

Purification and Characterization of Human Recombinant IgE-Fc Fragments That Bind to the Human High Affinity IgE Receptor*

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Mitali Basu‡§, John Hakimi¶, Elizabeth Dharm‡, Jo Ann Kondas¶, Wen-Hui Tsien¶, Robert S. Pilson¶, Ping Lin¶, Alasdair Gilfillan¶, Paul Haring**, Emory H. Braswell‡‡, Mignon Y. Nettleton§§, and Jarema P. Kochan§§

From the Departments of ‡Protein Biochemistry, ¶Immunopharmacology, §§Molecular Genetics, and ||Bronchopulmonary Research, Roche Research Center, Hoffmann-La Roche Inc., Nutley, New Jersey 07110, **F. Hoffmann-La Roche and Co. Ltd., Basel, Switzerland, and the ‡‡Analytical Ultracentrifugation Facility, University of Connecticut, Storrs, Connecticut 06269

The Fc-region of immunoglobulin E (IgE) comprising C₂, C₃, and C₄ domains is sufficient for binding to the α chain of the high affinity IgE-Fc receptor (Fc ϵ RI α). In order to identify the smallest Fc fragment capable of binding to the Fc ϵ RI α with high affinity, various regions of the IgE-Fc molecule were expressed in COS cells and investigated for their ability to bind Fc ϵ RI α . The smallest fragment that showed Fc ϵ RI α binding activity spans amino acids 329–547 and lacks the entire C₂ domain. Two active fragments, viz. Fc_(315–547) (containing Cys³²⁸ which is responsible for interchain S–S bonding) and Fc_(329–547), have been overexpressed in CHO cells and purified to homogeneity. The purified proteins bind to the Fc ϵ RI α with high affinity, similar to native IgE. SDS-polyacrylamide gel electrophoresis analyses indicate that Fc_(315–547) is an S–S-linked dimer of apparent molecular mass of 68 kDa. Fc_(329–547) appears on SDS-gel as three distinct bands at ~32 kDa, both under reducing and nonreducing conditions. However, size exclusion chromatography and analytical ultracentrifugation studies suggest that Fc_(329–547) also remains associated as a dimer. The presence of N-linked glycosylation was detected in both proteins. The deglycosylated form of Fc_(315–547) was isolated after Endo F/N-glycosidase F digestion and demonstrated to have binding activity comparable to that of the mock-digested protein. These results suggest that the presence of N-linked sugars is not necessary for Fc ϵ RI α binding. Both proteins blocked the release of histamine from RBL cells expressing human Fc ϵ RI α in a dose-dependent manner. The availability of these recombinant IgE-Fc proteins will be helpful in elucidating the key epitopes essential for the binding of IgE to its high affinity receptor.

Immunoglobulin E (IgE) is responsible for mediating the Type 1 immediate hypersensitivity. It binds to the α chain of the high affinity receptor (Fc ϵ RI α) present on mast cells and basophils, thereby sensitizing these cells for antigen-induced

degranulation and release of histamine and other mediators of the allergic condition. One important strategy for designing therapeutic agents for allergic diseases is inhibiting the binding of IgE to Fc ϵ RI α . Identification of the structural entities on the IgE molecule that interact with Fc ϵ RI α is an essential prerequisite for this purpose. Although it is known that IgE binds to Fc ϵ RI α through its Fc region comprising the C₂–C₄ domains, the exact sites that are involved in the binding are not yet known. Several approaches have been undertaken to define the binding region of IgE. Circular dichroism measurements of reduced and heat-inactivated IgE suggest that the C₃ and C₄ domains are involved in the binding of Fc ϵ RI α (1, 2). Studies by Perez-Monfort and Metzger (3) reported that a region between the C₂ and C₃ domains of rodent IgE was protected from proteolysis when bound to Fc ϵ RI α . Recently, efforts have been made to identify the binding site on the IgE molecule utilizing recombinant DNA technology. One of the earliest studies reported that *Escherichia coli*-derived IgE-Fc fragments containing the C₂–C₄ domains were able to bind to basophils (4, 5) and were active in the passive sensitization of cultured basophils (6). Further studies reported that a monomeric 76-amino acid fragment expressed in *E. coli* was also capable of inhibiting the *in vivo* passive sensitization of mast cells (7, 8). This fragment spans amino acids 301–376 (encompassing C₂–C₃), implicating this inter-domain region as the site of Fc ϵ RI α interaction. There are also several reports in which the receptor-binding site of IgE has been probed by studying the behavior of several IgE mutants (9) and chimeras (10–12) expressed in eukaryotic cells. All of these studies assigned the C₃ domain of IgE as the principal region involved in the interaction with Fc ϵ RI α .

We have attempted to study the interactions of IgE-Fc with Fc ϵ RI α in greater detail. Our goal is to identify the smallest IgE-Fc fragment that retains binding activity and to resolve its three-dimensional crystal structure. The expression of the IgE-Fc fragments in *E. coli* has been found to be unsuitable for large-scale purification, because the proteins accumulate in inclusion bodies and can be solubilized only under denaturing conditions. The refolding of the denatured protein is inefficient (13, 14).¹ Therefore, a eukaryotic expression strategy was devised, whereby different IgE-Fc fragments were fused to the signal peptide of the p55 subunit of the IL-2 receptor in order to facilitate secretion. The ability of the recombinant Fc fragments to bind to Fc ϵ RI α was assayed in

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§ To whom correspondence and reprint requests should be addressed: Dept. of Protein Biochemistry, Hoffmann-La Roche Inc., 340 Kingsland St., Nutley, NJ 07110. Tel.: 201-235-5789; Fax: 201-235-3805.

¹ M. Basu and J. Kochan, unpublished observations.

a receptor-based sandwich-type ELISA.² Two IgE-Fc fragments capable of binding to Fc ϵ RI α were identified in this manner. The fragments have been overexpressed in CHO cells and purified to homogeneity, and their interactions with Fc ϵ RI α have been characterized.

MATERIALS AND METHODS

Human myeloma IgE (IgE-PS) and rat monoclonal IgE were purified as previously described (15). Monoclonal antibodies, 22E7 and 29C6, were raised against the human Fc ϵ RI α subunit (16) and kindly provided to us by Dr. R. Chizzonite, Hoffmann-La Roche. E48, a mouse anti-human IgE monoclonal antibody (IgG1/K), was purified from the ascites fluid by following the procedure of Reik *et al.* (17). The anti-NIP (4-hydroxy-3-iodo-5-nitrophenylacetic acid) IgE (Tan-IgE), a chimeric human antibody (9), was purified on an E48 affinity column from culture supernatants of transfected cells. These cells and NIP-BSA were kindly provided by Dr. Z. Eshhar of The Weizmann Institute, Israel. N-Glycanase and neuraminidase were obtained from Genzyme. Endoglycosidase F/N-glycosidase F, Endoglycosidase H, Endoglycosidase F (N-glycosidase F-free), and endo-N⁴-acetylgalactosaminidase were purchased from Boehringer Mannheim. Iscove's modified Dulbecco's medium, amethopterin, and glutamine were obtained from Sigma. Penicillin-streptomycin mixture and fetal bovine serum (FBS) were from Gibco and Hyclone, respectively.

DNA Constructions and Transfections—The cDNA for the human IgE-Fc (C₂–C₄) was isolated by the polymerase chain reaction from total peripheral blood lymphocytes cDNA (18). The different IgE-Fc fragments (Fig. 1) were isolated by polymerase chain reaction and cloned in-frame into the expression vector pBC12-pSP36 (19). This expression vector allows the cloning of cDNA fragments adjacent to the IL-2R signal peptide, so that the polypeptides can be secreted. The resulting cDNA expression constructs were verified by sequence analysis. The fusion resulted in the presence of the sequence Glu-Leu at the amino terminus of the constructs. The Fc₁(315–547) construct which was introduced into CHO cells contained the sequence Glu-Leu-Gln-Ser at the amino terminus. The two extra amino acids were derived from the oligonucleotides used for generating the polymerase chain reaction amplicons and were not cleaved by the restriction enzyme ScaI during cloning. The desired Fc₁(315–547) containing only Glu-Leu at the amino terminus was expressed in COS cells and was found to be similar to the CHO-derived Fc₁(315–547) in its behavior. The sequences of the IgE-Fc region were identical with the sequences previously reported (2), except for threonine 387, which was found to be arginine, and glutamine 482, which was glutamic acid.

