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Efficient expression of recombinant soluble human FcγRI in mammalian cells and its characterization

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ABSTRACT

The extracellular domain of human FcγRI which interacts with a human IgG was expressed as recombinant soluble human FcγRI (rshFcγRI) by Chinese hamster ovary (CHO) cell. Stable CHO cell clones with efficient expression of rshFcγRI were established based on a dihydrofolate reductase (DHFR)/methotrexate (MTX) gene-amplification system. The CHO clones efficiently produced rshFcγRI under high-density continuous culture in a bioreactor. After 53 days of culture, the number of cells had reached approximately 4×10^6 cells/mL in the bioreactor and the average production of rshFcγRI had reached 7.4 mg L-medium^{-1} day^{-1}. Secreted rshFcγRI was purified to a homogeneous state using cation exchange and affinity chromatographies. The binding affinities of rshFcγRI to human IgG subclasses were determined using surface plasmon resonance analysis. The binding affinities of rshFcγRI to human IgG1/κ and IgG3/κ were high (1.59×10^{-10} and 2.81×10^{-10} M, respectively), whereas that of rshFcγRI to human IgG4/κ was lower binding affinity (1.41×10^{-8} M). Binding to IgG2/κ was not detectable. Examination of circular dichroism spectra indicated that rshFcγRI was rich in β-structures and loop or turn structures, but there were few α-helices. These results may be valuable for further studies of the structure and function of human FcγRI.

Introduction

Human FcγRI (hFcγRI)[1] is an integral membrane glycoprotein and is constitutively expressed on macrophages, monocytic cells, and dendritic cells [1,2]. Human FcγRI expression can be induced with IFNγ or various elicitors on neutrophils and glomerular mesangial cells [3,4]. The basic structure of hFcγRI consists of three conserved extracellular immunoglobulin C2-type domains, a transmembrane region, and a cytoplasmic tail. Binding of hFcγRI to IgGs initiates phagocytosis, endocytosis of IgG-opsonized particles, clearance of immune complexes, and antibody-dependent cellular cytotoxicity (ADCC) [1]. The glycosylated full-length hFcγRI from a human monocyte cell line has a relative molecular mass of 70–72 kDa on SDS–PAGE [5,6]. Human FcγRI exhibits high binding affinities to human IgG1 and human IgG3 subclasses, whereas those to human IgG4 and IgG2 are lower binding affinity or negligible [7,8]. Mutagenesis studies on IgG molecules have shown that the hinge region of IgG at amino acid residues 234–237 is the major site of interaction with hFcγRI [9,10].

Efficient expression of recombinant soluble human FcγRI (rshFcγRI) is extremely important for the elucidation of protein structure and function. Various expression systems have been developed. The extracellular domain of hFcγRI was obtained by refolding from inclusion bodies expressed by *Escherichia coli* [11]. Mammalian cells are often chosen to obtain correct protein folding and post-translational modifications such as glycosylation. In previous studies, rshFcγRI has been expressed by the human embryonic kidney (HEK) cell line and Chinese hamster ovary (CHO) cell [8,12,13]. However, efficient production of rshFcγRI has been difficult and yields have been very low.

Here, we focused on achieving efficient expression of rshFcγRI in mammalian cells. On the basis of a dihydrofolate reductase (DHFR)/methotrexate (MTX) gene-amplification system [14], we were able to successfully establish stable CHO clones efficiently expressing rshFcγRI. The stable CHO clones were cultured at high density in a bioreactor. Expressed rshFcγRI was purified by cation exchange and affinity chromatographies, and the purified rshFcγRI had a homogenous state. The binding affinities of purified rshFcγRI to human IgG subclasses were measured by surface plasmon resonance (SPR) analysis. The secondary structure of purified rshFcγRI was determined by circular dichroism (CD) spectrum analysis.

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[1] *Abbreviations used:* CD, circular dichroism; CHO, Chinese hamster ovary; DHF, dihydrofolate; DHFR, dihydrofolate reductase; hFcγRI, human FcγRI; MTX, metho trexate; rshFcγRI, recombinant soluble human FcγRI.

