

Secretion of Active Fc Fragments of Immunoglobulin E Directed by the Yeast Invertase Signal Sequence in Mammalian Cells

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KAMIYA, T., SUGIO, S., YAMANOUCHI, K. and KAGITANI, Y. *Secretion of Active Fc Fragments of Immunoglobulin E Directed by the Yeast Invertase Signal Sequence in Mammalian Cells*. Tohoku J. Exp. Med., 1996, **180** (4), 297–308 — The human Fc fragments of immunoglobulin E (IgE-Fc) expressed in the mammalian COS cells, those mainly consisting of C ϵ 3-C ϵ 4 chains with or without Cys-328 known to be responsible for interchain disulfide bonding, were secreted into the culture medium directed by the yeast *Saccharomyces cerevisiae* invertase (SUC2) signal sequence (SUC2/Ig $_2$ or SUC2/Ig, respectively) as well as by the human interleukin 2 receptor α chain signal sequence (IL2R/Ig $_2$ or IL2R/Ig, respectively). For the binding activity to the human soluble α chain of high affinity receptor for IgE, IgE-Fc with Cys328, SUC2/Ig $_2$ and IL2R/Ig $_2$, were active but had smaller activity than native IgE. By comparison IgE-Fc without Cys328, SUC2/Ig and IL2R/Ig, showed lower activity than SUC2/Ig $_2$ and IL2R/Ig $_2$. Immunoprecipitation analysis revealed both SUC2/Ig and SUC2/Ig $_2$ had molecular weights (M_r s) and degrees of glycosylation similar to IL2R/Ig and IL2R/Ig $_2$, respectively. These results suggested that in mammalian cells the SUC2 signal sequence was functional in directing the heterologous multimeric proteins to the endoplasmic reticulum, resulting in secretion of the active proteins. This finding might show merits on heterologous protein secretion systems. — endoplasmic reticulum; heterologous expression; IgE-Fc; signal sequence; yeast invertase

Immunoglobulin E (IgE)/the α chain of its high affinity receptor (Fc ϵ RI α) binding is a key regulator of efferent phase of type I allergy, and the human Fc

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Abbreviations used: CHO, Chinese hamster ovary; CMV/T7 Pro, cytomegalovirus/T7 RNA polymerase promoter; DMEM, Dulbecco's modified Eagle's medium; D-PBS, Dulbecco's phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; Fc ϵ RI α , α chain of high affinity Fc receptor for IgE; hIL-2R α , α chain of human interleukin 2 receptor; N-, asparagine-linked; SUC2, *Saccharomyces cerevisiae* invertase; SV40 intron/pA, simian virus 40 splice and polyadenylation signals.

fragment of IgE (IgE-Fc) is shown to be capable of inhibiting the in vitro and in vivo passive sensitization of mast cells, which means that IgE-Fc could abolish effluent phase of type I allergy (Basu et al. 1993; Sutton and Gould 1993; Young et al. 1995).

In eukaryotic cells a protein destined for secretion, but not exclusively, is usually synthesized as a precursor with an amino-terminal endoplasmic reticulum (ER) signal peptide because the signal sequence is required for protein targeting to the ER membrane and for initiation of protein translocation across the membrane followed by cleavage by signal peptidases (Gilmore 1993; Dobberstein 1994; Walter and Johnson 1994; Jungnickel and Rapoport 1995; Karaplis et al. 1995). As a common feature of signal peptides, three domains have been identified, and are considered to be necessary for carrying their functions, although the primary structures are poorly conserved. Recent studies, however, indicate that there is not only a striking similarity between the yeast and mammalian cell's signal recognition and protein targeting apparatus (signal recognition particle/signal recognition particle receptor) but also between the translocation machinery (Dobberstein 1994). Not all of mammalian proteins, nevertheless, were secreted in yeast by using the native signal sequence (Romanos et al. 1992), even if the yeast *Saccharomyces cerevisiae* α -mating factor signal sequence or invertase signal sequence (SUC2) was used to direct expression (Wedlock et al. 1993), which has been used most widely for heterologous protein secretion in yeast. In mammalian cells, on the other hand, expression of yeast glycoproteins revealed different degrees of conservation of secretory function. For example, yeast carboxypeptidase Y expressed in monkey kidney COS cells was inefficiently targeted to the rough ER because of the relative paucity of hydrophobic residues within its signal sequence (Bird et al. 1987). Expression of yeast prepro- α -factor in GH₃ rat pituitary cells, however, resulted in the efficient targeting to the ER and the processing, but in the degradation in ER or pre-Golgi compartment due to the post-translational modifications as asparagine-linked (N-) glycosylation at the potential sites within its proregion (Su et al. 1993). Furthermore, expression of invertase cDNA in Ψ -2 mouse fibroblast cells resulted in the efficient intercompartmental transport and secretion of a signal peptide-cleaved, heavily glycosylated, and active product (Bergh et al. 1987). However, it remains to be clarified whether a foreign protein is successfully secreted from mammalian cells using the yeast signal sequence. Therefore, using monomer and disulfide-linked dimer of IgE-Fc, we have tried to determine whether the active IgE-Fc fragments are secreted in mammalian cells by the SUC2 signal sequence compared with the human interleukin 2 receptor α (hIL-2R α) signal sequence. Here, we show the usefulness of the yeast signal sequence in mammalian expression system for protein production.