Cell Culture—The conditions for COS cell growth and transient DNA transfection were as described (20) except that the Dulbecco's modified Eagle's medium (Gibco) was supplemented with 10% Nutridoma-SP (Boehringer Mannheim), 50 μ g/ml chloroquine (Sigma), 500 μ g/ml DEAE-dextran (Pharmacia LKB Biotechnology Inc.), and 2 μ g/ml purified plasmid DNA. The conditions for CHO cell growth and permanent transfection have been described previously (21). Permanent cell lines expressing Fc₁(315–547)[pMJ2004] and Fc₁(329–547)[pMJ2005] were amplified by stepwise increasing concentrations of 0.1 and 1.0 μ M amethopterin. Stable clones expressing high levels of recombinant IgE-Fc fragments were isolated following limited dilution cloning. The levels of IgE-Fc fragment expression were quantitated by using a solid-phase IgE-binding assay (see below). For large-scale purification, CHO cells expressing Fc₁(315–547) [clone 2004A] and Fc₁(329–547) [clone 2005–2C7] were cultured in 2-liter roller bottles using Iscove's modified Dulbecco's medium supplemented with 10% FBS, 2 mM glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin. The supernatants were harvested from the confluent culture and the cells were replenished with fresh medium. Harvesting of the conditioned medium was continued at 3–5-day intervals for about 2 weeks.

Metabolic Labeling of Transfected COS Cells with [³⁵S]Cysteine—

² The abbreviations used are: ELISA, enzyme-linked immunosorbent assay; RBL, rat basophilic leukemia; PAGE, polyacrylamide gel electrophoresis; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonic acid; BSA, bovine serum albumin; FBS, fetal bovine serum; NIP, 4-hydroxy-3-iodo-5-nitrophenylacetic acid; SPEBA, solid-phase IgE-binding assay.

COS cells were transfected with the indicated constructs and, 48 or 72 h post-transfection, were starved of cysteine by incubation in 1 ml of cysteine-free Dulbecco's modified Eagle's media (Gibco) at 37 °C in a humidified atmosphere of 95% air, 5% CO₂. After 30 min of cysteine starvation, 200 μ Ci of [³⁵S]cysteine (specific activity > 600 Ci/mmol; Amersham) was added to each well and incubated for an additional 2–3 h. Labeled cells were extracted with 1 ml of 10 mM CHAPS in phosphate-buffered saline containing 30 μ g/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin. Cell extracts were clarified by centrifugation and then precleared with GammaBind G agarose beads (Genex, Gaithersburg, MD, or Sigma). IgE-Fc fragments were immunoprecipitated by the addition of 1.5 μ l of a rabbit polyclonal anti-human IgE antibody (7.8 mg of IgG/ml; Dako, Carpinteria, CA) and incubated for 30 min at room temperature. Immune complexes were precipitated by the addition of 35 μ l of a 50% suspension of GammaBind G agarose and incubated for 60 min at room temperature. Bound proteins were eluted from washed beads by boiling in Laemmli buffer (22). Proteins were electrophoresed through 12.5% SDS-polyacrylamide gels and subjected to fluorography.

Solid-phase IgE-binding Assays (SPEBA)—Samples were assayed for receptor binding activity by a sandwich-type ELISA in which Fc ϵ RI α protein was captured on 96-well assay plates with a noninhibitory anti-receptor monoclonal antibody. 96-well Nunc Maxisorb plates were coated overnight at 4 °C with 1 μ g/ml monoclonal antibody 22E7 in PBS. The wells were blocked with 1% BSA in PBS for 1 h at room temperature, followed by three washes with PBS-containing 0.05% Tween 20. Each well was incubated with a 1:10 dilution of a stock of CHO cell-conditioned media (No. 1429, a cell line³ secreting a truncated form of Fc ϵ RI α) for 2 h at room temperature. All dilutions were made in a diluting buffer of PBS/0.05% Tween 20/0.1% BSA. The wells were washed, and serial dilutions of 100- μ l samples (standard IgE-PS, cell culture supernatants, or lysates containing IgE fragments) were added to wells and incubated overnight at 4 °C. The wells were then washed and incubated with rabbit anti-human IgE (0.5 μ g/ml; Dako) for 2 h at room temperature. After washing the wells, peroxidase-conjugated donkey anti-rabbit IgG (1:1000 dilution; Jackson ImmunoResearch, West Grove, PA) was added to the wells and incubated for another 2 h at room temperature. The plates were developed with 1 mM 2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid) (Sigma) in 0.05 M sodium citrate buffer, pH 4.2, containing 0.03% hydrogen peroxide. The plates were read at 405 nm.

ELISA—The purification of Fc₁(315–547) and Fc₁(329–547) proteins was followed by an ELISA which utilized monoclonal antibody E48 (0.1 μ g/well) to capture IgE-Fc fragments in 96-well plates and a peroxidase-conjugated anti-human IgE antibody (1:1000 dilution; Kirkegaard and Perry, Gaithersburg, MD) for detecting the bound IgE-Fc fragments. IgE-PS was used as a standard.

Purification of Fc₁(315–547) and Fc₁(329–547)—The monoclonal antibody E48 was coupled to NuGel P-AF poly-NHS gel (Separation Industries, Metuchen, NJ) via the primary amine groups according to the manufacturer's instructions. The coupling density was 1–1.5 mg/ml. The recombinant proteins were purified from the culture supernatants of CHO cells by immunoaffinity chromatography on the E-48-coupled NuGel column (bed volume 150 ml). After sample loading, the column was washed extensively with PBS/0.5 M NaCl and PBS, respectively, and the bound protein was eluted with 0.2 M acetic acid/0.2 M NaCl. The eluted protein was immediately neutralized by adding 1/2 volume of 0.5 M Na₂HPO₄. It was then concentrated by ultrafiltration (Omegacells™ with a 10K membrane; Filtron Technology, Clinton, MA) to a final concentration of 2 mg/ml and equilibrated with 0.1 M Tris-HCl, pH 7.2, containing 0.25 M NaCl. The protein was then passed through a Blue-Sepharose column (Pharmacia LKB Biotechnology Inc.) equilibrated with the same buffer. The proteins were further purified by gel filtration on a Sephacryl S-300 column (Pharmacia) (4 × 95 cm) equilibrated with PBS/0.5 M NaCl.

Binding Assays—IgE-PS and Fc₁(315–547) were radiolabeled with ¹²⁵I-sodium (Amersham) by the ENZYMOBEADS method (Bio-Rad), and Fc₁(329–547) was iodinated by the chloramine T method (23). The specific radioactivity was approximately 1–2 × 10⁶ cpm/ μ g of protein. Bindability of the radiolabeled protein was determined as described previously (16). It ranged between 30 and 40% for IgE-PS and 60 and 75% for Fc₁(315–547) and Fc₁(329–547). CHO-3D10 cells expressing 4–6 × 10⁵ chimeric Fc ϵ RI α (α subunit) were used for all binding assays (15). For saturation binding assay, cells were resus-

³ N. Tobkes and J. Kochan, unpublished observations.

pended (1.6×10^6 cells/ml) in RPMI-1640 supplemented with 10% FBS, 5 mM HEPES, pH 7.4, and incubated at 25 °C for 2 h with varying concentrations of ^{125} I-labeled ligand. Nonspecific binding was determined in the presence of 100-fold molar excess of IgE-PS. Bound radioactivity was separated from the free ligand by centrifugation through a mixture of *n*-butyl phthalate and olive oil (80:20). Nonspecific binding was 5–10% of the total binding. Concentrations of free ^{125} I-labeled ligands were corrected for bindability, and K_d was determined from Scatchard analysis (24) of the binding data. For competition assays, cells (2×10^6 cells/ml) were preincubated with IgE-Fc samples of various dilutions prior to the addition of ^{125} I-IgE as described previously (15).

Sedimentation Equilibrium Studies—The sedimentation experiments were performed, and the data were accessed and analyzed essentially as described by Arakawa *et al.* (25) except that cells with longer solution column heights (26) were used (2.6 mm versus 0.7 mm) in this study. The centrifuge was run at 20,000 rpm at 4 °C until sedimentation equilibrium had been attained. After the data were taken, the centrifuge was accelerated to 28,000 rpm and run until equilibrium was again achieved. Data analysis was performed using a nonlinear least squares program (27). The analysis proceeds by assuming first that only a single ideal species is present. Next, a nonideal single species model is assumed followed by a number of self-association models. The program will yield the molecular weight of the material for the ideal and nonideal fits and the virial coefficient for the latter model. For the self-association models, the molecular weight of the smallest associating molecule, the association stoichiometry, and the appropriate equilibrium constants are produced. A fit is considered good when the root mean square error of the fit is in the same range as that for a blank run and little or no systematic error is evident.