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Materials and methods

Construction of rshFcγRI expression plasmid

Human FcγRI cDNA [15,16] (Human cDNA clone TC119841 plasmid vector) was purchased from Origene Technologies, Rockville, MD. The rshFcγRI gene encoding a signal and an extracellular domain of hFcγRI (1–289 amino acids) (Fig. 1A) was amplified by PCR using forward (hFcR-F) and reverse (hFcR-6His-R) primers, with the TC119841 plasmid as a template cDNA. 5′-GAAGATCTATGT GGTTCTTGACAACTCTGCTCC-3′ was employed as the hFcR-F primer and 5′-CGTCTAGACTAGTGGTGGTGGTGGTGGACAGGAGTTGG TAACTGGAGGC-3′ was the hFcR-6His-R primer. The underlines show the sites for the restriction enzymes *Bgl*II and *Xba*I. A 6-His sequence was linked to the hFcR-6His-R primer for detection and purification of rshFcγRI. The amplified rshFcγRI gene was digested with *Bgl*II and *Xba*I and inserted into the *Bgl*II and *Xba*I sites of pECEdhfr [17] to construct the recombinant plasmid vector (pECE-rshFcRdhfr) for expression of rshFcγRI (Fig. 1B). The sequence was confirmed based on the dideoxy chain-termination method.

Transformation of CHO cells and gene amplification

A dhfr-deficient CHO cell line (DBX-11) [18] was transfected with pECE-rshFcRdhfr by using lipofectamine 2000 (Invitrogen, Carlsbad, CA). Transformants were cultivated at 37 °C under a 5% CO_2 atmosphere in alpha-MEM (Invitrogen) supplemented with 10% dialyzed fetal calf serum (FCS) and 50 μg/mL kanamycin. After the rshFcγRI genes in the transformants had been amplified through multiple rounds of screening in the presence of different concentrations of methotrexate (MTX) (5 nM to 100 μM), the amplified transformants were cloned based on a limiting dilution technique and the best rshFcγRI expression clone was selected.

Culture of recombinant CHO cells

A bioreactor (BelloCell-500P; Cesco Bioengineering, Taichung, Taiwan) consisting of two chambers was used to culture the recombinant CHO cells. The upper chamber held 5.5 g of BioNOCII carriers, with inward and outward-flowing connectors, and the lower compressible chamber contained the medium (Fig. 2A). The carriers were made of polyethylene terephthalate. The carriers provided approximately 2400 cm^2/g specific surface area for cell growth.

The bottle was mounted on a control console named the BelloStage, which was placed in a CO_2 incubator. The temperature was kept at 37 °C, and the CO_2 was initially controlled at 5% and then adjusted to 0% after 3 days. The bottle was filled with 470 mL pre-warmed DMEM/F12 (Invitrogen) culture medium supplemented with 5% FCS and 50 μg/mL kanamycin; 10^7–10^8 cells suspended in 30 mL DMEM/F12 were added. The BelloStage controlled the up and down speed to 2.0 mm/s and the top holding time to 1 min until the cells were attached to carriers. After the completion of cell attachment, the up and down speed was reduced to 1.0 mm/s; the down holding time was changed to 30 min and the top holding time was changed to 0 min. After 3 days, the bottle was connected to a reservoir vessel kept at 4 °C; the vessel contained 2 L of culture medium with DMEM (Invitrogen) and ExCell 302 (SAFC Biosciences, Brooklyn, Australia) at a ratio of 1:1, supplemented with 5% FCS, 50 μg/mL kanamycin, and 2 mM L-glutamine. The culture medium was exchanged at 50 mL/h (Fig. 2B). The reservoir vessel was changed every 4 days.

The attached cells were quantified by use of a crystal violet dye (CVD) nucleus-staining method. Three carriers were picked from three different locations in the bottle and were placed in a 1.5-mL tube; 1 mL of CVD reagent was then added. After vortexing and incubation at 37 °C for 1 h, the cell membranes were ruptured and the nuclei were released. The nuclei were counted with a hemocytometer and the cell population was calculated.

Purification of rshFcγRI

The culture supernatant was dialyzed against 20 mM sodium acetate (pH 4.5) and loaded onto a 400 mL Streamline SP XL packed C-50 column (50 mm i.d. × 1000 mm) with expanded bed adsorption (GE Healthcare) at a flow rate of 100 mL/min. The contracted absorbent bed was equilibrated with 20 mM sodium acetate (pH 4.5) and the solution was eluted with 50 mM potassium phosphate

