

Characterization of Truncated α Chain Products from Human, Rat, and Mouse High Affinity Receptor for Immunoglobulin E*

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The high affinity receptor for immunoglobulin E (IgE) is a tetrameric structure ($\alpha\beta\gamma_2$) consisting of non-covalently associated subunits: one IgE-binding α chain, one 4-fold membrane spanning β chain, and two disulfide-linked γ chains. Here, we have engineered α cDNA constructs (α_{trunc}) encoding exclusively the leader peptide and the extracellular domain of the α subunit. Transfection of human α_{trunc} into COS-7 cells resulted in the secretion of soluble IgE-binding polypeptides. By contrast, the polypeptides generated from rat and mouse α_{trunc} transfections were sequestered in the endoplasmic reticulum and degraded even though they appeared to fold properly as judged by their capacity to bind IgE. Stable transfectants with human α_{trunc} were obtained from a dihydrofolate reductase-deficient Chinese hamster ovary cell line. Several clones secreted substantial amounts ($0.1 \mu\text{g/ml}/10^6$ cells) of IgE-binding polypeptides. The dissociation rate of bound IgE from this soluble truncated α ($k_{-1} = 4.9 \times 10^{-6} \text{ s}^{-1}$ at 25°C) was characteristic of receptors on intact cells. After treatment with tunicamycin, the transfectants secreted unglycosylated 18-kDa polypeptides which could also bind IgE. These unglycosylated products had a tendency to form dimers and higher oligomers which were resistant to treatment by sodium dodecyl sulfate and reducing agents. These data demonstrate unequivocally that the extracellular domain of the α subunit is sufficient to mediate high affinity binding of IgE. Furthermore, posttranslational addition of carbohydrates is not required for proper folding and function of the receptor binding site. The truncated human α should be a suitable reagent for crystallographic analysis and for detailed analysis of the receptor binding site.

is one of the key molecules involved in triggering the allergic reaction (1, 2). This receptor binds monomeric IgE with high affinity. When receptor-bound IgE is challenged by a multivalent allergen, the consequent redistribution of IgE-receptor complexes on the cell surface induces cellular degranulation and the release of factors responsible for allergic reactions. Triggering of these cells through Fc ϵ RI also induces the synthesis and secretion of various cytokines among which are interleukin 3, interleukin 4, and interferon γ (3-5).

Fc ϵ RI is a tetrameric complex of one α , one β , and two identical disulfide-linked γ subunits. cDNAs for α (6-9), β (9, 10), and γ (9, 11, 12) have now been isolated from different species and a schematic model for the receptor has been proposed recently (1, 11). Past reports have indicated that the receptor is univalent (13, 14) and that the high affinity receptor binding site is localized on the α subunit (1). However, the role and importance of the respective domains of α was not assessed. The possible role of N-linked sugar on the receptor binding site was investigated by treating Fc ϵ RI-expressing cells with tunicamycin. In these conditions, the surface-expressed receptor could bind IgE (15). However, the possible role of O-linked sugar associated with the receptor in these cells (16) was not analyzed.

In the present study we have engineered truncated cDNAs of α (α_{trunc}) from three different species, which consist only of sequences encoding the leader peptide and extracellular domain. After transfection with these constructs, the truncated product of human α is secreted in a soluble form, whereas the truncated products of mouse and rat α are retained in the endoplasmic reticulum or in the cis-Golgi. The truncated human α is fully functional in binding IgE with high affinity even after deglycosylation.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis and Subcloning—The human α_{trunc} construct was engineered by restricting the full length cDNA with *Eco*RI and *Sma*I (7). The resulting 672-bp fragment was purified and ligated with the complementary oligonucleotides 5'-CCTCAACAT-TACTGTAATAAAGCTTAAG-3' and 5'-GATCCTTAAGCTTT-TATTACAGTAATGTTGAGGGGC-3'. The TAA stop codon is underlined. These oligonucleotides also provided a *Bam*HI site for convenient subcloning into the pGEM-3Z vector (Promega Biotech, Madison, WI). The mutation and the integrity of the remaining sequence were assessed by completely sequencing the new construct. Sequencing was performed as before (10). The *Eco*RI-*Bam*HI fragment of the human α_{trunc} construct was excised from pGEM-3Z, filled in with Klenow enzyme (New England Biolabs, Beverly, MA) and cloned into the *Sma*I site of the SV40 late promoter-driven pSVL vector (Pharmacia, Piscataway, NJ). The *Eco*RI-*Pst*I fragment of the α_{trunc} construct was excised from pGEM-3Z and was subcloned into the corresponding sites of the polylinker of the SV40 early promoter-driven pKC3 vector (17).

The truncated rat and mouse α mutants were constructed using the polymerase chain reaction, as described (18). The rat α full length cDNA in pGEM-3Z (6) and mouse α in pBluescript SK (9) (Strata-

The receptor for IgE (Fc ϵ RI)¹ on mast cells and basophils

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¹ The abbreviations used are: Fc ϵ RI, high affinity receptor for IgE; α_{trunc} , truncated Fc ϵ RI α cDNA; DHFR, dihydrofolate reductase; BBS, borate-buffered saline; bp, base pair(s); DNP, dinitrophenyl; SDS, sodium dodecyl sulfate; Endo H, endo- β -N-acetylglucosaminidase H; RBL, rat basophilic leukemia; CHO, Chinese hamster ovary.

gene, La Jolla, CA) were used as templates. Mutant complementary oligonucleotides were: 5'-GTAAAGATTAGACAATTGAGT-3'; 5'-ACTCAATTGTCTAATCTTTTAC-3' for rat α and 5'-GTAAAG-CTTAGAAATGCAAGT-3'; ACTTGCATTCTTAAGCTT-TTAC for mouse α . Outer primers (Sp6 and T7 for rat α ; T3 and T7 for mouse α) were employed to amplify two fragments with the sense- and antisense-oligonucleotide, respectively. The two fragments were gel-purified and mixed together in a second polymerase chain reaction amplification using the two outer primers. Rat α -amplified products were then restricted with *EcoRI*-*HindIII* and cloned into the respective sites of pGEM-3Z. Mouse α -amplified products were restricted with *EcoRV*-*XbaI* and cloned into the *XbaI*-*HincII* sites of pGEM-4Z. The *EcoRI*-*HindIII* fragment of the rat α construct and the 863-bp *PstI* fragment of the mouse α construct were excised from their corresponding pGEM-vectors, filled in with Klenow or T4 polymerase (New England Biolabs) and cloned into the *SmaI* site of pSVL.