The partial specific volume (v) for the protein portion of Fc₁(329–547) was calculated as 0.728 ml/g from the amino acid composition data (28). Since there is an estimated 3 kDa of sugar for 24.8 kDa of protein, an error in the estimated v of the sugar would not be serious. If the v of the sugar portion was estimated to be about 0.625 (considering the value for glucose (29)), then the effective v for the complete molecule can be calculated to be about 0.717. The densities of the solvents were estimated from density tables to be 1.007 and 1.02 g/ml at 20 °C, which when corrected to 4 °C become 1.009 and 1.023 mg/ml, respectively.

Digestion with Endoglycosidases—For *N*-glycanase digestion, 60 µg of the purified protein was first denatured by boiling for 5 min in the presence of 0.5% SDS and 50 mM 2-mercaptoethanol as recommended by the supplier. The denatured protein (25 µg) was then made 2.5% with Triton X-100 and incubated with 15 units/ml *N*-glycanase in a final volume of 60 µl. The purified proteins were treated with Endo F and Endo H in 0.1 M sodium acetate buffer, pH 6.0, containing 10 mM Na₂EDTA and 100 mM 2-mercaptoethanol. The reduced protein was incubated overnight at 37 °C with either 1.5 units/ml Endo F or 50 milliunits/ml Endo H. In the sequential deglycosylation experiment, 45 µg of each protein was first digested with neuraminidase (1 unit/ml) for 2 h at 37 °C in 0.1 M sodium acetate buffer, pH 6.0. The reaction mixture was then divided into three equal aliquots (A, B, and C). Aliquots B and C were made 10 mM with Na₂EDTA and 0.2% with Triton X-100. Endo F (1.5 units/ml) was added to tube B, and tube C received a combination of Endo F (1.5 units/ml) and endo-*N*-acetylgalactosaminidase (20 milliunits/ml). All three samples were incubated overnight at 37 °C. Aliquot A was used as a “no enzyme” control.

Histamine Release Assay—Freshly trypsin-treated RBL-48 cells (RBL-2H3 cells transfected with the α subunit of the human IgE-receptor; Ref. 39) were resuspended in minimum essential medium with Earle's salts supplemented with 15% FBS and plated in 12-well tissue culture dishes (0.4×10^6 cells/well). To evaluate the ability of Fc proteins to inhibit sensitization, cells were incubated at 37 °C with appropriate dilutions of inhibitors [IgE-PS, Fc₁(315–547), and Fc₁(329–547)] for 1 h at 37 °C. Tan-IgE was then added to each well to a final concentration of 0.75 µg/ml, and the cells were incubated for 1 h at 37 °C. The adherent cells were washed with 25 mM Tris-HCl, pH 7.6, containing 120 mM NaCl, 5 mM KCl, 0.6 mM CaCl₂, 1 mM MgSO₄, and 0.05% FBS (Tris-ACM) and triggered by incubation with NIP-BSA (5 µg/ml) for 30 min at 37 °C. The supernatants were then harvested, and the cells were lysed with distilled water and clarified by centrifugation. The histamine content in the supernatants (S) and the cell lysates (C) was assayed using the Leukocyte Histamine Release Test (LHRT) method (30). Percent histamine released was calculated as $[S/(S + C)] \times 100$.

For direct sensitization studies, the format of the assay was modified slightly. In this case, RBL-48 cells were incubated for 1 h with various concentrations of IgE-PS, Fc₁(315–547), and Fc₁(329–547), and then the cells were then triggered with a 1:500 dilution of a rabbit anti-human IgE antibody (Dako).

Gel Electrophoresis and Immunoblot—SDS-PAGE and electroblotting procedures have been described before (31). The blot was probed with a monoclonal anti-huIgE antibody E48 (1 µg/ml) and developed with alkaline phosphatase-conjugated anti-mouse antibody (1:1000; Sigma) following the procedure of Blake *et al.* (32).

Protein Sequence Analysis and Amino Acid Composition—Amino-terminal sequence analysis was performed by automated Edman degradation using an Applied Biosystems Model 470A gas-phase Sequencer (33). Phenylthiohydantoin-amino acid derivatives were identified on-line with an ABI Model 120A PTH analyzer.

Samples for amino acid analysis were hydrolyzed with 6 N HCl containing 4% thioglycolic acid at 110 °C for 20–24 h *in vacuo*, and analyses were performed as described by Pan *et al.* (34) using post-column reaction with fluorescamine.

RESULTS

In order to identify the smallest IgE-Fc fragment that will retain its ability to bind to Fc ϵ RI α , a series of IgE-Fc fragments were expressed in eukaryotic cells, and tested to determine whether they were capable of binding to Fc ϵ RI α . The expression and secretion of such fragments were facilitated by cloning the appropriate Fc₁ sequence into the expression vector pBC12-SP36 (19). This vector allows for the in-frame fusion of polypeptide fragments to the IL-2 receptor (p55) signal peptide, such that the recombinant polypeptides are expressed and secreted. Fig. 1 describes all of the constructs that were prepared in this study. COS cells were transfected with these constructs and examined for the presence of immunoreactive proteins. The initial screening was done by an immunofluorescence technique utilizing a polyclonal antiserum specific for human IgE. The presence of all of the recombinant IgE-Fc polypeptides was detected in this manner (data not shown). The recombinant proteins were further characterized by an immunoprecipitation analysis. COS cells were transfected with the different IgE-Fc constructs, radiolabeled with [³⁵S]cysteine, and then the radiolabeled polypeptides were immunoprecipitated with an anti-IgE antiserum. The immunoprecipitates were analyzed by SDS-PAGE under reducing conditions. Results from such an analysis are presented in Fig. 2, where both cell extracts (Fig. 2A) and cell supernatants (Fig. 2B) were immunoprecipitated. All of the recombinant IgE-Fc fragments were readily detected in the cell extracts and most were secreted. Judging by the signal observed in the immunoprecipitations, the amounts of the recombinant fragments which were synthesized did not differ substantially. For most of the proteins, at least three distinct bands (and sometimes four) were detected by SDS-PAGE. These bands may represent recombinant IgE-Fc polypeptides containing different levels of glycosylation. The only recombinant IgE-Fc polypeptides that could not be detected as secreted products were Fc₁(235–547), Fc₁(315–527) (Fig. 2B), and Fc₁(329–527) (results not shown).

Binding of Recombinant IgE-Fc Fragments to Fc ϵ RI α —To determine whether the various IgE-Fc fragments were capable of binding to the Fc ϵ RI α subunit, a solid-phase IgE-binding assay (SPEBA) was developed. In this assay, soluble Fc ϵ RI α is captured on ELISA plates with a non-neutralizing anti-Fc ϵ RI α monoclonal antibody (16). IgE-PS or recombinant IgE-Fc fragments are added to wells at different dilutions, and the relative levels of IgE bound to the receptor are quantitated by developing with a peroxidase conjugated antibody. The binding of IgE to Fc ϵ RI α which is determined in this assay as absorbance at 405 nm is proportional to the IgE concentration and is inhibited by rat IgE or soluble Fc ϵ RI α .

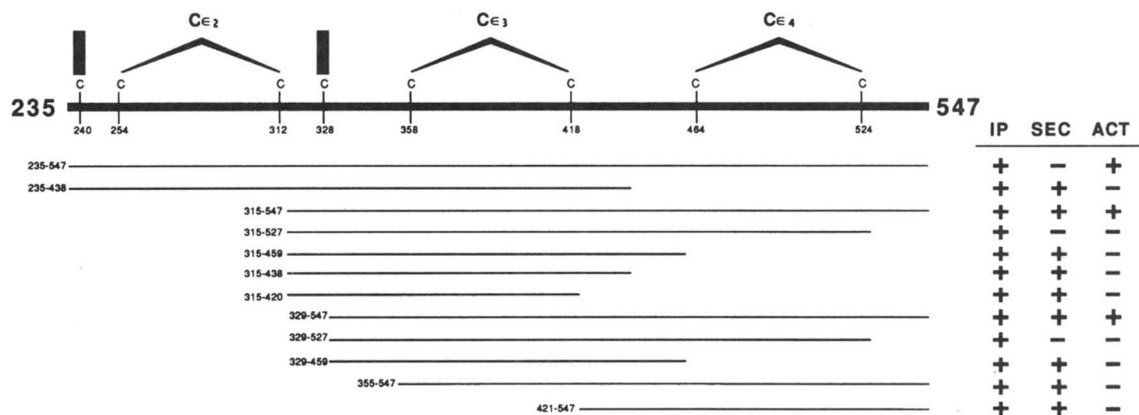


FIG. 1. Schematic representation of the IgE-Fc molecule and the different fragments constructed. The upper map indicates a schematic representation of the IgE-Fc region. The cysteine residues (Cys²⁴⁰ and Cys³²⁸) which form intermolecular disulfide bonds are not paired. The cysteine residues forming the intramolecular disulfides are paired, and their residue numbers are indicated. The regions corresponding to the different C_ε domains are indicated on top. The amino acid residues of the fragments which were analyzed in this study are presented below the IgE-Fc map.

