

Mutagenesis Within Human FcεRIα Differentially Affects Human and Murine IgE Binding¹

Graham A. Mackay,^{2*} Mark D. Hulett,^{3†} Justin P. D. Cook,^{*} Halina M. Trist,[†] Alistair J. Henry,^{4*} James M. McDonnell,[‡] Andrew J. Beavil,^{*} Rebecca L. Beavil,^{*} Brian J. Sutton,^{*} P. Mark Hogarth,[†] and Hannah J. Gould^{5*}

Soluble fragments of the α-chain of FcεRI, the high-affinity receptor for IgE, compete with membrane-bound receptors for IgE and may thus provide a means to combat allergic responses. Mutagenesis within FcεRIα is used in this study, in conjunction with the crystal structure of the FcεRIα/IgE complex, to define the relative importance of specific residues within human FcεRIα for IgE binding. We have also compared the effects of these mutants on binding to both human and mouse IgE, with a view to evaluating the mouse as an appropriate model for the analysis of future agents designed to mimic the human FcεRIα and attenuate allergic disease. Three residues within the C-C' region of the FcεRI α2 domain and two residues within the α2 proximal loops of the α1 domain were selected for mutagenesis and tested in binding assays with human and mouse IgE. All three α2 mutations (K117D, W130A, and Y131A) reduced the affinity of human IgE binding to different extents, but K117D had a far more pronounced effect on mouse IgE binding, and although Y131A had little effect, W130A modestly enhanced binding to mouse IgE. The mutations in α1 (R15A and F17A) diminished binding to both human and mouse IgE, with these effects most likely caused by disruption of the α1/α2 interface. Our results demonstrate that the effects of mutations in human FcεRIα on mouse IgE binding, and hence the inhibitory properties of human receptor-based peptides assayed in rodent models of allergy, may not necessarily reflect their activity in a human IgE-based system. *The Journal of Immunology*, 2002, 168: 1787–1795.

The interaction between allergen-specific IgE and its high-affinity receptor, FcεRI, is key to the onset and propagation of allergic disease (1). The tight association between these molecules enables multivalent allergen to readily cross-link and activate inflammatory cells bearing FcεRI with the consequent secretion of numerous inflammatory and immunomodulatory molecules (2).

FcεRI consists of three subunits: α, which has two domains (α1 and α2) and provides the binding site for IgE, β, and homodimeric γ (3). These latter subunits are responsible for transducing the initial cross-linking stimuli into the cellular functional responses of immediate mediator secretion, synthesis of proinflammatory arachidonic acid metabolites, and the transcriptional regulation of numerous genes (4, 5). Some studies also suggest a role for the ex-

tracellular region of the γ-chains in the effective high-affinity membrane display of FcεRIα (6), a feature paralleled in the homologous FcγRI (7).

Interruption of the first step of this proallergic pathway, and blocking formation of the complex between IgE and FcεRIα, should prevent inflammatory cell activation and alleviate allergic symptoms. This has been borne out by the clinical success of a humanized anti-IgE Ab (8) that interacts with nonreceptor-associated IgE to prevent binding to FcεRIα, although other mechanisms such as the inhibition of IgE synthesis and down-regulation of cellular FcεRIα levels are thought to contribute to the efficacy of the Ab (8, 9). We have chosen to explore another means of producing a similar effect by investigating the potential of soluble forms of FcεRIα, or rationally designed peptides derived from the receptor, to bind and prevent IgE association with cell-expressed FcεRIα (10). Such an approach would potentially have the added benefit of inactivating the anti-FcεRIα autoantibodies that are clearly involved in the etiology of chronic idiopathic urticaria and perhaps other immunological disorders (11, 12).

A soluble form of FcεRIα (sFcεRIα)⁶ has been shown in numerous *in vivo* and *in vitro* studies to block IgE association with membrane-bound FcεRIα (13–16) and, moreover, to displace existing endogenously bound IgE (13). We have partially duplicated these effects using a cyclized 13-mer peptide derived from the C-C' loop of α2 (16). This peptide was able to inhibit both human IgE binding to immobilized sFcεRIα, and the sensitization of rat

*Randall Centre, New Hunt's House, King's College London, Guy's Campus, London, United Kingdom; †Helen M. Schutt Laboratory for Immunology, Austin Research Institute, Austin Repatriation Medical Center, Heidelberg, Victoria, Australia; and ‡Laboratory of Molecular Biophysics, Department of Biochemistry, University of Oxford, Oxford, United Kingdom

Received for publication June 22, 2001. Accepted for publication December 10, 2001.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by the Medical Research Council of the United Kingdom, by grants from the National Asthma Campaign (to H.J.G. and B.J.S.) and the National Health and Medical Research Council of Australia (to P.M.H.), and by a Union Chimique Belge Institute of Allergy Traveling Fellowship (to G.A.M.).