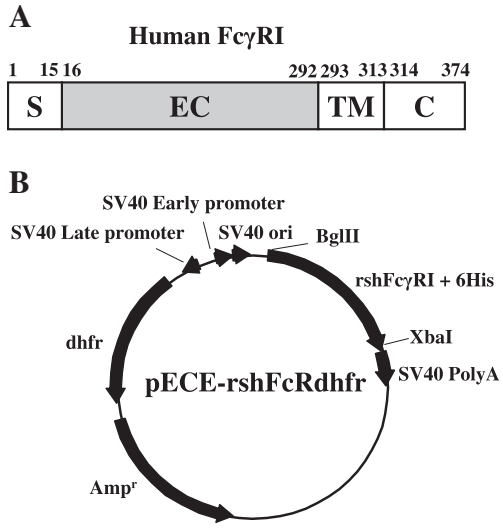

Fig. 1. Construction of recombinant pECE-rshFcRdhfr vector. (A) Schematic illustration of hFcγRI. Vales above S (signal sequence), EC (extracellular domain), TM (transmembrane domain), and C (cytoplasmic domain) indicate numbers of amino acid residues. (B) Recombinant pECE-rshFcRdhfr vector was constructed by inserting rshFcγRI gene encoding a signal and an extracellular domain of hFcγRI containing a 6-His sequence in its C-terminus into the pECEdhfr vector using *Bgl*II and *Xba*I restriction sites.

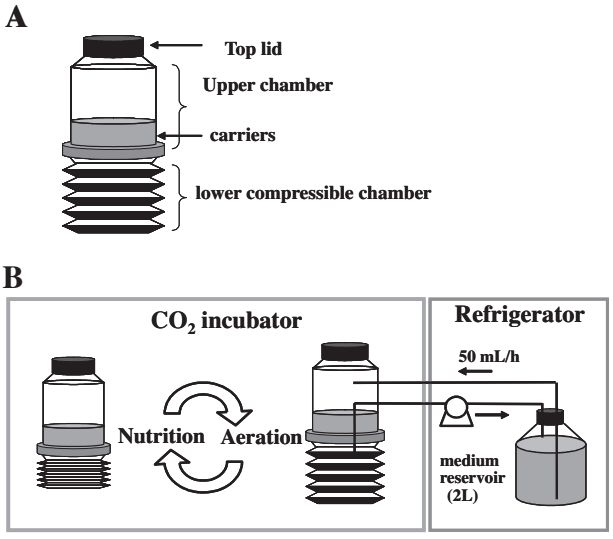

Fig. 2. Schematic illustration of the BelloCell (A), and production of rshFcγRI in this system (B). (A) The reactor bottle consists of an upper chamber containing the carriers for cell immobilization, with inward- and outward-flowing connectors and a lower compressible chamber. The top lid was equipped with a 0.22-μm filter for gas exchange. (B) The bottle can be alternately compressed and released for nutrition and aeration by the BelloStage. The bottle was placed in a CO_2 incubator and connected to the reservoir medium in a refrigerator.

(pH 8.0) supplemented with 0.5 M NaCl and 5% (v/v) glycerol at a flow rate of 50 mL/min. The eluted solution was dialyzed against 50 mM potassium phosphate (pH 8.0) supplemented with 0.5 M NaCl and 5% (v/v) glycerol and filtered through a mixed cellulose ester type membrane filter (pore size 3 μm) (Advantec, Tokyo, Japan). The dialyzed solution containing 20 mM imidazole in final concentration was applied to an 20 mL Ni-IDA (Novagen, Merck, Darmstadt, Germany) packed HR 16/10 column (16 mm i.d. × 100 mm; GE Healthcare), equilibrated with 50 mM potassium phosphate (pH 8.0) supplemented with 0.5 M NaCl at a flow rate of 3 mL/min and then eluted with 50 mM potassium phosphate (pH 8.0) supplemented with 0.5 M NaCl, 0.5 M imidazole and 5% (v/v) glycerol at a flow rate of 3 mL/min. Next, the eluted sample was applied to a 12 mL IgG-Sepharose 6 Fast Flow (GE Healthcare) packed open column, equilibrated with 20 mM potassium phosphate (pH 7.4) supplemented with 150 mM NaCl and under gravitational flow; the product was then eluted with 20 mM citrate buffer (pH 3.0). The eluted solution was immediately neutralized by the addition of 1 M Tris–HCl (pH 8.0). The concentration of purified rshFcγRI was measured by using the Bradford method with a human IgG (Kaketsuken, Kumamoto, Japan) standard curve [19]. After the purified rshFcγRI had been dialyzed against 20 mM potassium phosphate (pH 7.6) supplemented with 150 mM NaCl and 30% (v/v) glycerol, it was stored at −20 °C.