Cell Lines—COS-7 cells (American Type Culture Collection (ATCC), Rockville, MD; CRL 1651) were maintained in Dulbecco's modified Eagle's medium supplemented with 16% fetal bovine serum, 2 mM glutamine and 100 units/ml penicillin-streptomycin (Biofluids, Rockville, MD).

The Chinese hamster ovary mutant cell line CHO (ATCC CRL 9096), lacking the dihydrofolate reductase (DHFR) gene was maintained in Iscove's modified Eagle's medium containing 2 mM glutamine, 100 units/ml penicillin-streptomycin, 10% fetal bovine serum, and supplemented with hypoxanthine (10^{-4} M), thymidine (1.6×10^{-5} M) supplement, (GIBCO). For selection, the hypoxanthine, thymidine supplement was omitted.

Transient Transfection—Before transfection, COS-7 cells were washed two times in ice-cold phosphate-buffered saline and resuspended at 1×10^7 cells/ml in phosphate-buffered sucrose (272 mM sucrose, 7 mM sodium phosphate, 1 mM $MgCl_2$, pH 7.4). 60 μ g of purified DNA of α_{trunc} -containing pSVL were incubated with 8×10^6 COS-7 cells in Genepulser cuvettes and transfected at 380 V, 25 microfarads by using an electroporation apparatus (Bio-Rad, Richmond, CA).

Stable Transfection and Selection of Clones—Two plasmids were cotransfected: the pSV2-DHFR vector (19) containing a cDNA copy of the mouse DHFR gene driven by the SV40 early promoter and the human α_{trunc} -containing pKC3 vector. DHFR-deficient CHO cells were cotransfected with both plasmids (25 μ g of human α_{trunc} -containing pKC3 DNA and 1 μ g of DHFR-containing pSV2 DNA) in the same conditions as above except that electroporation was performed at 500 V, 25 microfarads. 24 h after transfection, cells were placed in selective medium without hypoxanthine, thymidine supplement. Resistant colonies were subjected to stepwise increases of methotrexate concentration (15 nM, 60 nM, 240 nM, 1 μ M, and 2 μ M) over a 6–8-week period and finally maintained in 2 μ M methotrexate. Individual clones were obtained by diluting the cell population at 0.5 cells/well in 96-well culture dishes.

Detection of Secreted α —A cellular radioimmunoassay was used to measure the capacity of truncated α products to inhibit the binding of iodinated mouse IgE to rat basophilic leukemia cells (RBL-2H3). Supernatants from transfected cells, each in duplicate samples, were incubated with 100 ng/ml of ^{125} I-mouse IgE for 16–24 h at 4 °C. To 1×10^6 of the samples, 100 μ l of RBL cells at a concentration of $0.5\text{--}1 \times 10^6$ cells/ml were added and incubated at room temperature for 1–3 h. RBL cells were centrifuged at $200 \times g$ for 6 min and washed once in medium. Cell pellets, supernatants, and washes were counted in a gamma counter.

The maximum IgE binding (0% inhibition) was determined using culture media from COS-7 cells transfected with rat γ or from nontransfected CHO cells. For measurement of nonspecific binding, RBL cells were preincubated for 10 min with rat IgE (65 μ g/ml) before being incubated with 30 μ g/ml of rat IgE during the assay described above. The nonspecific counts were subtracted to determine the net specific binding.

Biosynthetic Labeling and Pulse-chase Experiments—Stable transfectants (CHO cells) were grown to confluence ($\approx 4\text{--}5 \times 10^7$ cells) in 162-cm² flasks. Transient transfectants (8×10^6 COS-7 cells) were kept in 75-cm² flasks for 40 h after transfection. Attached cells were washed twice in cysteine-free Dulbecco's modified Eagle's medium containing 2 mM glutamine, 100 units/ml penicillin-streptomycin, 3% dialyzed fetal bovine serum (GIBCO) and preincubated in cysteine-free medium. After 1 h the medium was replaced and the cells were incubated for 12–18 h in 10 ml (COS-7 cells) or 15 ml (CHO cells) of cysteine-free medium containing 50–150 μ Ci/ml of L-[³⁵S]-cysteine (Amersham Corp.). In selected experiments 83.3 μ Ci/ml of

D-[6-³H]glucosamine-HCl (ICN Biomedicals) was also added to the incubation mixture.

For pulse-chase experiments, transiently transfected COS-7 cells were preincubated in 8 ml of cysteine- and methionine-free medium before being pulse-labeled for 30 min with 3.75 mCi of a L-[³⁵S]-methionine-L-[³⁵S]-cysteine mixture (Amersham). Following the pulse, the cells (2.4×10^7 cells per time point) were incubated in complete medium supplemented with 1.8 mM L-cysteine and L-methionine. After each time point the cells were kept chilled on ice then processed together at the end of the experiment. The supernatants were collected, the cells treated with trypsin, washed once in medium, twice in phosphate-buffered saline, and lysed at 5×10^7 cells/ml in borate-buffered saline (BBS), pH 8.0, containing 0.5% Triton X-100, 10 mM aprotinin, 1 mM phenylmethanesulfonyl fluoride, 5 mg/ml leupeptin, and 5 mg/ml pepstatin (all reagents from Fluka, Ronkonkoma, NY).

Immunopurification Procedures—Cell culture supernatants were incubated with 1 μ g/ml of mouse anti-dinitrophenyl (DNP)-IgE (20) for 12–16 h at 4 °C, except in the experiment with tunicamycin in which the incubation was started during the biosynthetic labeling to avoid possible degradation of unbound glycosylated truncated α . The truncated α -IgE complexes were bound to a 5-fold excess of trinitrophenyl-lysyl-Sepharose beads (binding capacity, ≈ 1 mg/ml) as described (21) and then thoroughly washed with BBS. After elution with 10 mM DNP- ϵ amino-caproate (Sigma) in BBS, the eluate was immunoprecipitated as before (21) with rabbit anti-mouse IgE followed by Pansorbin (Calbiochem) or protein G-Sepharose (Pharmacia LKB Biotechnology Inc.). Centrifuged pellets were washed three times in BBS and extracted with sample buffer by boiling for 5 min as described previously (21).