² Current address: Division of Cell Pathology, Department of Pathology, University of New Mexico, Albuquerque, NM 87131.

³ Current address: Division of Immunology and Cell Biology, John Curtin School of Medical Research, Australian National University, Canberra Australian Capital Territory, Australia.

⁴ Current address: Celltech Therapeutics, Slough, Berkshire, U.K.

⁵ Address correspondence and reprint requests to Dr. Hannah J. Gould, Randall Centre, New Hunt's House, King's College London, Guy's Campus, London SE1 1UL, U.K. E-mail address: h.j.gould@noah.raai.umds.ac.uk

⁶ Abbreviations used in this paper: sFcεRIα, soluble FcεRIα; RBL, rat basophilic leukemia; WT, wild type; BMGY, buffered glycerol-complex medium; mWT, WT sFcεRIα expressed in a mammalian system; pWT, WT sFcεRIα expressed in a *P. pastoris* system; NIP, 4-hydroxy-3-nitro-5-iodo-phenylacetyl; CD, circular dichroism; SPR, surface plasmon resonance; EMEM, Eagle's MEM; HSA, human serum albumin.

basophilic leukemia (RBL) cells with mouse IgE. This latter approach will undoubtedly be further facilitated by the recently determined crystal structure of the complex between FcεRIα and an IgE fragment that unambiguously identifies the contact residues in both proteins (17).

The crystal structures of both FcεRIα (18) and the FcεRIα/IgE complex (17) have conclusively validated significant earlier studies. Domain exchange studies (19–21), together with phage display and soluble expression of single FcεRIα domains (22–24), had demonstrated the essential requirement of the α2 domain for IgE binding, and indeed the crystal structure has shown that the regions of FcεRIα that contact the Cε3 domains of IgE lie solely within α2. However, it was also clear from these earlier studies that α1 was required for optimal Ab binding. This can now be rationalized in light of the extensive interdomain contact seen in FcεRIα (18) and homologous FcγR (25–27). At a more detailed level, potential IgE binding residues had been identified in loop and strand exchange (19–21), peptide synthesis (16), and site-directed mutagenesis studies (28–31) within the α2 domain of FcεRIα. These studies had implicated several regions and highlighted specific residues within α2 as important in complex formation, including the C-C', C'-E, and F-G loops. Involvement of these regions, and many of the specific residues identified in these earlier studies, has now been confirmed by the crystal structure of the FcεRIα/IgE complex (17).

We have selected three residues within the α2 domain of FcεRIα for mutagenesis and expression as a soluble construct, to dissect out their relative importance to the formation and maintenance of the high-affinity complex. We previously reported that the mutation K117D in α2 greatly reduces binding of human IgE (29), and we now extend these studies with two additional mutants, W130A and Y131A, and use a wider range of in vitro assay methods. We also explore the possibility of disrupting the interface between α1 and α2 as a way to reduce FcεRIα affinity for IgE, beginning with independent alanine mutations at R15 and F17. Crystallographic (18) and modeling studies (32) reveal that these amino acids in the α2 proximal A-strand of α1 interact directly with residues of α2.

Previous studies from our laboratories have demonstrated that development of a soluble receptor with (modestly) enhanced affinity for IgE compared with wild-type (WT) FcεRIα is achievable through single point mutagenesis (29, 30). Such a soluble receptor fragment would presumably out-compete endogenous cell surface-expressed FcεRIα, and would consequently be more efficacious as a therapeutic agent than the native protein. Mouse models of allergic disease are well suited to testing the in vivo therapeutic potential of such higher-affinity receptor fragments or receptor-based synthetic peptide antagonists. Therefore, we investigated whether mutations in human sFcεRIα, which are known to affect human IgE binding, have a similar effect upon binding to mouse IgE. Thus, results from the present study have implications for the design of novel FcεRIα-based therapeutics and for the screening methodologies used in subsequent assessment of their potential therapeutic utility.