SDS–PAGE

Purified proteins were separated on 5–20% (w/v)-gradient polyacrylamide gels (ATTO, Tokyo, Japan) under reducing conditions. Proteins were visualized by staining with 0.25% (w/v) Coomassie Brilliant Blue R250. Protein molecular mass was determined by using the Mark 12 protein standard (Invitrogen).

Glycosidase digestion reaction

The purified rshFcγRI was diluted to 0.9 mg/mL with a solution containing 0.5% SDS and 40 mM dithiothreitol. The protein solution was heated at 100 °C for 10 min. N-linked sugar chains were digested with 1000 units of peptide-*N*(4)-(*N*-acetyl-beta-glucosaminyl) asparagine amidase (PNGase F) (New England Biolabs, Ipswich, MA). O-linked sugar chains were digested with 80,000 units of End-α-*N*-Acetylgalactosaminidase (New England Biolabs). One of the glycosidase, 2 μL of 10 × G7 buffer (0.5 M sodium phosephate, pH 7.5), 2 μL of 10% NP-40 and 14 μL of the protein solution were mixed in a final volume of 20 μL. The reaction mixtures were incubated at 37 °C for 1 or 2 h.

Enzyme-linked immunosorbent assay (ELISA)

A 96-well microplate (Nunc, Thermo Fisher Scientific, Rosklide, Denmark) was coated with human IgG (Kaketsuken) (1 μg/well) overnight at 4 °C. After being blocked with Starting Block (Pierce, Thermo Fisher Scientific, Rockford, IL) for 2 h at 30 °C, the wells were washed twice with TBST (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% (w/v) Tween 20). Serially diluted rshFcγRI (100 μL/well) was added to the wells, which were then incubated for 1 h at 30 °C. After the wells had been washed four times with TBST, 100 μL of 1/10,000-diluted horseradish peroxidase conjugated with anti-6-His antibody (Bethyl Laboratory, Montgomery, TX) was added to each well and incubated for 1 h at 30 °C. After the wells had been washed four times with TBST, specific binding antibody was detected by using TMB Microwell Peroxidase Substrate (KPL, Gaithersburg, MD). Absorption at a wavelength of 450 nm was measured. The concentration of rshFcγRI protein was determined from a standard curve based on that of FcγRI-6His (R&D Systems, Minneapolis, MN).

SPR analysis

Binding of rshFcγRI to human IgG1/κ, human IgG2/κ, human IgG3/κ, and human IgG4/κ (Sigma–Aldrich, St. Louis, MO) was measured with a Biacore T100 immunogenicity package (GE Healthcare) [20]. Human IgGs proteins were covalently coupled to sensor chips (CM5) by using a standard amine coupling kit (GE Healthcare). The carboxylated dextran matrix was activated by injecting a mixture of 50 mM *N*-hydroxysuccinimide (NHS) and 0.2 M *N*-ethyl-*N*'-(3-diethylaminopropyl)carbodiimide (1:1). After human IgG subclasses (20 μg/mL in 10 mM sodium acetate buffer, pH 4.5) had been injected into the activated sensor chip, 1 M ethanolamine hydrochloride (pH 8.5) was injected to block the remaining NHS ester groups. The surfaces of the sensor chips were coupled to human IgG1/κ (773.0 RU (resonance units)), human IgG2/κ (642.1 RU) human IgG3/κ (707.3 RU) or human IgG4/κ (754.5 RU).

The purified rshFcγRI was diluted from 0.19–3.00 nM with HBS-EP+ (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.05% (v/v) Surfactant P20) (pH 7.4) running buffer and then injected at 30 μL/min onto the sensor chips on which human IgG1/κ, IgG2/κ, or IgG3/κ were immobilized. The contact time was 210 s and the dissociation time was 400 s at 25 °C. The samples were exposed to both active and control surfaces on the sensor chips. After the response was measured in RU, the sensor chips were regenerated by 10 mM glycine–HCl (pH 2.5). Regeneration of the chip surface was confirmed by repeating binding assays of the purified rshFcγRI five times and was sufficient. Simulation analysis for binding curve fitting (monovalent interaction model) was performed by using BIA evaluation software (version 1.1.1). Purified rshFcγRI was diluted from 1.6–203 nM with running buffer and then was injected at 30 μL/min onto the sensor chip on which human IgG4/κ was immobilized. The measurement was performed, as described above. The equilibrium dissociation constant (K_D) of rshFcγRI to human IgG4/κ was determined by plotting the equilibrium binding response versus concentration.