Intracellular truncated α was recovered by incubation of cell lysates with 20 μ g/ml mouse IgE anti-DNP for 12–16 h at 4 °C. The purification was performed as above except that the trinitrophenyl-lysyl-Sepharose beads were washed with BBS containing 0.1% Triton X-100 and protease inhibitors.

Deglycosylation Studies—A stock solution of 0.5 mg/ml tunicamycin (Fluka) (22) was prepared in phosphate-buffered saline/ethanol (1:1). Stable transfectants were preincubated in 0–4 μ g/ml tunicamycin for 6–18 h before and during biosynthetic labeling.

Digestion with peptide-N⁴-(N-acetyl- β -glucosaminyl) asparagine amidase (N-glycanase) (Genzyme, Boston, MA) was performed according to the supplier's instructions. Before migration on SDS gels, N-glycanase-treated samples were precipitated with 90% ethanol.

Treatment with endo- β -N-acetylglucosamidase H (Endo H) (Genzyme) was performed by incubating immunoprecipitates for 16 h at 37 °C in 50 μ l of 0.1 M sodium phosphate buffer, pH 6.1, containing 8 milliunits of Endo H, 0.1% Triton X-100, 0.03% SDS, and 20 mM EDTA (23).

Ammonium Sulfate Assay—The full length rat α -IgE complexes and truncated human α -IgE complexes were prepared as follows: after the binding of ^{125} I-mouse IgE to RBL-2H3 cells, the cells were washed extensively and full length rat α -IgE complexes were isolated by solubilization in the same lysis buffer as above; truncated human α -IgE complexes were generated by incubating the supernatant from transfected CHO 2.5 cells (see "Results") with 100 ng/ml of ^{125} I-mouse IgE for 16 h at 4 °C and the formation of complexes was assessed by using the cellular radioimmunoassay (see above).

The ammonium sulfate precipitation assay was performed as described (24) with minor modifications. Briefly, about 0.1–0.5 pmol of ^{125} I-mouse IgE or ^{125} I-mouse IgE- α complexes in 200- μ l medium and 100 μ l of normal rabbit serum (as a carrier) were added sequentially to 1 ml of BBS buffer containing $(NH_4)_2SO_4$ at the desired final concentration. The mixture was mixed on a vortex shaker and incubated for 1 h at 4 °C. After centrifugation at $16,000 \times g$ (10 min at 4 °C) the supernatant was removed. Precipitated pellets and supernatants were counted separately in a gamma counter.

Determination of the Dissociation Rate Constant— $2.5\text{--}5 \times 10^6$ CHO cells (2.5 or 2.5–33 transfectants) were biosynthetically labeled with [³⁵S]-cysteine and the culture medium was supplemented with ^{125} I-mouse IgE anti-DNP (1 μ g/ml) at a specific activity of about 6000 cpm/ μ g. After the labeling period, the IgE-truncated α complexes were bound to 2 ml of trinitrophenyl-lysyl-Sepharose beads. The beads were washed thoroughly and aliquoted into 12 microcentrifuge tubes so that triplicate samples could be assayed per time point. To each tube, 1 ml of BBS containing 500 μ g/ml of human IgE was added to prevent rebinding of dissociated IgE-binding polypeptide. The samples were kept at 4 or 25 °C for 0, 6, 12, and 24 h. After each time

point the beads were washed four times with 1 ml of BBS. Specifically bound material was eluted with 10 mM DNP- ϵ -amino caproate, immunoprecipitated, and applied to a 12.5% polyacrylamide gel under nonreducing conditions. The gel was dried, exposed to autoradiography, cut into 2-mm slices, and counted for ^{125} I. The gel slices were then further processed for determination of ^{35} S counts as described before (25). The ^{35} S counts corresponding to the peak of truncated human α were normalized to the ^{125} I-IgE counts in each lane. Dissociation rate constants were then determined as described (26).

RESULTS

Truncation of Human, Rat, and Mouse Fc ϵ RI α cDNAs—The cDNA sequences of human, rat, and mouse Fc ϵ RI α were mutated to generate cDNA constructs encoding exclusively the leader peptide and extracellular domain of α (α_{trunc}) (Fig. 1). The stop codon TAA was introduced by site-directed mutagenesis to replace the proline 173-CCG codon of human α . Similarly, the TAC codons of tyrosine 173 of both rat and mouse α were replaced by a TAG stop codon. The resulting constructs (human, rat, and mouse α_{trunc}) encode peptides of 172 amino acid residues after cleavage of the leader peptide. Sequencing of these constructs revealed an additional mutation in mouse α_{trunc} . This is commonly observed in constructs generated by polymerase chain reaction (27). However, the mutation encoded a valine residue, as in the original cDNA clone.

Analysis of the Truncated α Chains by Transient Expression—We then examined whether the transfected constructs could lead to a secreted form of the α chain which could bind IgE. The constructs were subcloned into the eucaryotic expression vector pSVL under the control of the SV40 late promoter, and the resulting plasmids were transfected into COS-7 cells. Two days after transfection, the cell supernatants were assayed for the presence of soluble IgE-binding α products. Mouse IgE (unlike human IgE) binds to both human and rodent Fc ϵ RI. Therefore, IgE-binding α chains from the three species should inhibit the binding of mouse IgE to rat basophilic leukemia (RBL) cells. Table I shows that supernatants of cells transfected with human α_{trunc} completely inhibited IgE-binding to RBL cells. As expected, preincubation of the supernatants with human IgE reversed the inhibitory effect. Surprisingly, the supernatants of cells transfected with rat and mouse α_{trunc} did not inhibit IgE-binding to RBL cells.