Materials and Methods

Cloning and site-directed mutagenesis within FcεRIα

Mammalian. The cloning of the soluble WT FcεRIα fragment (residues Val¹ to Lys¹⁷⁶) and the K117D mutant for mammalian expression in the mouse myeloma cell line NS0 has been described in a previous paper (29).

Pichia pastoris. A shorter construct comprising residues Val¹ to Ala¹⁷² was generated as previously described (14). WT sFcεRIα and mutants W130A and Y131A were generated by PCR from pKC3 templates that already contained the mutants but in a membrane-expressible form (30)

using the primers HT-11 (forward) 5'-GGCGTGAATTCGTCCTCA GAAACC-3' and GM-3 (reverse) 5'-GTACTTGAATTCCTAAGCTTTTAT TACAGTAATGTTGGGGG-3', which included *EcoRI* restriction sites (shown in bold) and stop codon (shown in italics). For the generation of R15A and F17A mutations, we used the splice overlap extension method (33) and the following primers alongside HT-11 and GM-3: R15A (forward) 5'-CCATGGAATGCAATATTTAAAGG-3', (reverse) 5'-CCTTTA AATATTGCATTCATGG-3', F17A (forward) 5'-GAATAGAATAGCA AAAGGAGAG-3', and (reverse) 5'-CTCTCCTTTGCTATTCTATTTC-3'; mutated sequences are underlined.

Constructs were generated and amplified using standard PCR methods and they were then cloned into the *EcoRI* site of the *Pichia pastoris* expression vector pPIC9 (Invitrogen, Groningen, The Netherlands) in frame with the *Saccharomyces cerevisiae* α-mating factor prepropeptide, which targets the expressed sFcεRIα to the secretory pathway (34). This peptide is later cleaved by the proteases KEX2 and STE13, although a residual peptide of Tyr-Val-Glu-Phe, the latter two residues formed from the introduced *EcoRI* site, is retained at the N terminus of all the sFcεRIα products expressed in *P. pastoris*. Integrity and orientation of the constructs were confirmed by PCR-based automated sequencing (ABI Prism 377; Applied Biosystems, Foster City, CA) of both strands using the pPIC9 primers (forward) 5'-CCAACAGCACAAATAACG-3' and (reverse) 5'-GACATCCTCTTGATTAG-3', which map to regions ~100 bp on either side of the multiple cloning site.

Expression and purification of the sFcεRIα proteins

Mammalian WT and K117D were expressed and purified as previously described (29). Expression of the *P. pastoris* clones largely followed the manufacturer's guidelines (Invitrogen). Briefly, ~10 μg of purified plasmid DNA (Qiagen, Valencia, CA) was electroporated (0.2-cm cuvette, 2500 V, 25 μF, 400 Ohms, 10 ms) into the *P. pastoris* strain SMD1168. To this was added 1 ml of sorbitol (1 M; Sigma, Poole, Dorset, U.K.) and cells were spread on histidine-free plates and left at 30°C for 2–3 days by which time resistant colonies were clearly visible. Approximately 100 colonies were picked and spread in parallel on either dextrose- or methanol-containing plates and they were left for an additional 2–3 days to grow. Around 5–10% of the clones were slow growing on methanol plates, indicating that in these clones homologous recombination of the pPIC9 vector and insert had occurred around the AOX1 yeast gene. These slow-growing colonies were selected for expansion and small-scale sFcεRIα production analysis.

Clones were expanded overnight at 30°C with constant shaking in growth medium (buffered glycerol-complex medium (BMGY; 10 ml) consisting of 1% yeast extract, 2% peptone, 1.34% yeast nitrogen base (all from Difco, Cowley, Oxfordshire, U.K.), 100 mM potassium phosphate (pH 6.0), 4×10^{-5} M biotin, and 1% glycerol (all from Sigma-Aldrich, St. Louis, MO). Cells were then pelleted ($3000 \times g$ for 5 min) and resuspended in buffered methanol-complex medium growth medium (3 ml) where 0.5% methanol replaced the glycerol. Cultures were induced for 3 days with methanol being added on a daily basis to compensate for evaporation. Cells were then sedimented and the media was assayed for sFcεRIα by ELISA.