CD spectroscopy

CD spectroscopy of purified rshFcγRI (100 μg/mL) was performed at 25 °C in 20 mM Tris–HCl (pH 7.5) by using a CD spectrometer (model J-805, Jasco, Tokyo, Japan). The CD spectra were analyzed by use of the software provided with the J-805 instrument.

Results

Construction of recombinant plasmid vector for expression of rshFcγRI

The rshFcγRI gene encoding a signal and an extracellular domain of hFcγRI (Fig. 1A) containing a 6-His sequence in its C-terminus was amplified by PCR and inserted into mammalian cell expression vector (pECEdhfr) [17] by using the restriction sites *Bgl*II and *Xba*I to construct an rshFcγRI expression plasmid vector, termed pECE-rshFcRdhfr (Fig. 1B). Constructed pECE-rshFcRdhfr harbored SV40 early and late promoters for the expression of rshFcγRI and DHFR, respectively.

Establishment of CHO stable clone expressing rshFcγRI

Although the dhfr-deficient CHO cell line (DXB-11) [18] was transfected with pECE-rshFcRdhfr, rshFcγRI production by the transformants was very low. We amplified integrated rshFcγRI gene to establish efficient expression by stable CHO clones. The dhfr gene encoding DHFR was employed as an amplifiable and

selectable marker gene. MTX is an analog of dihydrofolate; it inhibits its DHFR and causes cessation of rapid growth of cell. The transformants were exposed to MTX in increasing concentrations from 5 nM to 100 μM to co-amplify both the rshFcγRI and the dhfr gene. The CHO cells harboring the amplified genes were cloned based on a limiting dilution technique at each concentration of MTX. The stable clones were cultured until they became confluent monolayers in the dish. After confluence was reached, 10 mL of the culture medium was exchanged and the clones were further cultured. The next day, production of rshFcγRI in the culture medium was measured with ELISA. We were able to establish clones efficiently expressing rshFcγRI by stepwise selection. The established stable clones were termed 12D, 2–3B, and 4–12A and could grow in the presence of 5, 25, and 100 μM MTX, respectively. The productivities of 12D, 2–3B, and 4–12A in 10 mL culture medium were 3.2, 4.8, and 8.1 $\mu g\,mL^{-1}\,day^{-1}$, respectively. The highest productivity of rshFcγRI was obtained with 4–12A, which was exposed to the highest concentration of MTX.

High-density culture of cloned recombinant CHO cells

Although rshFcγRI production was increased by amplification of the rshFcγRI gene, the amount of rshFcγRI produced was still low. Next, we attempted high-density culture of established clones by using a bioreactor. Use of a bioreactor has previously led to successful culture of adherent cells such as HEK-293 and insect cells, resulting in efficient production of proteins [21,22].

After culture for 53 days in medium exchanged at 50 mL/h, the total cell numbers of the clones became constant at approximately 4×10^6 cells/mL in the bioreactor. The amount of rshFcγRI reached 99.6, 168.4 and 195.8 mg in clones 12D, 2–3B and 4–12A, respectively (Fig. 3). The average production of rshFcγRI reached approximately 3.8, 6.4, and 7.4 mg L-medium^{-1} day^{-1} in clones 12D, 2–3B, and 4–12A, respectively. We succeeded in the efficient production of rshFcγRI by high-density culture in the bioreactor and maintained the rshFcγRI stably in a refrigeration system. Production of rshFcγRI by clone 4–12A, which was exposed to the highest concentration of MTX, gave the best result in high-density culture.

Purification of rshFcγRI

The rshFcγRI was purified by use of cation exchange chromatography and Ni-IDA and IgG affinity chromatographies. From 10 L of culture supernatant, 20 mg of rshFcγRI was purified at an overall estimated yield of 34% (Table 1). The purity of rshFcγRI was confirmed by SDS–PAGE under reducing conditions. The purified rshFcγRI gave a relatively broad single band with a relative molecular mass of 40–50 kDa (Fig. 4). The molecular mass of rshFcγRI calculated based on the amino acid sequence which does not contain sugar chains was approximately 31.5 kDa. The relative molecular masses of rshFcγRI whose N-linked or O-linked sugar chains were removed were confirmed by SDS–PAGE (Fig. 4). The relative molecular mass of rshFcγRI whose N-linked sugar chains were removed was approximately 33–34 kDa on SDS–PAGE. It was almost same as the calculated molecular mass of rshFcγRI. The relative molecular mass of rshFcγRI whose O-linked sugar chains were removed was the little change.