To characterize these protein products, transfected COS-7 cells were biosynthetically labeled with L-[^{35} S]cysteine, and IgE-binding peptides were recovered from cell supernatants

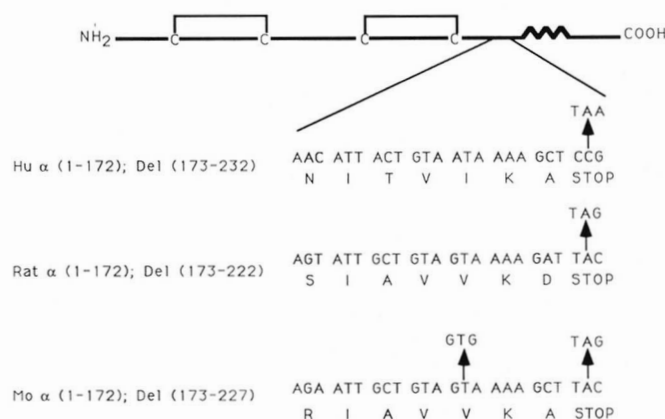


FIG. 1. Schematic representation of the structure of the α constructs. The sequence and localization of the mutated sites and stop codons is shown for the human, rat, and mouse constructs. Del refers to the amino acid residues deleted from the original protein sequence.

TABLE I

Detection of secreted α products by transfected COS-7 cells

Results are means $\pm$ S.D. obtained from two independent experiments, each in duplicate measurements.

Transfected construct	Supernatant ^a	Human IgE ^b	% inhibition of IgE-binding to RBL cells
Human α_{trunc}	+	—	99.2 $\pm$ 0.7
Mouse α_{trunc}	+	+	2.8 $\pm$ 0.6
Rat α_{trunc}	+	—	2.9 $\pm$ 1.3
Rat γ	+	—	6.8 $\pm$ 3.0
			~0

^a The cell supernatant from a 48-h culture of transfected COS-7 cells was used to inhibit the binding of ^{125} I-mouse IgE to RBL cells.

^b Supernatant was preincubated with human IgE in 500 molar excess over ^{125} I-mouse IgE.

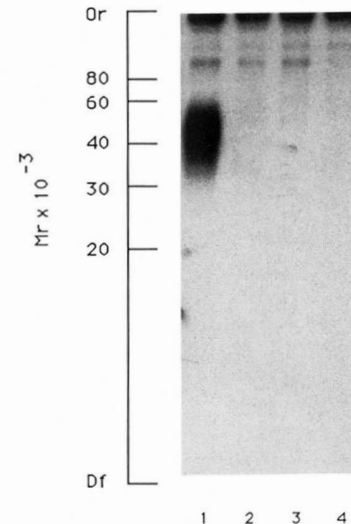


FIG. 2. Analysis of the truncated α products secreted by COS-7 cells transfected with the α constructs. The figure shows the autoradiography of a 12.5% polyacrylamide gel on which IgE-binding polypeptides, purified from the supernatant of transfected COS cells, were analyzed under nonreducing conditions. COS-7 cells transfected with human α_{trunc} (lane 1), rat α_{trunc} (lane 2), mouse α_{trunc} (lane 3), and Fc ϵ RI γ cDNA (lane 4). After transfection with the different α constructs, COS-7 cells were grown in L-[^{35} S]cysteine and IgE-binding polypeptides secreted in the culture medium were purified with mouse IgE using a double immunospecific protocol.

by using anti-DNP mouse IgE and a double immunospecific protocol (21). The immunoprecipitates were then analyzed by SDS gels and autoradiography (Fig. 2). Lane 1 shows that the secreted human α_{trunc} product appeared as a broad glycosylated band at around 45 kDa. The other protein bands observed around 90–120 kDa were present in the negative control (lane 4) and therefore considered as nonspecific. No specific peptides were detected in supernatants from cells transfected with either rat α_{trunc} (lane 2) or mouse α_{trunc} (lane 3). However rat α_{trunc} or mouse α_{trunc} products could have been secreted in undetectable quantities.

Intracellular Processing of Human and Rodent Truncated α Chains—To compare the fate of human and rodent truncated α chains, COS-7 cells transfected with human α_{trunc} (Fig. 3A) and rat α_{trunc} (Fig. 3B) were pulse-labeled with L-[^{35} S]cysteine and L-[^{35} S]methionine and chased for different time periods. The truncated α chains were then immunopurified with IgE from both cell lysates and cell culture supernatants and examined on SDS gels. Therefore, this study addressed only the fate of IgE-binding polypeptides. After a 30-min pulse labeling, soluble α chains from both species could be readily

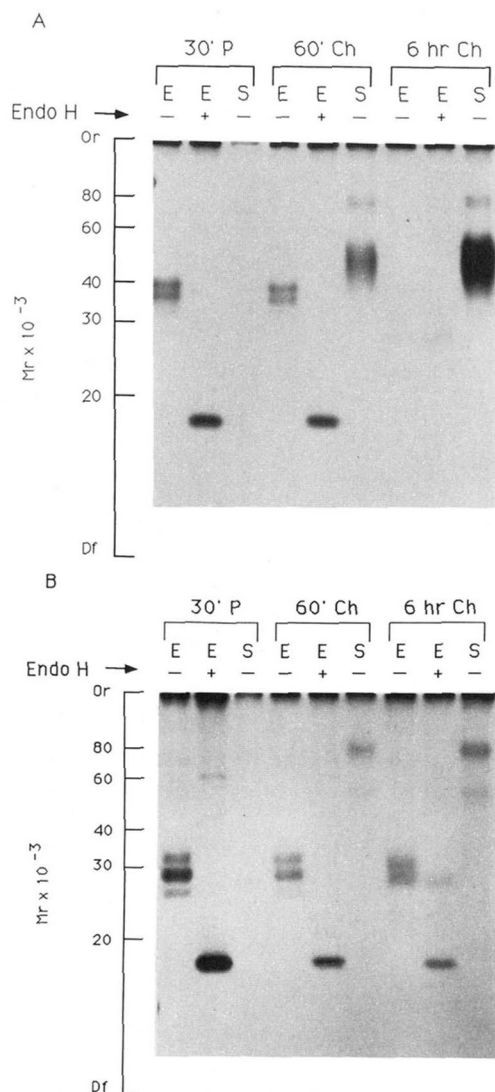


FIG. 3. Pulse-chase analysis of human (A) or rat (B) truncated α products in transfected COS 7 cells. Autoradiography of gels on which the purified material was analyzed. Transfected cells were pulse-labeled for 30 min (30' P) at 37 °C with L-[^{35} S]cysteine and L-[^{35} S]methionine and chased for 1 h or 6 h (6 hr Ch) in complete medium at 37 °C. Culture supernatant (S) and cellular extracts (E) were then immunoprecipitated with IgE, the immunoprecipitates treated (+) or not (–) with Endo H and analyzed as in Fig. 1.