Screening for expressing clones and scale-up production

An ELISA was developed for assaying production levels of sFcεRIα. Anti-FcεRIα α2-specific mAb 15.1 (a kind gift of Dr. J.-P. Kinet, Harvard Medical School, Boston, MA) was coupled to 96-well plates (Maxisorp; Life Technologies, Paisley, U.K.) at 2 μg/ml for 1 h at 37°C. Following blocking, dilutions of the supernatants were added and left for an additional 1 h at 37°C. The plate was washed extensively before the addition of the anti-FcεRIα α1-specific mAb 3B4 conjugated with HRP (2 μg/ml). Following incubation with tetramethylbenzidine (Sigma-Aldrich), substrate OD₄₅₀ values were taken and compared against a standard curve constructed using mammalian WT sFcεRIα (mWT). Typical expression levels were ~2–4 μg/ml.

High-expressing clones were selected for scale-up production following the manufacturer's guidelines (Invitrogen). A single yeast colony was used to inoculate 10 ml of BMGY medium and was grown overnight. This culture was used to seed a larger 2-L BMGY volume contained in a 5-L conical flask, which was again incubated at 30°C overnight. Yeast cells were harvested by centrifugation ($3000 \times g$ for 10 min), the supernatant was discarded, and cells were resuspended in 250 ml of buffered methanol-complex medium. Again, induction was allowed to continue for 3 days with daily addition of methanol to 0.5%. Yeast cells were sedimented as described above, and the supernatant containing sFcεRIα was further clarified by another centrifugation ($8,000 \times g$ for 30 min) followed by filtration to 0.45 μm.

Purification of sFcεRIα

Purification of mammalian expressed material was as reported previously (29). A similar method using the anti-FcεRIα mAb 3B4 was used for purification of sFcεRIα proteins expressed in *P. pastoris* (pWT) with some modifications. Briefly, the filtered yeast supernatant was partially purified by 66% ammonium sulfate precipitation for 30 min on ice followed by centrifugation ($8,000 \times g$ for 30 min). The protein pellet was reconstituted in 50 ml of PBS and was then extensively dialyzed against PBS. The protein solution was supplemented with BSA (0.5%) and leupeptin (2 μg/ml; both from Sigma-Aldrich) and was then filtered to 0.45 μm before recirculation on a 3B4-conjugated Sepharose column. After extensive washing with PBS, sFcεRIα was eluted from the column using 0.1 M glycine (pH 2.5). The eluate was neutralized with Tris (1 M), was concentrated to ~1 ml, and was then dialyzed into PBS. Proteins were quantified by evaluating absorbance at 280 nm using an extinction coefficient for sFcεRIα of $2.56 = 1 \text{ mg/ml}$. This value was adapted accordingly in receptor mutants where replacement of aromatic residues occurred. Protein purity was determined by 12% SDS-PAGE (35) under nonreducing conditions and staining with Coomassie brilliant blue and Western blotting using both Abs 3B4 and 15.1.

Human and mouse IgE and their specific Ags

Human anti-4-hydroxy-3-nitro-5-iodo-phenylacetyl (NIP)-IgE (36) was obtained from the cell line JW8 (European Cell Culture Collection no. 87080706). Supernatants from the cell line were recirculated over an IgG4-Fc-(sFcεRIα)₂ (37) Sepharose column. Following extensive washing with PBS, NIP-IgE was eluted with glycine (0.1 M, pH 2.5), neutralized with Tris (1 M), concentrated, and dialyzed against PBS. In degranulation assays using NIP-IgE, the antigenic stimulus used was NIP-BSA, which was prepared by conjugating BSA with NIP-cap-O-succinimide (Cambridge Research Biochemicals, Northwich, Cheshire, U.K.). Mouse anti-DNP-IgE and DNP-human serum albumin (HSA) were both purchased from Sigma-Aldrich and were used without further purification.

CD spectroscopy

Circular dichroism (CD) measurements were performed on a Jobin-Yvon CD6 spectrometer (Longjumeau, France). WT and mutant proteins were analyzed in circular quartz cells (path length of 0.05 cm) at concentration ranges between 250 and 650 μg/ml in 140 mM sodium perchlorate and 10 mM sodium phosphate (pH 7.2) at 20°C. Measurements represent an average of four repeated scans in steps of 0.2 nm with an integration time of 1 s. Buffer-only controls were used as blanks and were subtracted from all measurements. The spectrophotometer was calibrated for wavelength and ellipticity (ε) using *d*-10-camphor-sulfonic acid. The units of $\Delta\epsilon$ are $\text{M}^{-1} \text{cm}^{-1}/\text{mean residue weight}$.