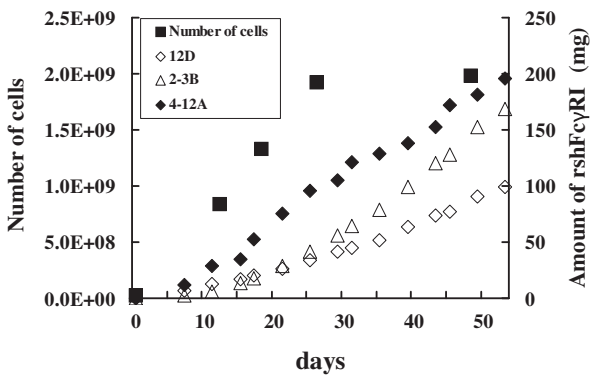

Fig. 3. Growth curve and production of rshFcγRI in high-density culture. The three different clones were cultured in a bioreactor. Production of rshFcγRI in the culture medium was determined by using the ELISA method.

Binding affinity of rshFcγRI

A quantitative analysis of the binding affinities of rshFcγRI for human IgG1/κ, IgG2/κ, IgG3/κ, and IgG4/κ was performed by use of SPR. The sensorgrams showed a simple monovalent interaction model (Fig. 5). We determined the association rate constant (k_a), dissociation rate constant (k_d), and K_D with human IgG1/κ, IgG2/κ, IgG3/κ, and IgG4/κ (Table 2). The rshFcγRI had no detectable affinity for human IgG2/κ. In contrast, the K_D with human IgG1/κ and IgG3/κ was 1.59×10^{-10} and 2.81×10^{-10} M, respectively. The affinity of rshFcγRI for human IgG4/κ was relatively low (K_D: 1.41×10^{-8} M). Thus the binding affinities of rshFcγRI to human IgG1/κ and IgG3/κ were high, whereas that of rshFcγRI to human IgG4/κ was low and that to IgG2/κ was not detectable.

Determination of secondary structure of rshFcγRI

To determine whether the purified rshFcγRI was correctly folded, we performed a CD analysis to determine the secondary structure of it. The CD spectrum showed maximum negative bands at a wavelength of approximately 218 nm (Fig. 6), suggesting the presence of a β-strand structure [23]. Analysis of the CD data by using the software provided with the J-805 instrument revealed 66–69% β-strand content and 30–33% random coils.

Discussion

For the study of structure and function of proteins such as cellular receptors that may not be easily produced, there is a need for efficient protein expression systems. In previous studies, production of rshFcγRI by HEK and CHO cells was insufficient for further detailed study of the structure and function of the receptor. Among the human Fc receptors studied, production of rshFcγRI was the lowest [24]. We succeeded in efficient expression of rshFcγRI in CHO cells. The critical points of this success must be attributed to the marked amplification of the rshFcγRI gene in the presence of MTX, the high-density continuous culture of recombinant CHO clones and stable preservation of expressed rshFcγRI in a refrigeration system.

The highest level of rshFcγRI expression, by the stable CHO clone 4–12A, was obtained in the presence of 100 μM MTX, which may cause rapid cessation of growth of normal cells. The productivity of clone 4–12A was 8.1 $\mu g\,mL^{-1}\,day^{-1}$ in 10 mL culture medium and was much higher than 30 $ng\,mL^{-1}\,day^{-1}$ in 5 mL culture medium in the previous study [24]. Furthermore, this clone could be cultured at high density. Average production of rshFcγRI reached 7.4 mg L-medium^{-1} day^{-1}, and productivity thus markedly improved. High-density culture is a serial culture system for the stable growth of large numbers of cells, and it resulted in an extremely high rate of production of rshFcγRI. Because rshFcγRI is labile at 30 to 37 °C, its function would be lost during culture at these temperatures. This system conserved the expressed rshFcγRI in a reservoir vessel at 4 °C during culture, therefore achieved

Table 1
Summary of purification. Concentrations of rshFcγRI were measured by use of ELISA. Total protein concentrations were determined by use of the Bradford method.