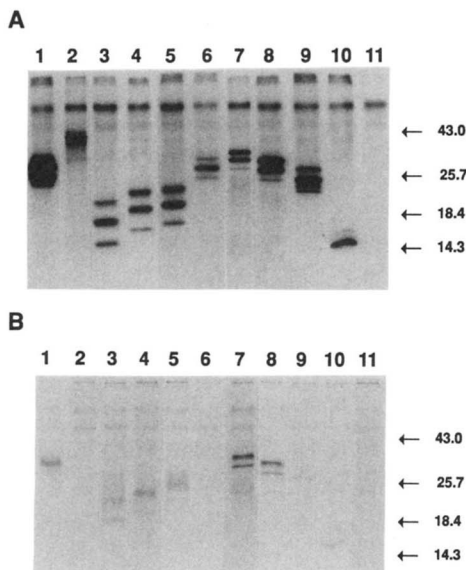


FIG. 2. Expression of recombinant IgE-Fc fragments in COS cells. COS cells were transfected with the different IgE-Fc constructs and radiolabeled with [³⁵S]cysteine 48 h post-transfection. Extracts or supernatants were prepared as described under "Materials and Methods." Recombinant IgE-Fc fragments were immunoprecipitated using a polyclonal anti-IgE antiserum, analyzed by SDS-PAGE, and autoradiographed. Panel A, precipitates from the extracts; panel B, precipitates from the supernatants. Lanes 1-11, respectively, are: Fc_ε(235-438), Fc_ε(235-547), Fc_ε(315-420), Fc_ε(315-438), Fc_ε(315-459), Fc_ε(315-527), Fc_ε(315-547), Fc_ε(329-547), Fc_ε(355-547), Fc_ε(421-547), and mock.

(data not shown). No reactivity is detected in the absence of human IgE.

Utilizing this assay, we analyzed the different recombinant IgE-Fc fragments for their ability to bind to the soluble receptor. Extracts or supernatants were prepared from transfected COS cells and assayed in the SPEBA. Analysis of the extracts indicated that only three of the recombinant IgE-Fc fragments were capable of binding to the FcεRIα (Fig. 3A). These are Fc_ε(235-547), Fc_ε(315-547), and Fc_ε(329-547). The first fragment contains the entire C_ε2-C_ε4 domains, while Fc_ε(315-547) contains the last 15 amino acids of C_ε2 and the entire C_ε3-C_ε4 domains. Fc_ε(329-547) contains only the C_ε3 and C_ε4 domains. The relative amounts of the recombinant IgE-Fc fragments in the extracts did not differ substantially

when examined by Western blot analysis performed with the same polyclonal antibody (results not shown). When the supernatants were examined for the presence of FcεRIα binding activity, only Fc_ε(315-547) and Fc_ε(329-547) were positive. The inability to detect any binding activity with the Fc_ε(235-547) supernatant is due to the fact that this fragment is not secreted from the cells (Fig. 3B).

Purification of Recombinant IgE-Fc Fragments—The two active fragments, Fc_ε(315-547) and Fc_ε(329-547), were over-expressed in Chinese hamster ovary cells to study their physical, biochemical, and biological properties. Stable cell lines secreting recombinant fragments (10–30 μg/ml) into the media were isolated. Fc_ε(315-547) and Fc_ε(329-547) were purified to homogeneity from the culture supernatants by a three-step sequential chromatography procedure. Initial enrichment was accomplished by immunoaffinity chromatography, and the proteins were relatively pure (>90%) after this step. Trace contaminants of albumin or other serum proteins from the conditioned media present in the protein samples (1–2% of the total protein) were removed by passing them through a Blue-Sepharose column equilibrated with 0.25 M NaCl. The contaminating proteins bound to the column under these conditions, and both Fc_ε(315-547) and Fc_ε(329-547) remained in the flow-through. Purification to homogeneity was achieved by gel filtration on a Sephacryl S-300 column to remove aggregates. The final yield was 50%, and a total of 150 mg of each fragment was purified. The purity of the final product was tested both by amino-terminal sequencing and amino acid analysis. A single amino-terminal sequence was detected for each protein, and the amino acid composition conformed with that deduced from the DNA sequence of the recombinant protein (data not shown).

Characterization of Recombinant IgE-Fc Fragments—The purified proteins were examined both by SDS-PAGE and size exclusion chromatography to check for homogeneity and molecular forms. On SDS-PAGE under nonreducing conditions, Fc_ε(315-547) and Fc_ε(329-547) migrated as doublets of apparent molecular mass of approximately 68 kDa and 32 kDa, respectively (Fig. 4A, lanes 1 and 2). On reduced gels, the Fc_ε(315-547) doublet was shifted to the 35-kDa region, indicating that the native protein exists as a disulfide-linked dimer (Fig. 4A, lane 4). There was no major change in the mobility of the Fc_ε(329-547) band, but its apparent molecular mass was slightly higher (Fig. 4A, lane 5). This difference in mobility probably reflects a more compact conformation of

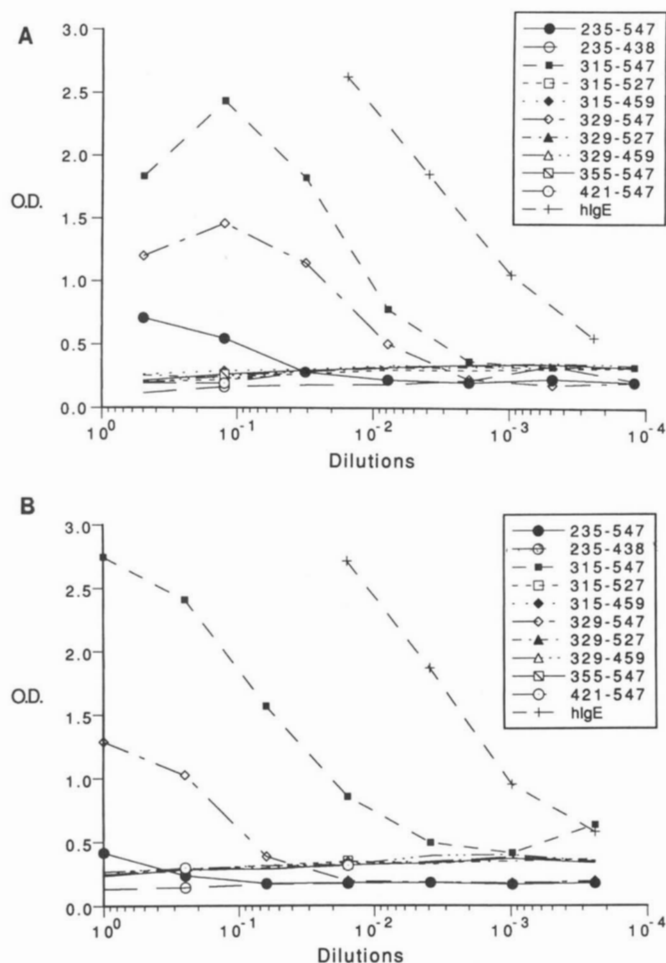


FIG. 3. $Fc\epsilon RI\alpha$ binding activity of recombinant IgE-Fc fragments expressed in COS cells. *A*, recombinant IgE-Fc fragments present in the cell extracts of transfected COS cells. *B*, recombinant IgE-Fc fragments secreted from transfected COS cells. Extracts from COS cells transfected with the various IgE-Fc constructs were prepared as described under "Materials and Methods." Serial dilutions of the cell extracts (*A*) or the supernatants from the transfected cells (*B*) were added to 96-well plates coated with soluble $Fc\epsilon RI\alpha$. The plates were developed as described under "Materials and Methods." Purified human IgE (IgE-PS) was included as an internal standard in the assay. The relative amounts of bound IgE-Fc fragments were determined as absorbance at 405 nm.

the native molecules which is stabilized by intramolecular disulfide bonds. Similar observations have been made for many other proteins; for example, the fibronectin receptor (35) and the mouse interferon γ receptor (36). Immunoblot analysis of the purified proteins using monoclonal antibody E48, which recognizes an epitope between residues 524 and 547, yielded a pattern similar to that observed in SDS-PAGE (data not shown). This observation together with the good agreement of the amino acid composition of the purified $Fc_\epsilon(315-547)$ and $Fc_\epsilon(329-547)$ proteins with the expected values (particularly that of the residues at the carboxyl-terminal end) suggest an overall integrity of the carboxyl-terminal end. The microheterogeneity seen in SDS-PAGE was subsequently found to be due to variation in *N*-linked glycosylation (described later). The apparent molecular masses of the two proteins in their native state were determined by size exclusion chromatography on a Superdex 75 HR column (Fig. 4*B*). Both proteins were eluted at an identical position that corresponds to a molecular mass of 68 kDa. These results suggest that $Fc_\epsilon(329-547)$, which lacks Cys³²⁸,

