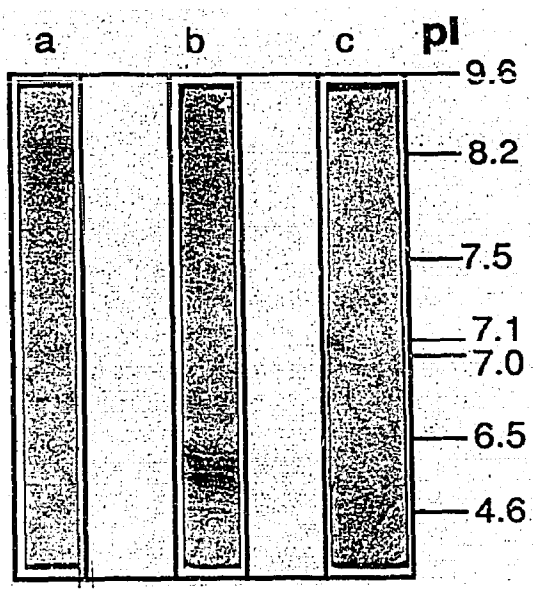


Fig. 4. Isoelectric point determination for soluble FcγR: 5 μg sFcγRII (a), FcγRIIb3 (b) and FcγRIII (c) were applied to 5% polyacrylamide gels and subjected to electrophoresis in the presence of pH 3-9 ampholines. Location of p*I* markers are shown on the right.

and sFcγRIII between pH 4 and 6. A high degree of charge was observed for each sFcγR, reflecting most probably glycosylation variability.

5. Biological activities of sFcγR

5.1. IgG-binding activity

The various sFcγR were shown to bind to the Fc portion–and not to Fab'2 fragments–of IgG, and to have the same isotypic specificity and reactivity towards mAbs as the EC domains of the entire receptors. Mouse sFcγRII binds 2.4G2 mAb and murine IgG1, IgG2a, IgG2b but not IgG3 [14,16], whereas human sFcγRIII binds human IgG1, IgG3, not significantly to human IgG2 and IgG4, and binds 3.G8 and BW209 mAbs [14,17].

5.2. Effect of sFcγR on antigen presentation

In the mouse, the addition of IgG antibodies directed to antigen increases the antigen presentation by antigen presenting cells. This phenomenon is due to the binding of the immune complexes to FcγRIIb2, allowing their endocytosis via coated pits, and the subsequent expression of peptides on membrane ClassII molecules [21]. We have recently shown that mouse sFcγRII as well as FcγRIIb3 inhibit antigen presentation by murine Langerhans cells and that this phenomenon is due to the masking of the Fc portion of IgG antibodies with sFcγR. Both sFcγRII and FcγRIIb3 had the same ability to inhibit this phenomenon, confirming that they most probably act via their identical EC regions (In preparation).

5.3. Effect of sFcγR on B cell functions in vitro and in vivo

For several years, the availability of pure recombinant sFcγRII allowed the demonstration that sFcγR exert potent inhibition of in-vitro antibody responses towards T-dependent antigens such as SRBC. When added on day 0, together with SRBC, to cultures of splenocytes from mice injected 9 days before with SRBC, sFcγRII inhibited, in a dose-dependent way, IgG-anti SRBC production measured on day 5 by an indirect plaque assay. A 400 ng/ml concentration of sFcγRII was required for reaching a 50% inhibition of the response and 1.5 μg/ml sFcγRII for achieving a complete inhibition [1,16].

In order to better define their mode of action in antibody responses, the effect of the recombinant sFcγR on pure resting B cells stimulated by LPS was investigated. Pure resting B cells were isolated by percoll gradient fractionation of anti-Thy-1 (Jlj.10 mAb) + complement treated splenocytes. The cells banding between 70 and 60% Percoll were collected. These cells were 95% surface IgM^+ and did not proliferate significantly when incubated with 2 μg/ml Concanavalin A. Fifty thousand resting B cells were cultured in 96-well microplates in the presence of 50 μg/ml LPS (Sigma, St-Louis, MO, USA), and of a range of concentrations of sFcγR in 0.2 ml culture medium (RPMI 1640 containing 25 m*M* Hepes, 2 m*M* L-glutamine, and supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, $5 \cdot 10^{-5}$ *M* 2β-mercaptoethanol and 10% FCS) at 37°C in a 5% CO_2 atmosphere. Ig secretion was measured on day 10 by isotype specific ELISA. As shown in Fig. 5, sFcγRII-enriched preparations inhibited, in a dose dependent manner, IgM and IgG3 secretion induced by LPS. The inhibition was almost complete for doses between 5–15 μg/ml and 2.5 μg/ml

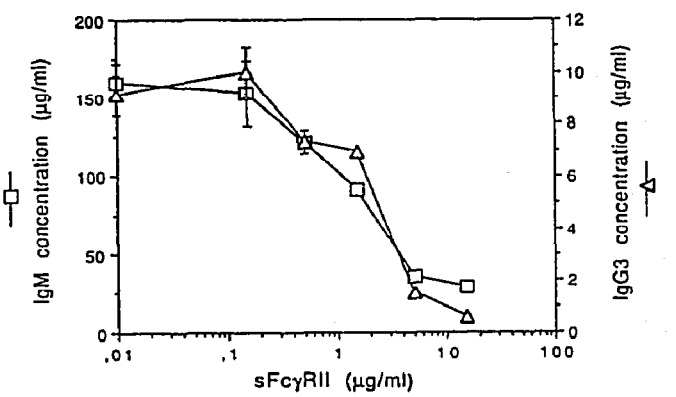


Fig. 5. Effect of sFcγRII-enriched preparation on Ig production by mouse B cells stimulated by LPS.

sFcγRII were required to reach 50% inhibition of IgM and IgG3 secretion.

Thus, sFcγRII seems to act directly on B cells, inhibiting antibody production induced by mitogen. The same range of concentrations of FcγRIIb3 also decreased polyclonal Ig production (data not shown).

In order to assess whether these observations are of physiological relevance, a 95% pure mouse sFcγRII prepared in endotoxin free conditions was injected into mice and its effect on anti-SRBC responses measured. Ten adult B6D2F1 mice were injected i.p. with 20 μg/day recombinant sFcγRII for 19 consecutive days, corresponding to a total dose of 380 μg sFcγRII. Ten control mice received, according to the same protocol, PBS. All mice injected with SRBC on days 7 and 16 and secondary antibody responses were measured on day 19. Anti-SRBC IgG1, IgG2b and IgG2a antibodies were titrated, using ELISA plates coated with SRBC, in the sera of individual mice. Results showed that sera of mice that had received recombinant sFcγRII contained less anti-SRBC IgG antibodies than controls. A mean of 75% inhibition was observed for IgG1, 56% for IgG2b and 71% for IgG2a [1]. These data suggest that recombinant sFcγR has *in vivo* immunoregulatory properties.

In the present manuscript we have described transfection, production and purification techniques which allow the preparation, at a laboratory scale, of high amounts of recombinant glycoproteins. The recombinant soluble FcγR have similar IgG-binding activities to the natural receptors and down regulate antibody production *in vivo* and *in vitro*. Further investigations are needed to know whether these molecules provide therapeutic tools to autoimmune disorders or to lymphoproliferative diseases. The recent demonstration of their binding to cell surface ligands distinct from IgG should help in solving the mechanisms by which sFcγR regulate immune functions.

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