detected in cellular extracts. They appeared as three different products migrating at 39, 37, 33 kDa for the human α_{trunc} (Fig. 3A, lane 1) and 33, 29, 26 kDa for the rat α_{trunc} (Fig. 3B, lane 1). A similar heterogeneity (36, 32, 28 kDa) was found for the mouse α_{trunc} (data not shown). The difference of molecular mass between the intracellular products of human and rodent α_{trunc} are likely due to differences of glycosylation. Digestion of these glycopeptides with Endo H produced a single product of approximately 18 kDa which corresponds to the core polypeptide of the truncated α chains (Fig. 3, A and B, lane 2). Endo H cleaves high mannose-type oligosaccharides (28) but not complex-type oligosaccharides which are added to proteins only after they have left the endoplasmic reticulum and entered the medial Golgi complex (29). Therefore the Endo H sensitivity of the three glycopeptides is indicative of their localization in the endoplasmic reticulum or in the *cis*-Golgi. After a 60-min chase, differences could be seen between the truncated human and rat α chains. Whereas the human product was further processed and appeared in the cell super-

natant (Fig. 3A, lane 6) the truncated rat α remained in the endoplasmic reticulum or in the *cis*-Golgi without being secreted (Fig. 3B, lane 6). After the 6-h chase, most of the counts were now associated with the 45-kDa secreted human α product (Fig. 3A, lane 9). By contrast, the corresponding 45-kDa product could not be purified in substantial amount from the supernatant of rat α_{trunc} transfected cells. However, it is possible that the faint band observed in this region (Fig. 3B, lane 9) corresponds to a small amount of secreted rat α product which would have bypassed the "filtering system" in a way similar to what we described previously for the full length rat α chain (9). The relative amount of truncated rat α remaining after a 6-h chase was determined by scanning densitometry of the autoradiograms (data not shown). While retained in the endoplasmic reticulum compartment, the truncated rat α chains are degraded with only a portion (21%) remaining after 6 h.

Production of a Stable Cell Line Secreting the Soluble Form of Human α —Because milligrams of proteins must be obtained before attempting to produce crystals suitable for analysis, we tried to generate stable transfectants with high level production of soluble α chains. The CHO cell line deficient for the *DHFR* gene was cotransfected with human α_{trunc} and the *DHFR* gene inserted into pKC3 and pSV2 vectors, respectively. This system of transfection presents two advantages: the transfectants can be stringently selected in hypoxanthine, thymidine-deficient medium and the copy number of the transfected genes can be amplified by using increasing doses of methotrexate over a 6–8-week period. When α_{trunc} transfected cells grew readily in 2 μ M methotrexate-containing medium, the supernatants were screened for the presence of secreted human α using the same assay as described above. Cell lines from three independent transfections were found to secrete truncated α , and the production remained stable over a period of 4 months. The 2.5 line showed the highest level of secretion and was cloned at 0.5 cell/well. Fig. 4 shows the quantitative inhibition of IgE binding to RBL cells by the 2.5 line and four of its subclones (2.5-9, 2.5-

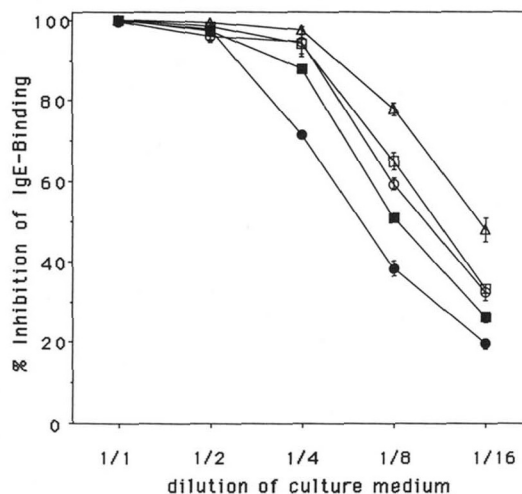


FIG. 4. Secretion of truncated α products by stable CHO transfectants. The cell supernatants were assayed for their capacity to inhibit the binding of mouse IgE to RBL cells. 1.25×10^6 cells/ml from four different clones were seeded in tissue culture flasks and cultured for 24 h. Dilutions of the cell supernatants were then preincubated with ^{125} I-mouse IgE (100 ng/ml). The binding capacity of ^{125} I-mouse IgE was 89.9%. The mixtures were then incubated with 1×10^7 RBL cells/ml for the binding assay. The figure shows the results of one representative experiment ($n = 5$). The mean values $\pm$ S.D. are from duplicate samples. $\circ$, cl.2.5; $\bullet$, cl.2.5-9; $\square$, cl.2.5-28; $\blacksquare$, cl.2.5-31; $\triangle$, cl.2.5-33.

28, 2.5-31, 2.5-33). At 50% inhibition the highest (2.5-33) and the lowest (2.5-9) producing clones differed only by a factor of 2. Assuming a 1:1 molar ratio between the α peptide and the bound IgE, one could calculate that the 2.5-33 clone produced about 2.5 ng/ml/h/ 10^6 cells of soluble α peptide. About 0.10–0.15 μ g/ml/ 10^6 cells can be produced routinely over 2 or 3 days of culture.

Determination of the Dissociation Rate of IgE from Soluble Truncated Human α —The interaction of the IgE molecule with membrane Fc ϵ RI is characterized by a single forward association rate constant (k_1) of about 10^5 M $^{-1}$ s $^{-1}$ for intact cells and a unimolecular dissociation rate constant (k_{-1}) of about 10^{-5} s $^{-1}$. Since we ultimately want to use the human α_{trunc} for crystallographic studies, it was important to examine whether the genetically engineered and expressed molecule had similar binding properties as the receptor on intact cells.

The association and dissociation rates of IgE from the α chain in detergent extracts of cells have previously been determined using an ammonium sulfate assay (24). The latter is based on a change in solubility of IgE when it becomes bound to the full length α chain. We investigated if this assay would also be suitable to study the binding characteristics of human truncated α . Fig. 5 shows a comparison between the precipitability of IgE and bound-IgE to full length α or truncated α . We confirmed that the solubility of IgE-full length α complexes and of unbound IgE can be easily distinguished. The maximum difference is seen in solutions saturated from 41 to 43% in ammonium sulfate. By contrast, the solubility of unbound IgE and of IgE-truncated α complexes is very similar. This suggests that either the transmembrane and/or the intracytoplasmic portion are critical for the change in solubility. These findings made it impractical to study the kinetics of binding of IgE to truncated α in solution.