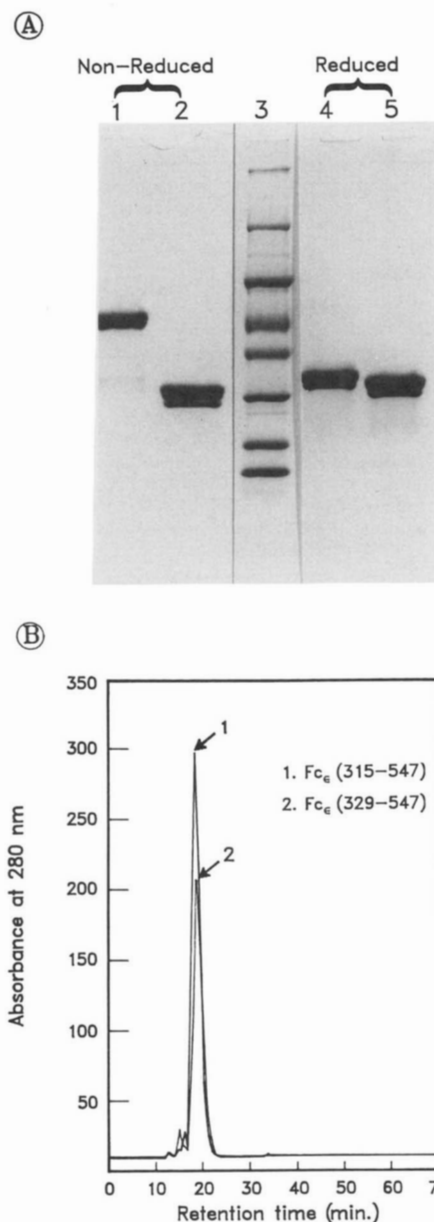


FIG. 4. *A*, SDS-PAGE analysis of recombinant IgE-Fc fragments. $Fc_\epsilon(315-547)$ and $Fc_\epsilon(329-547)$ proteins were analyzed by SDS-PAGE (4–20% gradient gel) both under nonreducing and reducing conditions. Lanes 1 and 4, $Fc_\epsilon(315-547)$; lanes 2 and 5, $Fc_\epsilon(329-547)$; and lane 3, standard protein mixture of molecular mass 200, 92, 69, 46, 30, 21.5, and 14 kDa. *B*, size-exclusion chromatography to determine the native molecular mass. Purified $Fc_\epsilon(315-547)$ and $Fc_\epsilon(329-547)$ proteins were fractionated on a Superdex 75 HR (10 mm $\times$ 30 cm) column in the presence of PBS/0.5 M NaCl. The flow rate was 0.5 ml/min. The column was calibrated with Bio-Rad's gel filtration molecular mass standards (data not shown).

exists in solution as a noncovalent dimer.

Sedimentation Equilibrium Studies—In order to determine the state of association of $Fc_\epsilon(329-547)$ in solution, the protein was studied by sedimentation equilibrium both in the presence of PBS/0.5 M NaCl (conditions used for size-exclusion chromatography) and PBS/0.15 M NaCl. Six sets of data (three loading concentrations of each sample in each of two cells run at two ultracentrifuge speeds) were analyzed using a nonlinear least squares program as described under "Materials and Methods" (27). The range of concentrations studied as a result of the centrifugal field was from a few micrograms/ml to over 2 mg/ml. The best fit found for both solvents was a strong

monomer-dimer association in which the monomer molecular mass and the natural logarithm of the association constant [$\ln k_{1,2}$ in which k is in units of liters/g] were, respectively, 28.9 ± 0.1 kDa and 5.6 ± 1.6 for the lower salt concentration. For the higher salt concentration, these values were 28.4 ± 0.2 kDa and 7 ± 3 . There was also evidence of a higher degree of association in which the dimer further dimerizes weakly to tetramers ($k_{1,4} = 8.2 \pm 0.6$ and 10.0 ± 0.2 , respectively, in which k is in units of liters³/g³). Although both sets of equilibrium constants are shifted to higher values at the higher salt concentration, the confidence limits of the monomer-dimer association are too large to strongly support the stabilizing effect of salt. From the values of the equilibrium constants, one can estimate that the dimer is the predominant species present over a protein concentration range from about 10 μ g/ml to several milligrams/ml.

Binding of Fc_ε(315–547) and Fc_ε(329–547) to FcεRIα—The functional integrity of the receptor-binding domain in the recombinant IgE-Fc proteins was examined and compared with that of IgE-PS both by direct binding assays with ¹²⁵I-labeled ligand and by competitive binding experiments. The results are tabulated in Table I. As a source of human FcεRIα, CHO-3D10 cells stably expressing a chimera of the extracellular domain of FcεRIα and the transmembrane and cytoplasmic domains of the IL-2 receptor were used (15). In direct binding experiments, Fc_ε(315–547) and Fc_ε(329–547) were indistinguishable from the whole IgE-molecule in their affinity for binding to FcεRIα. However, Fc_ε(329–547) consistently bound to fewer receptors (lower $B_{\max}$) than Fc_ε(315–547) and IgE-PS. This result was particularly evident in the experiments with CHO-3D10 cells where $B_{\max}$ values determined assuming the dimeric form of Fc_ε(329–547) as the active species were always almost half of that obtained with Fc_ε(315–547). In competitive binding inhibition assays, Fc_ε(315–547) and Fc_ε(329–547) competed effectively with IgE-PS for binding to FcεRIα. IC₅₀ values obtained for inhibition of ¹²⁵I-IgE binding to FcεRIα were comparable to that obtained with unlabeled IgE.

The binding properties of the two recombinant IgE-Fc fragments and IgE-PS were compared in greater detail by determining association and dissociation kinetics. The on-rate (k_1) and off-rate (k_{-1}) for the binding of each ligand to the chimeric FcεRIα were determined using CHO-3D10 cells as previously described (6, 15), and the binding affinities (K_a) were determined from the ratio k_1/k_{-1} (Table I). On-rates (k_1) for Fc_ε(315–547) and Fc_ε(329–547) binding were similar to that observed with IgE-PS. However, the off-rates for Fc_ε(315–547) and Fc_ε(329–547) were somewhat faster than that of IgE (2- and 5-fold, respectively).

Characterization of the Glycosylation Pattern in Recombi-

nant IgE-Fc Fragments—SDS-PAGE analysis of the purified proteins showed microheterogeneity. In addition, the apparent molecular weights determined by SDS-PAGE and gel filtration were higher than that expected from the amino acid sequence. Since the C_ε3 domain of IgE contains three potential *N*-glycosylation sites, attempts were made to determine the glycosylation pattern of the proteins by digesting them with different endoglycosidases and analyzing them by SDS-PAGE. Fig. 5A shows the modification of Fc_ε(315–547) after digestion with different endoglycosidases. On *N*-glycanase and Endo F digestion, there was a major reduction (5 kDa) in the apparent molecular mass of the protein (lanes 2, 3, and 4, respectively), suggesting the presence of *N*-linked oligosaccharides. Fc_ε(315–547) was only partly sensitive to Endo H as shown by a slight change in the proportion of the 35-kDa and 32-kDa species (lane 5). The protein was also tested for the presence of *O*-linked sugar by digesting sequentially with neuraminidase, Endo F/*N*-glycosidase F, and endo-*N*^α-acetylgalactosaminidase (*O*-glycosidase) (lanes 8–10) as described under "Materials and Methods." There was no further reduction in the size of the deglycosylated protein on *O*-glycosidase digestion. Interestingly, even though we could not detect any sensitivity of the protein toward neuraminidase treatment by this technique (lane 8), the sequential digestion with neuraminidase and Endo F gave a more complete deglycosylation than that obtained with Endo F or *N*-glycanase alone (lanes 3, 4, and 9). Fig. 5B represents the behavior of Fc_ε(329–547) after treatment with different endoglycosidases. After digestion with both *N*-glycanase and Endo F, Fc_ε(329–547), which appeared on a 12.5% gel as a triplet in the 32 ± 2 kDa region, was reduced to a single species of apparent molecular mass of 28 kDa. Similar to Fc_ε(315–547), Fc_ε(329–547) was only partly sensitive to Endo H and resistant to *O*-glycosidase digestion.