	Volume (mL)	rshFcγRI A (mg)	Total protein B (mg)	Purity (A/B)	Yield (%)
CHO culture supernatant	10,000	59.0	17,960.0	0.0033	100.0
Cation exchange (Streamline SP XL)	1000	27.9	12,950.0	0.0022	47.3
Affinity chromatography (Ni-IDA)	24	22.3	43.6	0.5115	37.8
Affinity chromatography (IgG-Sepharose)	45	20.1	20.3	0.9901	34.1

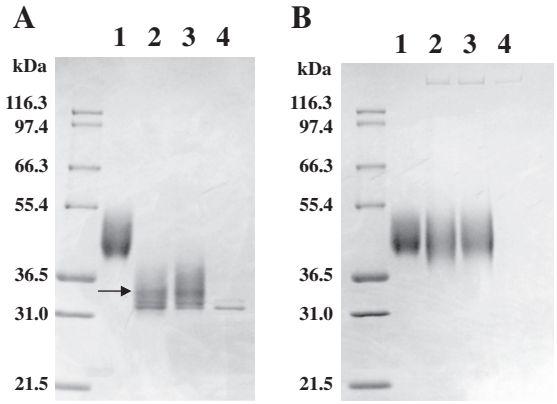

Fig. 4. Analysis of rshFcγRI whose N-linked or O-linked sugar chains were removed by SDS–PAGE. Purified rshFcγRI was analyzed by using SDS–PAGE under reducing conditions. (A) N-linked sugar chains of rshFcγRI were digested with PNGase F. (B) O-linked sugar chains of rshFcγRI were digested with End-α-*N*-Acetylgalactosaminidase. Lane 1 shows purified rshFcγRI. Lane 2 and lane 3 shows rshFcγRI digested by glycosidase for 2 and 1 h, respectively. The lane 4 shows that glycosidase reaction mixture without rshFcγRI. Molecular mass (kDa) standard is shown to the left. An arrow shows rshFcγRI without N-linked sugar chains.

successful production of a large amount of rshFcγRI and kept the protein molecules intact. If the culture period were extended, even greater amounts of rshFcγRI would be obtainable.

Table 2
Binding affinities of rshFcγRI to human IgG subclasses.

	k_a (1/Ms)	k_d (1/s)	K_D (M)
Human IgG1/κ	1.88×10^6	2.99×10^{-4}	1.59×10^{-10}
Human IgG2/κ	ND	ND	ND
Human IgG3/κ	2.41×10^6	6.78×10^{-4}	2.81×10^{-10}
Human IgG4/κ	NM	NM	1.41×10^{-8}[a]

ND, not detected.
NM, not measurable.
[a] K_D was determined from the plot of equilibrium binding response versus concentration.

In the purification of rshFcγRI, the yield of rshFcγRI by cation exchange was low. Expanded bed adsorption of Streamline SP XL was chosen as the first step of purification of rshFcγRI because it could be captured directly from the crude culture supernatant in a single step, without filtration or centrifugation. It was thought that other proteins might inhibit adsorption of rshFcγRI on the gel.

The relative molecular mass of the purified rshFcγRI was 40–50 kDa by SDS–PAGE analysis. This was larger than the molecular mass (approximately 31.5 kDa) of rshFcγRI calculated from the amino acid sequence 16–289 (UniProt ID: P12314) and 6 His. This discrepancy was caused by N-linked sugar chains of rshFcγRI (Fig. 4A). Indeed, rshFcγRI retained seven different N-glycosylation sites, at positions Asn (59), Asn (78), Asn (152), Asn (159), Asn