Since it is the slow rate of dissociation which accounts for the high affinity ($K_A \approx 10^{10}$) of the receptor (26), we looked at the rate of dissociation of ^{35} S-labeled soluble α from anti-hapten ^{125} I-IgE bound to haptenated beads. After different time periods, the dissociated α was removed by washing and

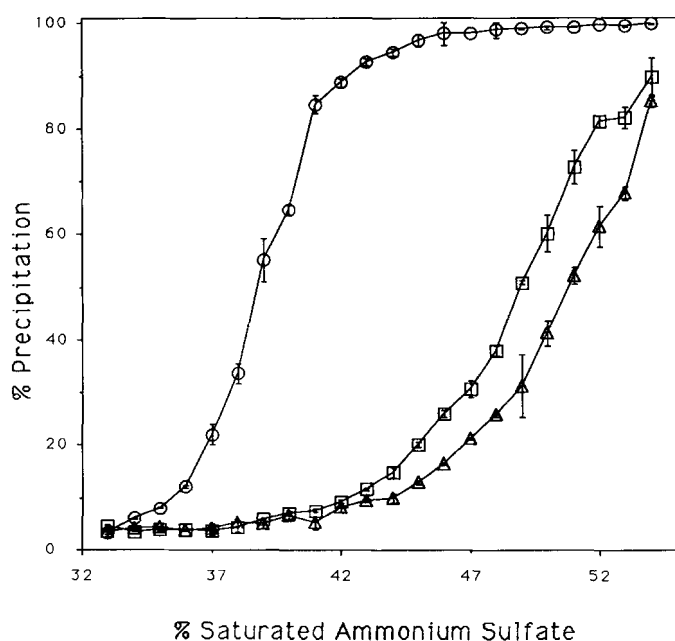


FIG. 5. Efficiency of precipitation of ^{125} I-IgE (Δ), ^{125} I-IgE-truncated α complexes ($\square$), and ^{125} I-IgE- α complexes from RBL cells ($\circ$) at various concentrations of $(\text{NH}_4)_2\text{SO}_4$. The figure shows the results of one representative experiment ($n = 3$). The mean values $\pm$ S.D. are from duplicate samples.

IgE-truncated α complexes were eluted by addition of excess hapten. After separation of the complexes on SDS gels, truncated α -associated counts (^{35} S) were quantified for each time point and normalized to IgE-associated counts (^{125} I). The fractional occupancy was then plotted as a function of time, and k_{-1} values were calculated at each temperature: $k_{-1} = 3.2 \times 10^{-6}$ s $^{-1}$ at 4 $^\circ$ C and $k_{-1} = 4.9 \times 10^{-6}$ s $^{-1}$ at 25 $^\circ$ C (Fig. 6). These dissociation kinetics (thick lines) are within the range of previously published data (dotted lines) for Fc ϵ RI α in solution (24) or on intact cells (26, 30–32).

Studies with Unglycosylated Soluble α Chains—To analyze the ability of unglycosylated soluble α chain to bind IgE, 2.5 CHO transfectants were labeled with L- ^{35} S]cysteine in the presence of tunicamycin. The secreted products were then recovered from the supernatant via their capacity to bind IgE and analyzed on SDS gels under nonreducing conditions (Fig. 7). Untreated cells produced soluble α chains which appeared as a broad band around 50 kDa (Fig. 7, lane 1; see also Fig. 8, lane 2). This was slightly higher than in COS cells and could be due to a difference in glycosylation between these two cell lines. By contrast, tunicamycin-treated cells secreted two major peptides around 17 and 45 kDa, which could clearly bind IgE with sufficient affinity to allow purification with the double immunospecific protocol which involves extensive washing (see "Experimental Procedures"). No difference was observed by increasing the doses of tunicamycin from 1 to 4 μ g/ml (Fig. 7, lanes 2, 3, and 4). The 17-kDa peptide most

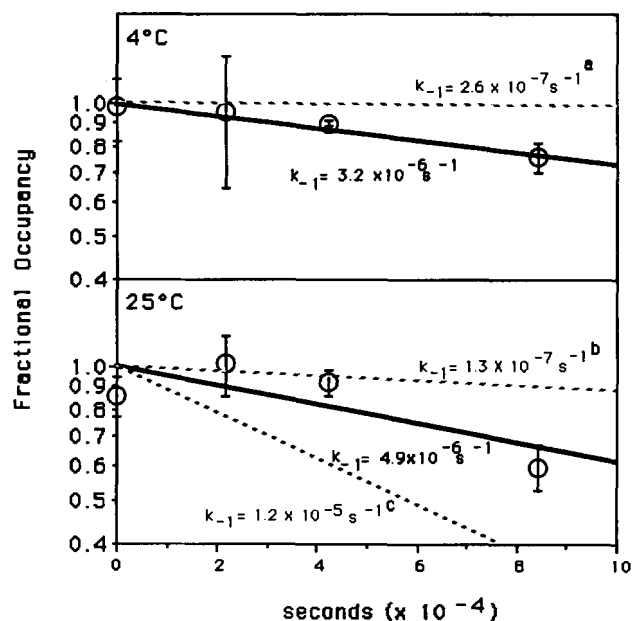


FIG. 6. Dissociation kinetics for the interaction of IgE with truncated human α at 4 $^\circ$ C and 25 $^\circ$ C. Transfected CHO cells (2.5 or 2.5-33 transfectants) were biosynthetically labeled with L- ^{35} S]cysteine and the culture medium was incubated with ^{125} I-mouse IgE. The IgE-truncated α complexes were bound to beads, washed, and assayed for dissociation of truncated α from bound IgE. After each time point the beads were washed, the bound material eluted, and separated by gel electrophoresis. The gel was dried, exposed for autoradiography, cut into 2-mm slices, and counted for ^{125} I and ^{35} S counts. The ^{35} S counts corresponding to the peak of truncated human α were normalized to the ^{125} I-IgE counts. Data points represent the mean of triplicate samples. Error bars represent the standard deviation of individual data points. The lines (thick lines) drawn through the data point represent first-order regression analysis for the entire data set. The regression lines (dotted lines) correspond to previously published data (references: a = 24; b = 31, c = 26, 32). Data for human basophils (30) are also available: when corrected for the temperature (25 $^\circ$ C), the $k_{-1} = 2.4 \pm 0.36 \times 10^{-5}$ s $^{-1}$.