MATERIALS AND METHODS

Antibodies and proteins

Recombinant human soluble Fc ϵ RI α (produced in Chinese hamster ovary [CHO] cells) was the gift of Dr. K. Naito (this laboratory). Human IgE and rabbit immunoglobulin to human IgE (ϵ -chains) were purchased from Chemicon International (Temecula, CA, USA) and Dako (Glostrup, Denmark), respectively. Affinity purified antibody goat anti-human IgE (ϵ -chains) and horseradish peroxidase-labeled goat antibody against rabbit IgG were purchased from Kirkegaard and Perry (Gaithersburg, MD, USA).

Cells and media

The human IgE (ϵ , λ)-producing plasmacytoma U266 and the monkey kidney COS-7 cells were the gift of Drs. K. Naito and K. Okabayashi (this laboratory), respectively. U266 cells were maintained in RPMI1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin, at 37°C in a 5% CO₂ atmosphere. COS cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 1x Nutridoma-SP (Boehringer Mannheim, Mannheim, Germany).

Construction of expression vectors

All DNA and RNA manipulations were carried out using standard techniques (Sambrook et al. 1989). The cDNA for human IgE-Fc (C ϵ 2-C ϵ 4) was cloned: Total RNA was extracted from U266 cells by Isogen (Nippon Gene, Tokyo), and a coupled reverse transcriptase (from avian myeloblastosis virus; Takara Shuzo, Otsu). PCR reaction was carried out with 0.26–3.6 μ g of total RNA as recommended by the manufacturers, and then the resultant 1005 bp product was cloned into the pCRII vector using a TA cloning kit (Invitrogen, San Diego, CA, USA) to yield a plasmid pCRIIhC ϵ 2-C ϵ 4.31. The first strand cDNA synthesis: oligo (dT)20-M4 adaptor primer; PCR primers: 5'-GTCGACAACAAAACCTTCAG (P1, from nucleotide 289 to 308, nucleotide number from G at GCC (Ala121) in C ϵ 1); 5'-AGTACGTCATTTACCGGGAT (P2, from nucleotide 1293 to 1274). The mammalian expression vector pCDM8 (Seed 1987; Linsley et al. 1991) was used for IgE-Fc expression in mammalian cells. The *Asu*II-*Hinf*I oligonucleotide consisting of the SUC2 signal sequence (corresponding to 19 amino acid residues, MLLQAFLFLLAGFAAKISA in one letter code) fused to Ala329 of C terminus of C ϵ 2 (C ϵ 2') was originally synthesized by a Model 392 DNA/RNA Synthesizer (Applied Biosystems, Foster, CA, USA). The *Hinf*I-*Spe*I fragment of the human entire C ϵ 3-C ϵ 4 cDNA sequence was removed from pCRIIhC ϵ 2-C ϵ 4.31. Then, the 0.7 kb *Asu*II-*Spe*I fragment containing the *Asu*II-*Hinf*I oligonucleotide and the *Hinf*I-*Spe*I fragment was blunt-ended and subsequently