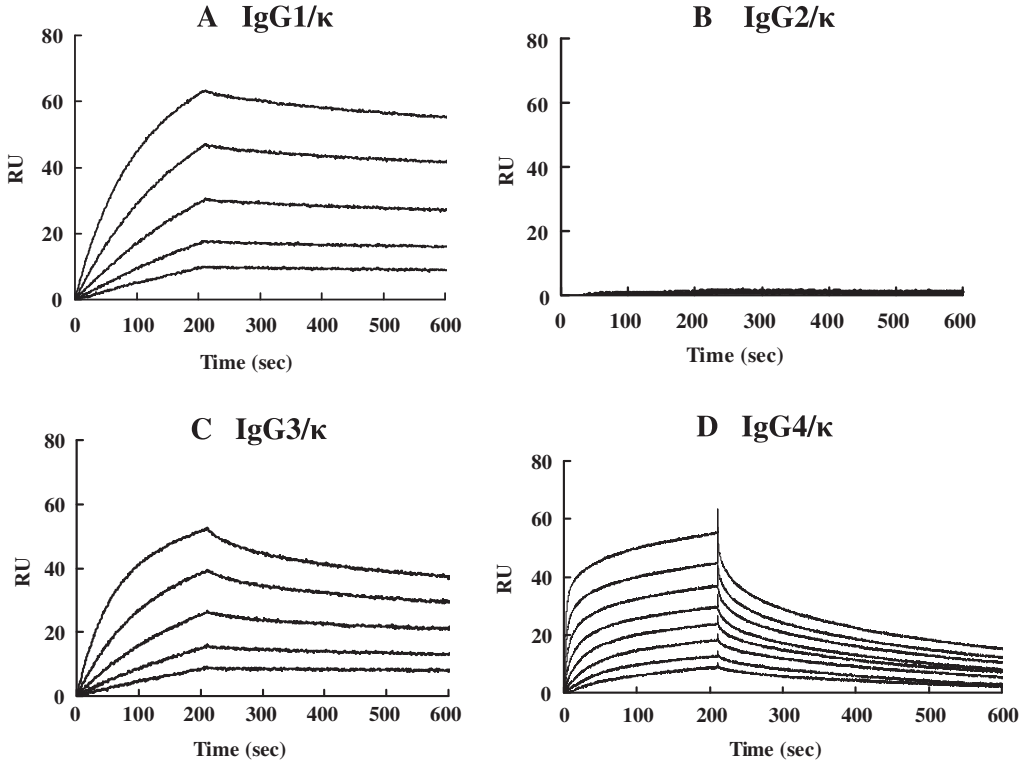

Fig. 5. Curves for binding of rshFcγRI to human IgG subclasses. Human IgG subclasses (A: IgG1/κ; B: IgG2/κ; C: IgG3/κ; D: IgG4/κ) were immobilized on the sensor chips (CM5). The binding curves of IgG1/κ and IgG3/κ were fitted to a monovalent interaction model and the parameters determined. Parameters for IgG4/κ were determined from the plot of equilibrium binding response versus concentration.

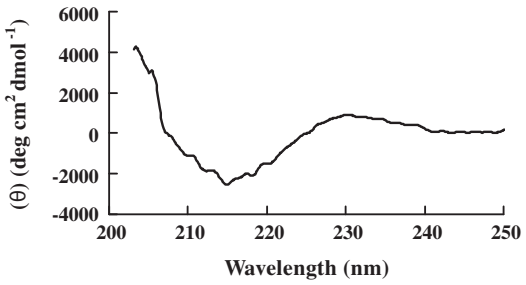

Fig. 6. Circular dichroism (CD) spectrum of rshFcγRI. Purified rshFcγRI (100 μg/mL) was dialyzed with 20 mM Tris–HCl (pH 7.5). The CD spectra of rshFcγRI were measured at 25 °C over a wavelength range of 200 to 260 nm. Measurements were reported as mean residue ellipticity (θ) (deg cm^2 dmol^{-1}).

(163), Asn (195), and Asn (240). In previous study, relative molecular mass of full-length hFcγRI whose N-linked sugar chains were removed was decreased [25]. The rshFcγRI was expressed as glycosylated protein.

The binding affinities of rshFcγRI toward human IgG subclasses have important biological implications. The K_D of rshFcγRI for human IgG1/κ was 159 pM. In a previous study, the extracellular domain of hFcγRI expressed by HEK cells showed almost the same affinity for human IgG1/κ, at 110 pM [13]. The K_D of rshFcγRI for human IgG3/κ was approximately equal to that of human IgG1/κ. In contrast, the affinity of rshFcγRI for human IgG2/κ was not detectable, as in a previous study [8], and that of rshFcγRI for human IgG4/κ was one-hundredth of that for human IgG1/κ or IgG3/κ.

The secondary structure of Fc receptors consists mainly of β-strands [26]. rshFcγRI is a member of the Fc receptor family, and we showed that the secondary structure of rshFcγRI was also dominated by β-strands in our CD spectral analysis.

Recent elucidation of the crystal structures of human FcγRII [27,28] and FcγRIII has provided information on the modes of IgG–Fcγ receptor interaction [29,30]. Although hFcγRI plays a key role in immune system, its crystal structure has not yet been determined. Our results may therefore prove valuable for the further study of hFcγRI structure and function. In addition, our study method is likely to be applicable to the efficient expression of other membranous proteins with low stability.

In terms of therapeutic applications, antagonism of soluble Fc receptors reduces immune complex-mediated inflammation and autoimmune diseases [31–33]. The extracellular domain of hFcγRI has been reported to be an effective inhibitor of type III hypersensitivity as well as of inflammation in a model of arthritis in mice [13]. The rshFcγRI might have high potency for a medicine similar to the antibody. Our present results could help to further study of rshFcγRI for therapeutic applications.

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