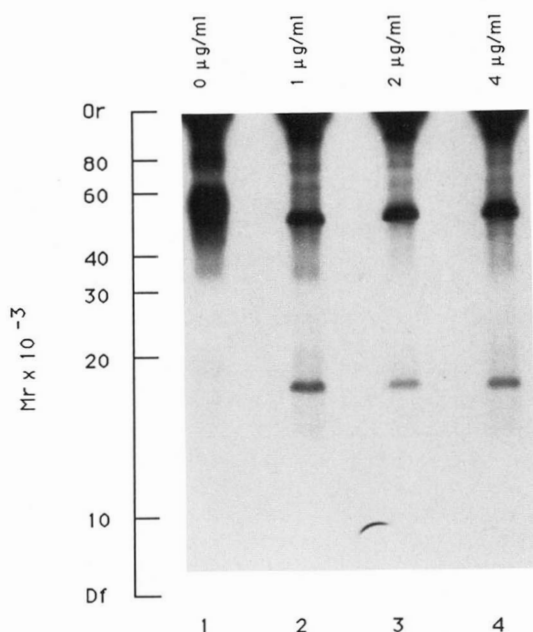


FIG. 7. Secretion of deglycosylated truncated α by tunicamycin-treated 2.5 CHO transfectants. Autoradiography of a polyacrylamide gel on which samples of purified truncated α were analyzed (as in Fig. 1). The cells were preincubated with 0, 1, 2, and 4 μ g/ml tunicamycin for 16 h before biosynthetic labeling with L-[^{35} S]-cysteine. The truncated human α was purified from cell supernatants.

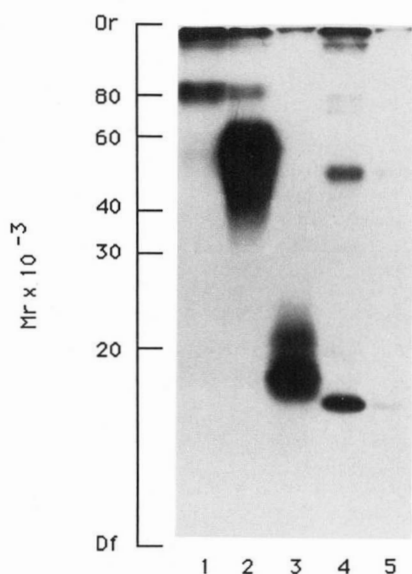


FIG. 8. Effect of *N*-glycanase digestion on secreted human truncated α . Nontransfected CHO cells (lane 1) and 2.5-33 CHO transfectants (lanes 2-5) were biosynthetically labeled in the absence (lanes 1-3) or presence of 2 μ g/ml tunicamycin (lanes 4 and 5). When purified, IgE-binding polypeptides were further treated with (lanes 3 and 5) or without (lanes 1, 2, and 4) 0.3 units of *N*-glycanase before gel electrophoresis in the presence of reducing agents. The figure shows an autoradiogram of the gel. Note: the two bands from lane 5 are more faint than the corresponding bands from lane 4. Although the same number of counts were used for these experiments, the additional processing of the sample analyzed on lane 5 resulted in the loss of counts. However, these bands were clearly seen on the original autoradiogram.

likely corresponds to the unglycosylated form of truncated α . The 45-kDa IgE-binding peptide may result from the incomplete action of tunicamycin or the presence of *O*-linked sugar.

It could also be the result of dimerization or aggregation of the unglycosylated molecules.

To address this question, labeled product purified from the supernatant of untreated and tunicamycin-treated 2.5 CHO transfectants was digested with *N*-glycanase and analyzed on SDS gel in the presence of reducing agents. This procedure converted the 50-kDa band of soluble α chains from untreated cells to a single band around 18 kDa (Fig. 8, lane 3). The latter is slightly higher than the lower band obtained from tunicamycin-treated cells (lane 4). This may be due to a difference in migration induced by the presence of Triton X-100. This detergent was added to the sample during *N*-glycanase digestion and could have been incompletely removed by the subsequent ethanol precipitation. More importantly, the 45-kDa band obtained from tunicamycin-treated cells was insensitive to reducing agents and to *N*-glycanase digestion (Fig. 8, lane 5). Therefore, incomplete action of tunicamycin and dimerization through disulfide linkage were unlikely factors in the formation of this 45-kDa band. To rule out the presence of *O*-linked sugar in the 45-kDa band, 2.5-33 cells were treated with tunicamycin after biosynthetic incorporation of D-[^{3} H]glucosamine and [^{35} S]cysteine (Fig. 9). In untreated cells, the ^{3} H and ^{35} S patterns were indistinguishable and the ^{3} H/ ^{35} S molar ratio of incorporation into the 50-kDa secreted α chains was 13.6. In tunicamycin-treated cells several L-[^{35} S]cysteine-labeled peaks of approximately 19.5, 45, 75, and 145 kDa were observed. The ^{3} H/ ^{35} S molar ratios of incorporation for the 45-kDa and 19.5-kDa bands were 1.18