cloned in-frame into the blunted *Xba*I site of pCDM8 to yield a mammalian expression vector, pCDM.SUC2/Ig, resulting in IgE-Fc fragment of C ϵ 2' (Ala329)-C ϵ 3-C ϵ 4. On the other hand, the *Asu*II-*Hinf*I oligonucleotide consisting of the SUC2 signal sequence fused to the region encompassing 15 amino acids of C ϵ 2' was originally amplified in a two-step PCR procedure using two primers encompassing the SUC2 signal sequence (SUC2P1 and P3, see below for the primers) and two primers encompassing C ϵ 2' (P4 and P5). Then, the 0.7 kb *Asu*II-*Spe*I fragment containing the *Asu*II-*Hinf*I oligonucleotide and the *Hinf*I-*Spe*I fragment of the human entire C ϵ 3-C ϵ 4 cDNA sequence was blunt-ended and subsequently cloned in-frame into the blunted *Xba*I site of pCDM8 to yield pCDM.SUC2/Ig₂, resulting in IgE-Fc fragment of C ϵ 2' (Val314-Ala329)-C ϵ 3-C ϵ 4. *Escherichia coli* MC1061/p3 cells (kind gift of Dr. T. Masuko, Tohoku University) were transformed with the ligated DNA. These constructs were verified by DNA sequencing. The primers were as follows (with restriction enzyme sites underlined): 5'-CGTTCGAAGCT ATGCTTTTGCAAGCTTTCCT (SUC2P1, a gift of K. Murakami of this laboratory); 5'-GATAGGTGAC TGCAGATATCTTGGCTG (P3); 5'-GATATCTGCA GTCACCTATCAAGG (P4); 5'-CCGCGGGTTG GAATC TGCACACTTCTTGG (P5). PCR amplifications were carried out using a Takara PCR amplification kit with a recombinant Taq DNA polymerase (Takara Shuzo) as recommended by the manufacturer. pCDM.IL2R/Ig and pCDM.IL2R/Ig₂ were constructed in similar fashion, using P6 or P8, and P10 as forward primer, and P7 or P9, and P5 as reverse primer, respectively. The resulting construct (pCDM.IL2R/Ig or pCDM.IL2R/Ig₂) encodes a protein comprising the human IL-2 receptor α chain signal sequence (MDSYLLMWGLLTFFIMVPGCQA), two amino acids introduced (EL), followed by either residues 329-547 or 315-547 of IgE-Fc, respectively. The 5' PCR primer P6 was designed such that they contained a *Hind*III restriction enzyme site, followed by an in-frame initiating methionine and a 45 bp sequence in the hIL-2R α signal sequence (P6, 5'-AAGCTT ATGGATAGCTACCTGCTGATGTGGGACTGCTCACGTTTCATCATGGTG); the 3' primer P7 consisted of the hybridizing sequence (18 bp) and a 15 bp sequence in the hIL-2R α signal sequence plus Glu-Leu, Ala329, and a *Hinf*I and a *Sac*II restriction site (P7, 5'-CCGCGGGTTGGAATC TGCGAGCTCTGCCTGGCAGCCAGGCACCATGATGAACGTGAG); P8: 5'-AAGCTT ATGGATTCATACCTGCTGATGTGGGGA; P9: 5'-GAATC TGCGAGCTCTGCCTGGCAGCCAGGCA; P10: 5'-GAGCTC ACCTATCAAGGTCACACCTTTG. DNA sequences were determined by Sanger's dideoxy chain termination method (Sanger et al. 1977) using alkali-denatured, double-strand DNA as templates either on manual or on an A.L.F. DNA Sequencer (Pharmacia, Uppsala, Sweden). In case of manual, [α -³²P] dCTP (specific activity ~3,000 Ci/mmol; Amersham, Little Chalfont, England) and Sequenase (version 2.0; United States Biochemical, Cleveland, OH, USA) or BcaBEST Dideoxy Sequencing Kit (Takara Shuzo) were used. In case of an A.

L.F. DNA Sequencer, AutoRead Sequencing Kit (Pharmacia Biotech, Uppsala, Sweden) and either fluorescein-labeled FHSA01, FHSA11 primer (the gifts of M. Miura, of this laboratory), fluorescein-labeled T7 primer (Takara Shuzo), fluorescein-labeled M13 reverse primer (Pharmacia Biotech), or sets of both M13 reverse primer RV (Takara Shuzo), M13 primer (—40) M4 (United States Biochemical), P2, or P11 and Fluore-dATP Labelling Mix (Pharmacia Biotech) were used. The primers were as follows: 5'-TTCGGATCAACTGTATTGAC (FHSA01); 5'-GAAGCCTGCATCTCTCAGGC (FHSA11); 5'-CGGTCCCCTTGCTGGGTGCCA (P11, from nucleotide 751 to 731).