Role of *N*-Linked Glycosylation in Receptor Binding Activity—A combination of neuraminidase and Endo F treatment is capable of removing all of the *N*-linked sugars from Fc_ε(315–547) and Fc_ε(329–547) even under nondenaturing conditions (data not shown). This procedure of deglycosylation was used to determine whether the *N*-linked sugars make any contributions toward the receptor binding activity of the IgE-Fc fragments (Fig. 6). Fc_ε(315–547) was deglycosylated as described in the legend and then fractionated by size exclusion chromatography. Fig. 6A shows the peaks of mock-digested Fc_ε(315–547) (peak A) and Endo F-digested material (peaks B₁ and B₂). The peak fractions were analyzed by SDS-PAGE (inset, Fig. 6A). Peaks B₁ and B₂ looked identical on a SDS-gel. However, the appearance of peak B₁ in the void volume suggests that it probably represents aggregates of the deglycosylated protein. Aggregation of a protein after deglycosyla-

TABLE I
Binding characteristics of the recombinant IgE-Fc fragments

Protein	K_d	IC ₅₀	$k_1 \times 10^{-8}$	$k_{-1} \times 10^6$	$K_a = k_1/k_{-1}$
	nM ^a	nM ^b	M ⁻¹ s ^{-1c}	s ^{-1c}	M ⁻¹
IgE-PS	1.2 → 3.9	18.2 ± 11.0	1.1 ± 0.3	1.1 (1.1/1.1)	1.0 × 10 ¹⁰
Fc _ε (315–547)	1.4 → 4.8	7.7 ± 4.5	1.4 ± 0.3	2.1 (1.9/2.2)	0.67 × 10 ¹⁰
Fc _ε (329–547)	1.7 → 8.3	32.9 ± 25.8	1.2 ± 0.5	4.9 (3.7/6.2)	0.24 × 10 ¹⁰

^a K_d was determined from saturation binding assays as described under "Materials and Methods." In four different experiments, the K_d values varied within the range shown above. The K_d referred to here is the apparent dissociation constant as determined by the method of Scatchard (24).

^b IC₅₀ represents the concentration at which 50% inhibition of ¹²⁵I-IgE-PS (1 nM) binding was obtained with each protein (see "Materials and Methods"). The results shown are averages with standard deviations from seven different experiments.

^c The on-rates (k_1) and off-rates (k_{-1}) were determined as described previously (15). k_1 values represent the average on-rates (±S.D.) obtained from three different experiments. k_{-1} is the mean from two different experiments. The numbers in parentheses represent the actual k_{-1} values determined in the two experiments.

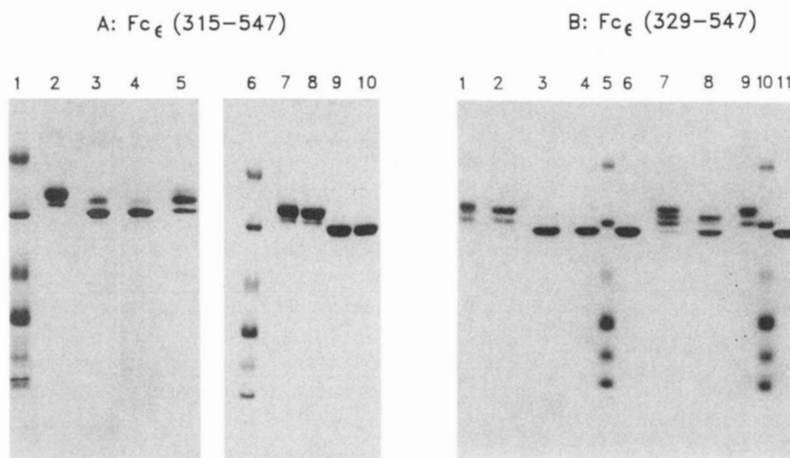


FIG. 5. **Enzymatic deglycosylation of Fc $_{\epsilon}$ (315-547) and Fc $_{\epsilon}$ (329-547).** The purified proteins were digested with the appropriate enzymes as described under "Materials and Methods." The reactions were terminated by adding 1/2 volume of 3 $\times$ the strength of the Laemmli buffer (38) with 15% 2-mercaptoethanol and boiling for 5 min. Approximately 3-5 μ g of protein was loaded per lane on a 12.5% polyacrylamide gel (15 $\times$ 20 cm). In A: 1 and 6, molecular mass standard; 2, mock digestion; 3, N-glycanase; 4, Endo F/N-glycosidase F; 5, Endo H; 7, no enzyme control; 8, neuraminidase; 9, neuraminidase plus Endo F; and 10, neuraminidase plus Endo F and O-glycosidase. In B: 1, no enzyme control; 2, neuraminidase; 3, neuraminidase plus Endo F; 4, neuraminidase plus Endo F plus O-glycosidase; 5 and 10, molecular mass standard; 6, Endo F/N-glycosidase F; 7, Endo F (N-glycosidase F-free); 8, Endo H; 9, mock digestion; and 10, N-glycanase. Molecular size standards (RainbowTM markers, Amersham): 46, 30, 21.5, 14, 6, and 3 kDa, respectively.

tion is not uncommon and has been observed with other proteins, *e.g.* erythropoietin (37). All three peak materials were tested for their ability to inhibit the binding of ¹²⁵I-IgE-PS to Fc $_{\epsilon}$ RI α (Fig. 6B). Peaks A (control) and B₂ (monomeric deglycosylated protein) showed comparable activity in the assay, but peak B₁ (the putative aggregated material) was at least 10-fold less active than the control sample. These results suggest that the presence of N-linked oligosaccharides is not important for the receptor binding activity of Fc $_{\epsilon}$ (315-547), but removal of the sugars probably exposes hydrophobic regions on the protein which leads to aggregation of the protein.

Biological Activity—The purified proteins were tested for their ability to inhibit sensitization of RBL-48 cells by IgE-Tan (Fig. 7A). Cells were preincubated with increasing concentrations of IgE-PS, Fc $_{\epsilon}$ (315-547), and Fc $_{\epsilon}$ (329-547) and then sensitized with a constant amount of Tan-IgE. The recombinant fragments inhibited the sensitization of RBL-48 cells in a dose-dependent manner, which was measured in terms of percent histamine release after triggering the cells with NIP-BSA. The fragments were also found to be capable of sensitizing RBL-48 cells directly which could then be triggered with various IgE antibodies for histamine release. Fig. 7B represents the results from one such representative experiment in which a polyclonal anti-human IgE antibody was used for triggering.

DISCUSSION

The ultimate goal of our studies is to resolve the three-dimensional structure of the IgE-Fc molecule and to understand the molecular mechanism of its interaction with the Fc $_{\epsilon}$ RI α . These studies require the identification of the smallest IgE-Fc fragment that retains binding activity and producing this fragment in large quantities for x-ray crystallography studies. To this end, we have expressed a series of truncated IgE-Fc fragments in COS cells and tested their ability to bind to the Fc $_{\epsilon}$ RI α . Utilizing this eukaryotic expression system, we avoided the uncertainty associated with the refolding of the denatured protein, a common problem for proteins expressed in bacteria. The level of expression of the different recombinant IgE-Fc fragments in COS cells was monitored by three different methods, *viz.* (i) immunofluorescence, (ii) immuno-

precipitation of the various ³⁵S-labeled polypeptides from transfected COS cells, and (iii) Western blotting. To determine if any of these fragments were able to bind the Fc $_{\epsilon}$ RI α , we developed a rapid and sensitive receptor-binding assay (SPEBA). This assay enabled us to determine which IgE-Fc fragments could bind to the Fc $_{\epsilon}$ RI α directly.

In this study, the smallest IgE-Fc fragment that was found to be capable of binding to the Fc $_{\epsilon}$ RI α was Fc $_{\epsilon}$ (329-547). This fragment spans amino acid residues 329-547, encompassing the C₃ and C₄ domains, and lacks Cys³²⁸ which forms the interchain disulfide bond in the IgE-Fc molecule. The two other fragments which showed Fc $_{\epsilon}$ RI α binding activity were Fc $_{\epsilon}$ (235-547) and Fc $_{\epsilon}$ (315-547). Fc $_{\epsilon}$ (235-547) represents the whole IgE-Fc region spanning the entire C₂-C₄ region. Fc $_{\epsilon}$ (315-547) contains only 15 amino acids from C₂ including Cys³²⁸, in addition to C₃ and C₄. None of the other fragments that were examined, including those that contained C₂ and C₃, or those that contained only C₃ or only C₄ bound to the Fc $_{\epsilon}$ RI α . The possibility remains that some of these smaller fragments are actually capable of binding to Fc $_{\epsilon}$ RI α but escape detection in the SPEBA due to their reduced reactivity to the polyclonal anti-IgE antibody. However, we could not detect any binding of these fragments to Fc $_{\epsilon}$ RI α when we tested them using two other criteria, *viz.* (i) the ability of ³⁵S-labeled IgE-Fc fragments to bind to Fc $_{\epsilon}$ RI α immobilized on silica gel and (ii) the ability to inhibit the binding of IgE in the SPEBA (data not shown).