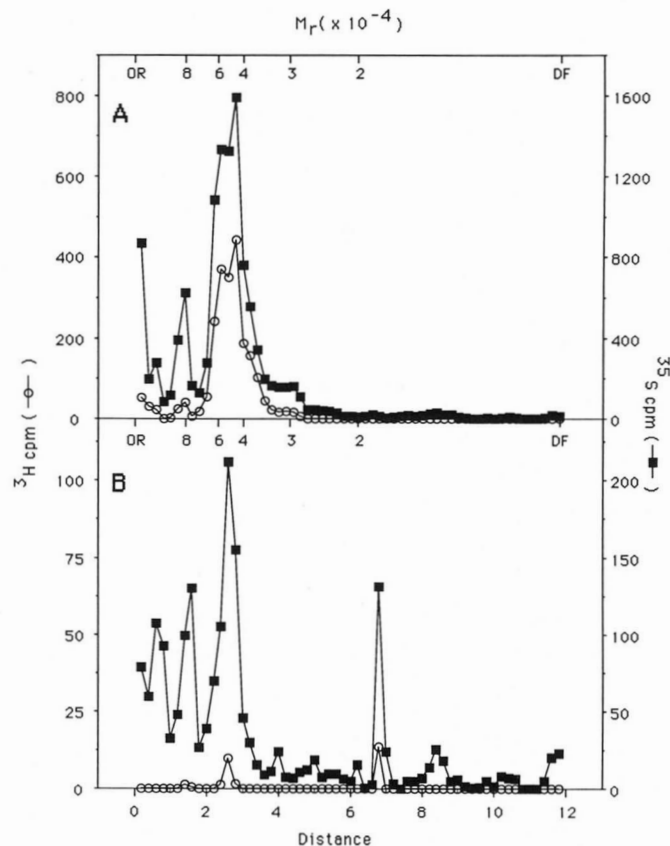


FIG. 9. Effect of tunicamycin on the relative incorporation of L-[^{35} S]cysteine and D-[^{3} H]glucosamine into human truncated α secreted from CHO transfectants. The CHO cells were labeled biosynthetically with L-[^{35} S]cysteine and D-[^{3} H]glucosamine in the absence (A) or presence (B) of 2 μ g/ml tunicamycin. IgE-binding polypeptides were then purified from cell supernatants and analyzed as in Fig. 8. The gel was cut into 2-mm slices and the slices counted for ^{3} H and ^{35} S. The counts were corrected for overlap.

and 1.87, respectively. These results demonstrate that the tunicamycin-induced inhibition of [^{3} H]glucosamine incorporation was very effective (91.3 and 86.3%, respectively). Therefore the 45-kDa species could not have been derived from incomplete action of tunicamycin or from residual *O*-glycosylation. Taken together these data strongly suggest that unglycosylated soluble α tends to form dimers and probably higher oligomers (see Fig. 9B) which cannot be disrupted by treatment with sodium dodecyl sulfate. In addition, resistance to reducing agents indicates that formation of these complexes is unlikely via disulfide linkages.

In one experiment similar to those presented in Fig. 6, the dissociation of IgE from the unglycosylated 18-kDa molecule was found to be insignificant after 60 h at 4 °C (data not shown). These data confirm that posttranslational addition of carbohydrates is not critical for the IgE-binding function of this polypeptide.

DISCUSSION

The major goal of this study was to produce a soluble form of Fc ϵ RI with binding characteristics of receptors on intact cells in order to facilitate future analysis of the receptor binding site. Our strategy was to engineer Fc ϵ RI α cDNA constructs (α_{trunc}) encoding exclusively the leader peptide and extracellular domain of the α chain. A similar strategy was used successfully (33) to produce a soluble form of mouse Fc γ RII, a receptor homologous to the Fc ϵ RI α chain. However, because Fc γ RII is thought to be a single-chain receptor, it was not known *a priori* whether similar results would be obtained with the α chain of Fc ϵ RI. In fact, only the transfection with the human α_{trunc} construct resulted in secretion of a soluble form of IgE-binding α chain. The truncated α chains derived from mouse and rat constructs, although capable of binding IgE, were surprisingly sequestered in the endoplasmic reticulum and degraded over time. This is interesting in light of our previous results showing that human, in contrast to rodent, receptors do not require the β subunit for surface expression (12, 32). The differential processing by the cell of the rodent and human truncated α suggests that the extracellular portion of the α subunit might play an important role in controlling the fate of this subunit. It is possible, for example, that a specific sequence in rodent α , but not in human α , acts as a retention signal for the α chain in the endoplasmic reticulum, as reported for other membrane proteins (34). The interaction between α and β would then "mask" this specific sequence thereby allowing further processing, assembly of the $\alpha\beta$ complex with γ , and surface expression of the $\alpha\beta\gamma_2$ complex. This "masking" would not necessarily require a direct interaction between α and β . The absence of the retention signal in human α would permit the nonassembled α to be exported out of the endoplasmic reticulum and would explain why human receptors can be expressed by transfection as $\alpha\gamma_{(n)}$ complexes on the cell surface. We are currently testing this hypothesis by using human/rodent chimeric receptors. Hopefully, a better understanding of this interesting difference between human and rodent receptors will help us in "rescuing" the rodent truncated α chains, for example, by mutating the putative retention signal. This would help us in achieving our original goal of producing soluble α chains from the three species, the rationale being that it might be easier, as for other proteins, to crystallize a soluble protein from one particular species.

The data on the truncated α chains demonstrate that the extracellular domain of α is sufficient to mediate high affinity binding of IgE, and that the conformation of the receptor binding site is not influenced by the other domains of α and

by the associated β and γ subunits. Our estimates of the dissociation rate constant of human IgE from the truncated α at two different temperatures are within the range of the published data for Fc ϵ RI on intact cells. Furthermore, the experiments with tunicamycin indicate that posttranslational *N*-glycosylation is not required for proper folding and formation of the receptor binding site. Previous data on Fc ϵ RI purified from tunicamycin-treated RBL cells support our conclusions (15). In these studies, however, the unglycosylated α subunit had an apparent mass of 38 kDa on polyacrylamide gels, well above the 27 kDa of the α core peptide. The residual heterogeneity after tunicamycin treatment of RBL cells, together with other studies (16), indicates that Fc ϵ RI from RBL cells also contains *O*-linked sugars which could play a significant role in the receptor binding function. Our studies are more clear in this regard. The truncated α from tunicamycin-treated CHO cells appears as a 17–19.5-kDa band on polyacrylamide gels, a value close to the exact molecular mass of 19,931 Da. In addition, the data from the double labeling study with [^{35} S]cysteine and [^{3} H]glucosamine rule out that the 18-kDa band contains any residual sugar, while still preserving its ability to bind IgE. If the heterogeneity of glycosylation appears as an obstacle for crystallization of the polypeptide, deglycosylation might be a way to overcome the problem, although the tendency of the unglycosylated truncated α to form aggregates may then present another problem. In any case, the truncated human α should be a suitable reagent to further characterize the receptor binding site by crystallographic analysis or nuclear magnetic resonance. Since the stable transfectants are able to secrete 100 μ g of truncated α per liter of culture, producing the several milligrams required to grow crystals should be a feasible task.

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