Transient expression

COS-7 cells were harvested by treatment with trypsin (Boehringer Mannheim), and cells ($2.5\text{--}3 \times 10^5$ cells per 60 mm dish) were seeded 18–24 hr before transfection and were transfected with the mammalian expression vectors (pCDM.IL2R/Ig, pCDM.IL2R/Ig₂, pCDM.SUC2/Ig, pCDM.SUC2/Ig₂, or pCDM8 as mock) using DEAE-dextran (Promega, Madison, WI, USA) according to the manufacturer's instructions except that DMEM was used for washing before transfection and for 3 hr-incubation with chloroquine after transfection. Transfected cells were then treated with 10% DMSO in Dulbecco's phosphate-buffered saline (D-PBS) at room temperature for about 2 min, and incubated in culture media at 37°C in a 5% CO₂ atmosphere for 2 or 3 days. The cell-free culture supernatants were stored at –70°C.

Measurement of IgE activity

Human soluble Fc ϵ RI α /human IgE binding activity was determined by enzyme-linked immunosorbent assay (ELISA) using a modification of solid-phase IgE-binding assays described by Basu et al. (1993). In brief, polystyrene microtiter plates (Nunc, Kamstrup, Denmark) were coated with recombinant human soluble Fc ϵ RI α : the wells were incubated with aliquots (50 μ l, 0.25 μ g) of human soluble Fc ϵ RI α (5 μ g/ml) in D-PBS at 4°C overnight, and then blocked with 0.2 ml of 2% BSA in D-PBS at room temperature for 2 hr, followed by four washes with the buffer consisting of 0.05% Tween 20 in D-PBS. The culture supernatants were 10-fold diluted with the buffer, and added into the wells (50 μ l/well) of the human soluble Fc ϵ RI α -coated plates. After 2 hr-incubation at 37°C and washing with the buffer, the wells were incubated with rabbit antibody against human IgE (1,000-fold diluted, 50 μ l/well) at room temperature for 2 hr, and then washed with the buffer and incubated with horseradish peroxidase-labeled goat antibody against rabbit IgG (1,000-fold diluted, 50 μ l/well) at room temperature for 1 hr. The plates were developed with 7.4 mM *o*-phenylenediamine (Nakarai Kagaku [Nacalai Tesque], Kyoto) in 44 mM citrate/103 mM phosphate buffer, pH 5, containing 0.006% hydrogen peroxide. The plates were read at 414 nm. The specific activity (arbitrary unit) is shown which

is the value obtained by this assay divided by the value of the IgE antigen activity (determined by the following assay). $-$, $+$, and $\#$ denote specific activity of less than 0.04; less than 0.2; and more than 0.2 but less than 0.5 (arbitrary unit), respectively. The specific activity of the native IgE is 1 (arbitrary unit) by this definition. For human IgE antigen activity (concentration), a sandwich ELISA was used using the intact native human IgE as a standard and anti-human IgE-coated plates. Aliquots ($50\ \mu\text{l}$, $0.5\ \mu\text{g}$) of affinity purified antibody goat anti-human IgE ($10\ \mu\text{g/ml}$) in D-PBS were added onto 96-well plates, incubated at 4°C overnight, and blocked with $0.2\ \text{ml}$ of 2% BSA in D-PBS at room temperature for 2 hr. The culture supernatants were 10-fold diluted with the buffer consisting of 0.05% Tween 20 in D-PBS, and added into wells ($50\ \mu\text{l/well}$) of the anti-human IgE-coated plates, and then probed and processed with rabbit antibody against human IgE and horseradish peroxidase-labeled goat antibody against rabbit IgG as above. Dose-response standard curve was obtained by the least squares method and significant linearity between several ng/ml and hundreds ng/ml was confirmed by analysis of variance/linear regression analysis.