The results from our studies differ from those obtained by Helm *et al.* (7, 8) who reported that a region on IgE-Fc spanning amino acids 301-376 constitutes the binding site for Fc $_{\epsilon}$ RI α , and thus the C₄ domain is not required for binding to Fc $_{\epsilon}$ RI α . There are several potential explanations for this discrepancy. Perhaps under the conditions used to refold the polypeptide from *E. coli*, the molecule regains the proper conformation, but when expressed in eukaryotic cells, the C₃ domain polypeptide once folded incorrectly cannot regain its proper conformation. We attempted to study the behavior of the 301-376 fragment expressed in *E. coli*. The protein was refolded under a variety of conditions, including the procedure of Helm *et al.* (7, 8). However, this material did not show any

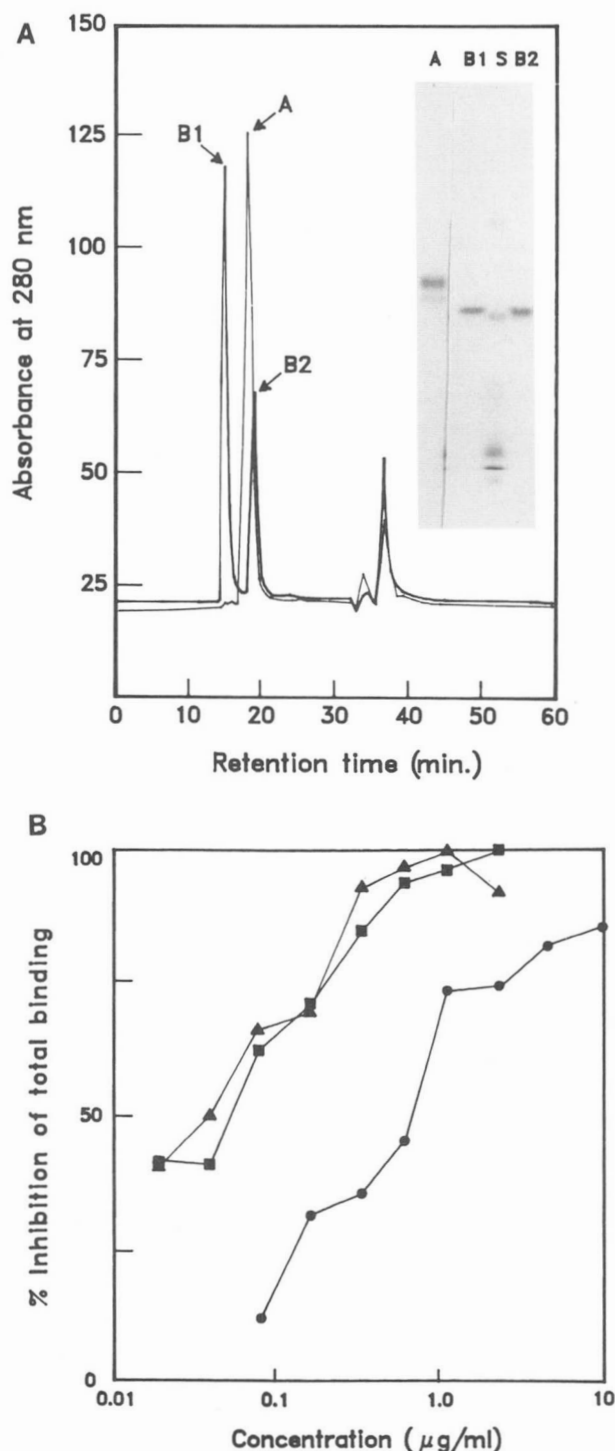


FIG. 6. Effect of removal of N-linked sugars on the binding activity of $Fc_{\epsilon}(315-547)$. Purified $Fc_{\epsilon}(315-547)$ protein (150 μ g) was incubated at 37 °C with neuraminidase (1 unit/ml) in 0.1 M sodium acetate buffer, pH 6.0. After 2 h, Triton X-100, EDTA, and Endo F/N-glycosidase F were added to the reaction mixture at a final concentration of 0.2%, 5 mM, and 2 units/ml, respectively. It was then incubated overnight at 37 °C. Control sample was mock-digested in exactly the same manner without adding any enzymes. Both reaction mixtures were fractionated on a Superdex 75 HR column equilibrated with PBS/0.5 M NaCl. Peak A ($R_t = 18.35$ min) corresponds to mock-digested protein; peaks B₁ and B₂ ($R_t = 15.22$ and 19.31 min) correspond to Endo F-treated sample. The peak fractions were analyzed by SDS-PAGE (12.5%) under reducing conditions (inset; S, molecular size markers of 46, 30, 21.5, and 14 kDa, respectively) and were also assayed for competitive inhibition of ^{125} I-IgE-PS binding to $Fc_{\epsilon}RI\alpha$. Briefly, CHO-3D10 membrane was preincubated with various dilutions of the control (peak A) and Endo F-

receptor binding activity even at very high concentration.⁴ Secondly, the assay used for characterizing the recombinant IgE-Fc fragments differs between the two studies. In our studies, we can clearly demonstrate the specific binding of the IgE-Fc fragments to the $Fc_{\epsilon}RI\alpha$, whereas in the other studies, the inhibition of a biological assay was used to monitor $Fc_{\epsilon}RI\alpha$ binding. Direct binding of the recombinant IgE-Fc fragments was never demonstrated. Similar explanations may account for the discrepancy seen between the $Fc_{\epsilon}(235-438)$ fragment of the present study and rE2-3 (residues 219-439) previously described (7). $Fc_{\epsilon}(235-438)$ did not show any receptor binding activity, but rE2-3 showed a 40% potency compared to IgE-PS in inhibiting the Prausnitz-Kustner reaction.

Our results are consistent with those obtained by Schwarzbach *et al.* (9) who observed a loss of $Fc_{\epsilon}RI\alpha$ binding activity in an IgE mutant that lacks the functional C₄ domain. There are also reports in which the contribution of the C₄ domain was examined by constructing chimeric IgE. Nissim *et al.* (11) replaced the C₃ domain of human IgE with the murine counterpart and demonstrated species-specific $Fc_{\epsilon}RI\alpha$ binding. The chimera bound mouse $Fc_{\epsilon}RI\alpha$ with high affinity. A chimera of mouse IgE and human IgG has been constructed by Weetall *et al.* (10) in which the C₄ domain was replaced with the C₃ domain. The binding and cell activating properties of mouse IgE was unaffected by this change. Since there is strong homology between mouse and human C₄ (51%) and between C₄ and C₃ (31%), these data cannot rule out the possibility that the substituted domain in the chimera is actually compensating for the role played by C₄. However, it is possible that the C₄ domain is not a direct part of the binding site, but it is essential for maintaining an active conformation of the C₃ domain which is necessary for binding to $Fc_{\epsilon}RI\alpha$.