Immunoprecipitation and Western blotting

Standard methods were used for protein manipulations (Harlow and Lane 1988). The culture supernatants of COS cells were passed through a $0.2\ \mu\text{m}$ filter and aliquots ($100\ \mu\text{l}$ to $3\ \text{ml}$) were incubated with 10 to $300\ \mu\text{l}$ (3 to $100\ \mu\text{g}$ equivalents) of goat anti-IgE polyclonal antibody-conjugated Sepharose CL 4B beads (Pharmacia Biotech, prepared with CNBr-activated beads as recommended by the manufacturer) overnight at 4°C . The beads were washed extensively with D-PBS and then with D-PBS containing $0.4\ \text{M}$ NaCl. For digestion with endoglycosidase H (Genzyme, Cambridge, MA, USA) (Fig. 2, lanes 2–5), aliquots of the beads were treated exactly according to the method described by Tanaka et al. (1995). In Fig. 2B (lanes 9 and 10: the IgE-Fc fragments of SUC2/Ig and SUC2/Ig₂, respectively, under nonreducing condition), samples were prepared as follows. The culture supernatants of COS cells (about 10 – $15\ \text{ml}$) were processed as above. After rocking and washing, the resin was collected into a column and eluted with $0.1\ \text{M}$ glycine (pH 2.0) (collection buffer, $1/10$ volume of $2\ \text{M}$ Tris), and the eluate was concentrated. The samples were then subjected to SDS-PAGE and electroblotting essentially according to the method described by Tanaka et al. (1995) except that the blot was blocked with 5% nonfat dry milk (Morinaga, Tokyo) in D-PBS, probed (for at least 1 hr) with $1\ \mu\text{g/ml}$ of rabbit anti-IgE polyclonal antibody and 5% milk in D-PBS, and developed with $5,000$ -fold diluted peroxidase-conjugated goat anti-rabbit IgG (for at least 30 min) and $3,3'$ -diaminobenzidine (Wako Pure Chemical, Osaka) ($10\ \text{mg}$ in $16.7\ \text{ml}$ of $0.1\ \text{M}$ Tris-HCl (pH 7.6) containing 0.03% hydrogen peroxide) or Renaissance Western blot chemiluminescence reagent (DuPont NEN, Boston, MA, USA). The rabbit anti-human IgE polyclonal antibody crossreacted with bovine carbonic anhydrase

(molecular marker) (data not shown) and the goat anti-human IgE polyclonal antibody.

RESULTS AND DISCUSSION

IgE-Fc expression vectors

The construction procedure of human IgE-Fc expression vectors is described under "MATERIALS AND METHODS." cDNA encoding IgE-Fc fused to the SUC2 signal sequence was cloned into the mammalian expression vectors: pCDM.SUC2/Ig and pCDM.SUC2/Ig₂, resulting in IgE-Fc fragments of C ϵ 2'(Ala329)-C ϵ 3-C ϵ 4 and C ϵ 2'(Val314-Ala329)-C ϵ 3-C ϵ 4 (SUC2/Ig and SUC2/Ig₂), respectively (Fig. 1). The fusion resulted in the absence of the extra amino acid sequence at the amino terminus of the constructs. Construction of an expression vector for IgE-Fc of C ϵ 2'(Thr315-Ala329)-C ϵ 3-C ϵ 4 fused to the SUC2 signal sequence failed at the first trial. According to studies by Basu et al. (1993), in which a mammalian expression strategy was devised whereby IgE-Fc fragments were fused to the hIL-2R α signal sequence in order to facilitate secretion, cDNA encoding IgE-Fc fused to the hIL-2R α signal sequence was also cloned into the mammalian expression vectors: pCDM. IL2R/Ig and pCDM. IL2R/Ig₂, resulting respectively in IgE-Fc fragments of C ϵ 2'(Ala329)-C ϵ 3-C ϵ 4 (lacking Cys328 but known to exist as a noncovalent dimer) and C ϵ 2'(Thr315-Ala329)-C ϵ 3-C ϵ 4 (containing Cys328 known to be responsible for interchain disulfide bonding) (IL2R/Ig and IL2R/Ig₂) (Fig. 1).

Expression of IgE-Fc by COS cells

IgE-Fc was transiently expressed in COS cells following transfection with various vectors. The concentration of expressed IgE-Fc secreted into the culture supernatant was determined by ELISA (Table 1). Cells transfected with 10 μ g of mock (pCDM8), pCDM.IL2R/Ig, pCDM.IL2R/Ig₂, pCDM.SUC2/Ig, and pCDM.SUC2/Ig₂ secreted IgE-Fc at less than 0.004, 0.32, 0.48, 0.19, and 0.27 μ g/ml, respectively, suggesting that the difference in the extent of IgE-Fc secretion is indistinguishable whether directed by the SUC2 signal sequence or the hIL-2R signal sequence. The concentration of IgE-Fc in the culture supernatant peaked at 2 days post-transfection, and transfection of cells with higher concentrations of plasmid (20 μ g) did not markedly increase the amount of IgE-Fc secreted (not shown). The values shown in Table 1 are mean $\pm$ s.d. from four experiments.

Comparison of recombinant IgE-Fc functional activity

The specific functional binding activity of IgE-Fc to the human soluble Fc ϵ RI α was assayed by ELISA. In this assay, soluble Fc ϵ RI α was coated on ELISA plates. Native IgE or recombinant IgE-Fc fragments were added to wells, and the relative levels of IgE bound to the receptor were quantitated by development with a peroxidase-conjugated antibody. The binding of IgE to