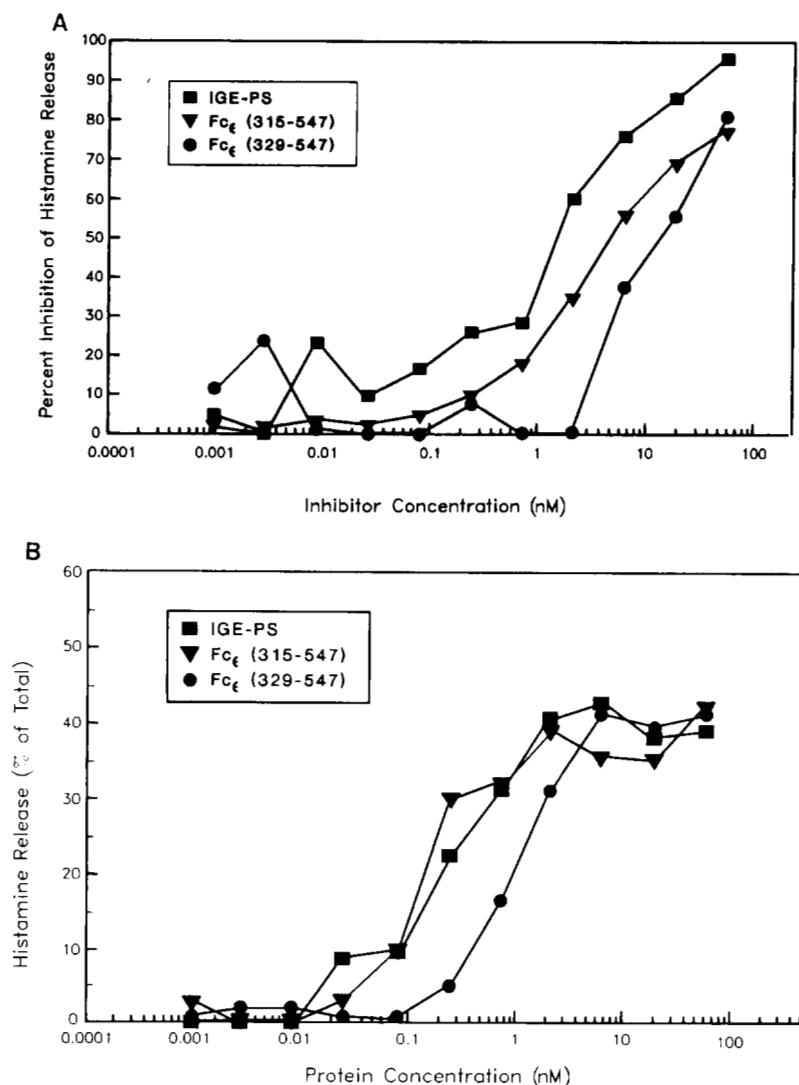
The two active fragments ($Fc_{\epsilon}(315-547)$ and $Fc_{\epsilon}(329-547)$) identified in this study have been overexpressed in CHO cells and purified to homogeneity. The $Fc_{\epsilon}(315-547)$ fragment exists as a disulfide-linked dimer, probably via residue Cys³²⁸. In contrast, Helm *et al.* (7, 8) observed interchain disulfide bond formation only with the intact Fc_{ϵ} fragment (residues 218-547). The fragment rE2-4 which spanned amino acids 301-547 was found to be predominantly a monomer even though it contained Cys³²⁸ (7). It is possible that this difference results from the incorrect folding of the fragment (rE2-4) during renaturation. Interestingly, size exclusion chromatography and sedimentation equilibrium studies indicate that $Fc_{\epsilon}(329-547)$, which lacks the entire C₂ domain and Cys³²⁸, also forms a dimer. The data from the sedimentation equilibrium studies do not exclude the possibility that the $Fc_{\epsilon}(329-547)$ polypeptide can exist as a monomer at very low concentrations (<1 μ g/ml), *i.e.* the conditions used for saturation binding assays. However, preliminary results from the characterization of preformed $Fc_{\epsilon}RI\alpha$ - $Fc_{\epsilon}(329-547)$ complexes, by amino-terminal sequencing and amino acid analysis, indicate a ratio of 1:2 for $Fc_{\epsilon}RI\alpha$ and $Fc_{\epsilon}(329-547)$ monomers.⁵ These data also suggest that the active species of the $Fc_{\epsilon}(329-547)$ polypeptide is most likely a dimer. We do not have any evidence that a monomer is capable of binding to $Fc_{\epsilon}RI\alpha$.

⁴ M. Basu, J. Hakimi, and J. Kochan, unpublished observations.

⁵ M. Basu and Y. C. Pan, unpublished observations.

treated samples (peaks B₁ and B₂) for 30 min at room temperature in Hank's balanced salt solution (HBSS) containing 0.1% BSA and 50 mM HEPES, pH 7.2. ^{125} I-IgE was added to a final concentration of 0.33 μ g/ml and incubated for 60 min at room temperature. The assay was terminated by filtration through a 0.22- μ m filter (GVWP, Millipore). Percent inhibition was calculated using total binding of ^{125} I-labeled IgE in the absence of any competitor as 100% binding, or 0% inhibition. ▲, peak A; ●, peak B₁; ■, peak B₂.

FIG. 7. A, dose-dependent inhibition of histamine release from Tan-IgE-sensitized RBL-48 cells by recombinant IgE-Fc fragments. Cells were incubated for 1 h with varying concentrations of either IgE-PS, $Fc_{\epsilon}(315-547)$, or $Fc_{\epsilon}(329-547)$ prior to their sensitization with Tan-IgE (0.75 $\mu\text{g}/\text{ml}$) as described under "Materials and Methods." Antigen (5 $\mu\text{g}/\text{ml}$ NIP-BSA)-induced histamine release from the sensitized cells in the absence of any inhibitors was 28% of the total histamine content. 50% inhibition in histamine release was observed with the three proteins at a concentration of 1.5 nM, 2.8 nM, and 7 nM, respectively. B, direct sensitization of RBL-48 cells with the recombinant IgE-Fc fragments. RBL-48 cells were incubated for 1 h with IgE-PS or $Fc_{\epsilon}(315-547)$ or $Fc_{\epsilon}(329-547)$. The cells were then triggered with a polyclonal anti-IgE antibody (1:500 dilution).



Both $Fc_{\epsilon}(315-547)$ and $Fc_{\epsilon}(329-547)$ proteins are glycosylated and contain only *N*-linked complex carbohydrates. After removal of sugars either by *N*-glycanase digestion (Fig. 5) or by chemical deglycosylation (data not shown), the two proteins showed a 4–5-kDa reduction in their apparent molecular sizes as determined by SDS-PAGE under reducing conditions. The expected molecular masses of the monomeric $Fc_{\epsilon}(315-547)$ and $Fc_{\epsilon}(329-547)$ proteins as deduced from their amino acid sequences are 26 kDa and 24.5 kDa, respectively. However, the apparent molecular masses of the deglycosylated form of the two proteins (30 kDa and 28 kDa, respectively) as determined by SDS-PAGE were somewhat higher than the expected values. The actual mass contribution from the sugar moieties in $Fc_{\epsilon}(315-547)$ is only 3 kDa per chain of monomer, since an accurate estimate of the molecular mass of the native protein by mass spectroscopy was only 58 kDa.⁶ The presence of *N*-linked oligosaccharides on $Fc_{\epsilon}(315-547)$ was not found to be necessary for $Fc_{\epsilon}RI\alpha$ binding activity. However, removal of the sugars caused a partial aggregation of the deglycosylated IgE-Fc fragment, and the aggregated population showed <10% of the original binding activity.

Purified $Fc_{\epsilon}(315-547)$ and $Fc_{\epsilon}(329-547)$ were tested for their ability to bind to $Fc_{\epsilon}RI\alpha$, both by direct binding assays and also in competitive binding assays. Both IgE-Fc frag-

ments were found to be indistinguishable from whole IgE with respect to their affinity for $Fc_{\epsilon}RI\alpha$ as determined by saturation binding assays and analyzing the data by the method of Scatchard (24). However, the K_d values determined here represent apparent dissociation constants and are useful only for relative comparisons within a study (9, 10, 12). The apparent dissociation constant is used because of the very slow dissociation of the receptor- Fc_{ϵ} complex, which does not allow for measurements to be made under equilibrium conditions. IC_{50} values obtained for the inhibition of ^{125}I -IgE binding were comparable to the values obtained with unlabeled IgE (Table I). Analysis of the on-rates (k_1) and off-rates (k_{-1}) indicated that the off-rates for $Fc_{\epsilon}(315-547)$ and $Fc_{\epsilon}(329-547)$ were about 2 and 5 times faster than that observed for IgE. No significant difference in the on-rates was observed between IgE and the recombinant IgE-Fc fragments. We also observed that the B_{max} values for $Fc_{\epsilon}(329-547)$ were always somewhat lower compared to that obtained with $Fc_{\epsilon}(315-547)$ or IgE. We do not have any explanation at present for this observation. Both $Fc_{\epsilon}(315-547)$ and $Fc_{\epsilon}(329-547)$ behaved as effective IgE-antagonists and inhibited the sensitization of RBL-48 cells with an antigen-specific IgE (Tan-IgE), in a dose-dependent manner (Fig. 7A). The fragments were also capable of eliciting a cellular response (degranulation) in RBL-48 cells when triggered with various anti-IgE antibodies (Fig. 7B).

⁶ M. Basu and S. A. Hefta, unpublished observations.

However, Fc_ε(329–547) was consistently 3–4-fold less effective than IgE-PS in direct sensitization or in the inhibition of sensitization of RBL-48 cells as determined by the histamine release assay. This difference became more pronounced as the sensitization period increased (data not shown). We are currently studying the phenomena in detail.

In conclusion, the present study suggests that both the C_ε3 and C_ε4 domains are important for maintaining the active conformation of IgE-Fc. The high affinity binding observed with Fc_ε(329–547) demonstrates that the C_ε2 domain, or amino acids amino-terminal to residue 329 are not necessary for binding. The fact that no detectable binding activity was observed with fragments truncated in the C_ε4 domain suggests that the C_ε4 domain plays an important role, either direct or indirect, in facilitating the binding of the IgE-Fc to the FcεRIα. The availability of large homogeneous preparations of Fc_ε(315–547) and Fc_ε(329–547) fragments will facilitate the three-dimensional characterization of the IgE-Fc molecule.

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