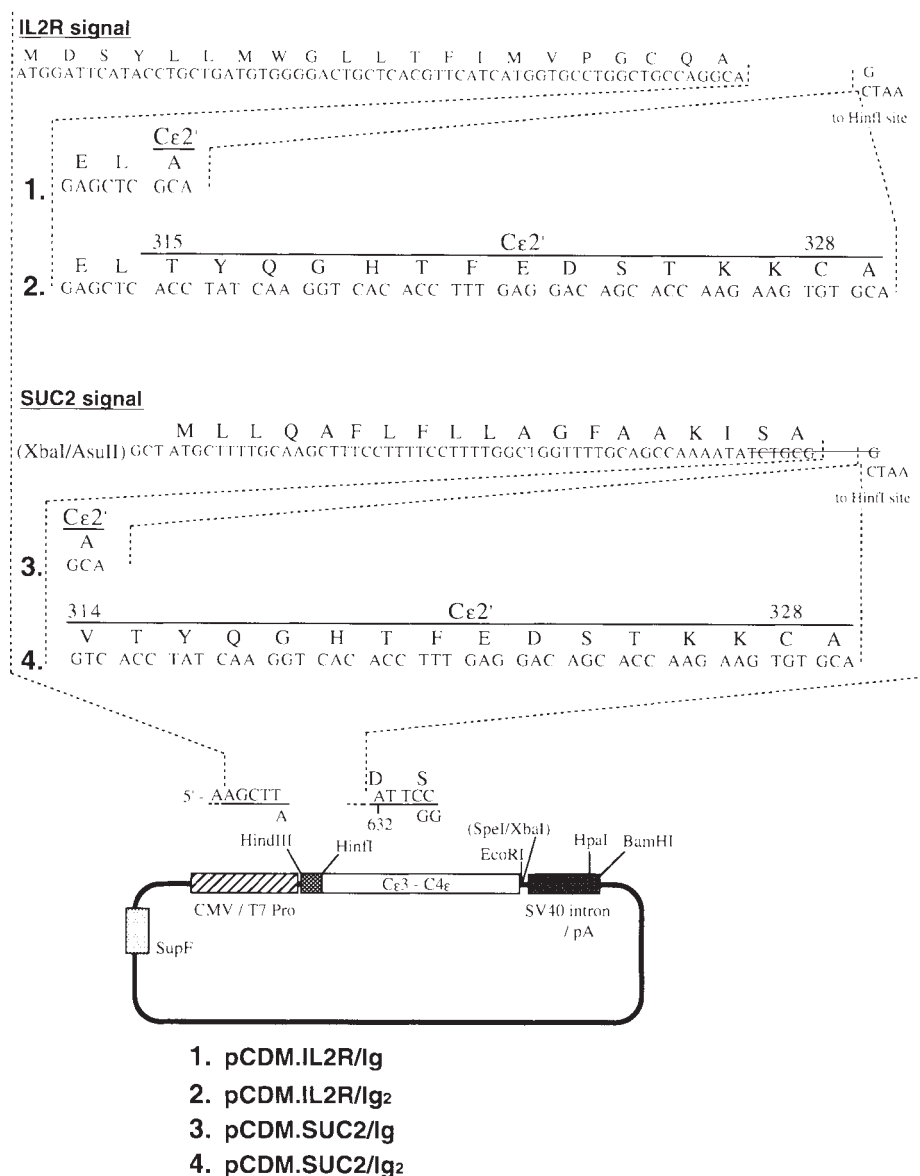


Fig. 1. Structure of human IgE-Fc expression vectors. The DNA sequence between the *Hind*III site and the *Hinf*I site of the IgE-Fc is shown. The predicted amino acid sequence of the amino-terminal region is also indicated above the DNA sequence. The mammalian expression vector pCDM8 carrying the cytomegalovirus/T7 RNA polymerase promoter (CMV/T7 Pro), the simian virus 40 splice and polyadenylation signals (SV40 intron/pA), and the suppressor tRNA *supF* gene, was used for IgE-Fc expression in mammalian cells.

$Fc\epsilon RI\alpha$, which was determined in this assay as absorbance at 414 nm, was proportional to the IgE concentration (not shown). No reactivity was detected in the absence of human IgE.

When the supernatants of the transfected COS cells were examined for the presence of $Fc\epsilon RI\alpha$ binding activity, SUC2/Ig₂ and IL2R/Ig₂ were active but had smaller activity than native IgE (Table 1). By comparison SUC2/Ig and IL2R/Ig showed lower activity than SUC2/Ig₂ and IL2R/Ig₂ (Table 1). Our results are consistent with those obtained by Basu et al. (1993), who observed that

TABLE 1 *Transient expression of human IgE-Fc fragments by COS cells*

IgE-Fc	IgE activity ^a	
	Concentration ^b (μ g/ml)	Fc ϵ RI α binding activity ^c
mock	<0.004	—
IL2R/Ig	0.32 $\pm$ 0.19	+
IL2R/Ig ₂	0.48 $\pm$ 0.28	‡
SUC2/Ig	0.19 $\pm$ 0.09	+
SUC2/Ig ₂	0.27 $\pm$ 0.16	‡

^aCOS cells were transfected with the indicated DNA constructs using the DEAE-dextran method, and cultured in medium at 37°C for 3 days. The culture supernatants were assayed for IgE activity.

^bThe values are mean $\pm$ s.d. from four experiments.

^c—, +, and ‡ denote specific activity (the value obtained by this assay divided by the value of the IgE concentration) of less than 0.04; less than 0.2; and more than 0.2 but less than 0.5 (arbitrary unit), respectively. The specific activity of the native IgE is 1 (arbitrary unit) by this definition.

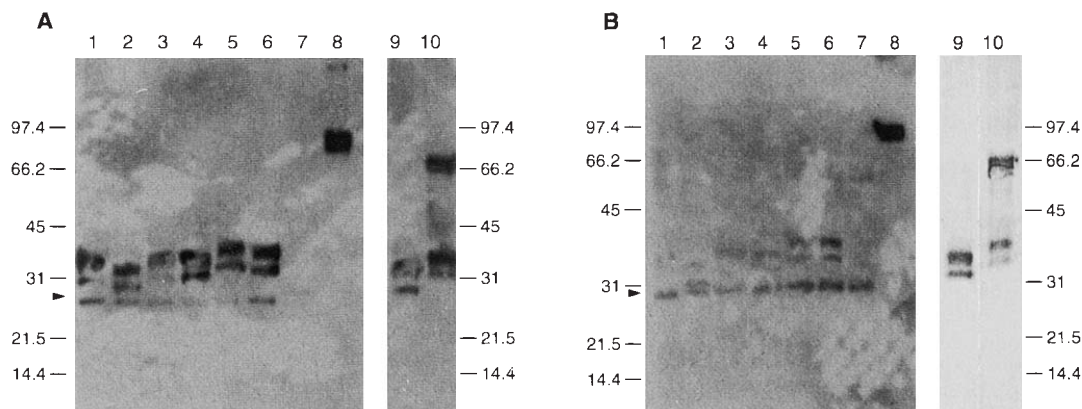


Fig. 2.(A) IgE-Fc fragments of IL2R/Ig (lanes 1-3, 9) and IL2R/Ig₂ (lanes 4-6, 10). The IgE-Fc fragments were immunoprecipitated from the culture supernatants of COS cells, and treated with (lanes 2, 4) or without (lanes 3, 5) endoglycosidase H. The proteins were then subjected to SDS-PAGE and analyzed by Western blotting (Renaissance Western blotting chemiluminescence reagent). IgE-Fc run under reducing (lanes 1, 6) and non-reducing conditions (lanes 9, 10) are shown. Endoglycosidase H alone (lane 7) and the native human IgE (lane 8, in the presence of 2-mercaptoethanol) were also run. (B) IgE-Fc fragments of SUC2/Ig (lanes 1-3, 9) and SUC2/Ig₂ (lanes 4-6, 10). Samples were prepared, loaded, and processed as above except that goat anti-human IgE alone (1 μ g) was run (lane 7) and that the blot (lanes 9, 10) was developed with 3, 3'-diaminobenzidine. Apparent molecular masses were determined on standard curves of marker proteins (molecular weights are shown in kDa) obtained by the least squares method (see "RESULTS AND DISCUSSION"). The arrowhead is a nonspecific band due to goat anti-human IgE antibody.

the smallest IgE-Fc fragment capable of binding to the $\text{Fc}\epsilon\text{RI}\alpha$ was $\text{Fc}\epsilon(329-547)$ (but less active than $\text{Fc}\epsilon[315-547]$ and much less than native IgE). However, it should be clarified whether the behavior is different between non-covalently associated and disulfide-linked IgE-Fc, especially on $\text{Fc}\epsilon\text{RI}\alpha$ binding activity.

Molecular properties of IgE-Fc

To analyze the molecular properties of IgE-Fc, the supernatants of the transfected COS cells were immunoprecipitated with an anti-IgE polyclonal antibody. The immunoprecipitates were treated with or without endoglycosidase H, resolved by electrophoresis on a polyacrylamide gel, and analyzed by Western blotting using another anti-IgE polyclonal antibody. For all of the proteins, multiple bands of doublets and narrower ones were detected by immunoprecipitation analysis (Fig. 2). On SDS-PAGE under nonreducing conditions, both SUC2/Ig and IL2R/Ig migrated as bands of apparent molecular masses of approximately 33- to 37-kDa (doublet) and 31-kDa. Both SUC2/Ig₂ and IL2R/Ig₂ did as those of 68- to 77-kDa (doublet), 36- to 39-kDa (doublet) and 34-kDa. On reduced gels, the bands of SUC2/Ig₂ and IL2R/Ig₂ shifted to the 37- to 40-kDa and 34-kDa region, indicating that these native proteins exist as disulfide-linked dimer or monomer. The latter remained probably by reason of being beyond the cell's potential for protein production, which is in particular characteristic for overproduction (Tuite and Freedman 1994). There was no major change in mobility of SUC2/Ig and IL2R/Ig, but their apparent molecular masses were slightly higher. This difference in mobility probably reflects a more compact conformation of the native molecules which is stabilized by intramolecular disulfide bonds. Moreover, SUC2/Ig, SUC2/Ig₂, IL2R/Ig, and IL2R/Ig₂ were only partly sensitive to endoglycosidase H as shown by slight reductions in their apparent molecular masses of 1- to 3-kDa (about 2-kDa) in the proportion of the respective bands (Fig. 2). Accordingly, these bands may represent recombinant IgE-Fc polypeptides containing different levels of glycosylation. Our results are consistent with those obtained by Basu et al. (1993). Comprehensively, the immunoprecipitation analysis revealed that both SUC2/Ig and SUC2/Ig₂ had M_r s and degrees of glycosylation similar to IL2R/Ig and IL2R/Ig₂, respectively.

These results indicate that in mammalian cells the N-glycosylated and active IgE-Fc fragments can be produced to almost the same extent whether COS cells are transfected with IgE-Fc fused to the SUC2 signal sequence or to the human IL-2R α signal sequence. However, the possibility of release by cell lysis is not excluded. To confirm that in mammalian cells the SUC2 signal sequence is critically functional in secretion of the active proteins, it should be elucidated whether the protein without the signal sequence is not secreted, and whether amino-terminal cleavage of the protein secreted occurs correctly. Furthermore, using CHO and other cells as host mammalian cells, it should be clarified whether

the SUC2 signal sequence is also functional in stable expression systems for protein production. Regardless, our finding might lead to develop a shuttle vector containing not only the powerful promoters but also the efficient common signal sequence between the yeast and mammalian cells, possibly making it convenient to construct expression vectors.

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