SPR analysis of binding

All surface plasmon resonance (SPR) measurements were conducted using a BIAcore 1000 (Pharmacia Biacore, Stevenage, Hertfordshire, U.K.). Extensive discussion of the SPR methodology and data analysis relating to FcεRIα and IgE interactions has been previously described (29, 38). Briefly, purified sFcεRIα was coupled to a BIAcore CM5 sensor chip using an aldehyde coupling technique, which uses protein carbohydrate to form stable linkage between the chip and protein. sFcεRIα immobilized through the more standard amine coupling methodologies was found to bind IgE poorly (data not shown). Runs were conducted in HEPES-buffered saline (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, and 0.005% surfactant P-20; Pharmacia Biacore) at a flow rate of 5 μl/min, with 750–1000 resonance units of immobilized protein. We have previously tested a range of immobilization levels with this flow rate and have shown that mass transport effects are only detectable at higher receptor densities (J. M. McDonnell, unpublished data).

Human or mouse IgE (15–250 nM) was injected, and binding and then dissociation of bound material was monitored before the surface was regenerated by three successive injections (60 s) of glycine (0.1 M, pH 2.5). Data were analyzed using BIAevaluation (Version 3.0; Pharmacia Biacore) with global monophasic fitting. In previous studies, we have shown that a biphasic analysis of this interaction is justified, and can provide information about binding mechanism (29, 38). However, because monophasic K_a values show the same trends (38), to simplify comparison of the effects of mutations in the mouse and human systems we report only these values in this study. Nonspecific binding, generated by passing IgE over a flow cell without any protein coupled, was subtracted from experimental data before kinetic evaluation.

Degranulation assays

Primary basophil cells. Human basophils (purity ~1%) were prepared by dextran sedimentation and washed twice in a PIPES-buffered saline (25 mM PIPES, 110 mM NaCl, 5 mM KCl, and 0.1% glucose, pH 7.3) containing 0.003% HSA but without Ca^{2+} and Mg^{2+} (39). Cells were then incubated with various concentrations of sFcεRIα and human anti-NIP-IgE (5 nM) for 1 h at 37°C. The cells were washed and resuspended in buffer containing both 1 mM Ca^{2+} and Mg^{2+} and they were then challenged with NIP-BSA (10 ng/ml) for an additional 40 min (37°C) and histamine release was measured using an automated fluorometric analyzer. The release of histamine was expressed as a percentage of total cellular histamine. Spontaneous histamine release (occurring in the absence of NIP-BSA) was typically ~3% and was subtracted from all other values. Control histamine release (obtained in activated samples without the presence of sFcεRIα) was in the range of 30–50% depending on the blood donor, and inhibition of secretion data are expressed as a percentage of this control release.

RBL cells. RBL-2H3 cells were cultured in Eagle's MEM (EMEM) with 10% FBS, L-glutamine (2 mM), penicillin (10 U/ml), and streptomycin (1 μg/ml; all from Life Technologies). On the day before experimentation, RBL cells were removed from the culture flasks using Versene (Life Technologies) and were seeded into 96-well plates (7×10^4 cells/well) and left overnight to attach. Wells were washed with EMEM and were then incubated (1 h at 37°C) with varying concentrations of sFcεRIα and mouse anti-DNP-IgE (0.1 nM) made up in EMEM but with 2% FBS. Cells were washed with PIPES buffer (25 mM PIPES, 120 mM NaCl, 5 mM KCl, 5.6 mM glucose, 1 mM CaCl_2 , 0.4 mM MgCl_2 , 0.1% BSA, pH 7.3) and were then activated by adding DNP-HSA (20 ng/ml; 40 min; 37°C) in the above PIPES buffer (200 μl). Samples (150 μl) were removed and analyzed for β-hexosaminidase release as previously described (16). Results are expressed as for the basophil experiments described above with spontaneous β-hexosaminidase release being typically 1–2%, and control release being in the range of 20–25%.

Molecular modeling

Structures of FcεRIα (18) and its complex with the Cε3 and Cε4 domains of human IgE Fc (17) were viewed using INSIGHT II (Molecular Simulations, Waltham, MA). A homology model of the corresponding Fc fragment of mouse IgE, based upon the structure of the human IgE Fc in the complex with FcεRIα, was generated using SWISS-MODEL (40).

Results

Expression of sFcεRIα in mammalian and yeast systems

We have previously expressed and purified both WT and mutant sFcεRIα from a mammalian expression system (mouse myeloma cell line NS0) generating clones that secrete in the range of 2–5 mg protein/L culture supernatant (29). To facilitate the more rapid production of sFcεRIα mutants for this and future studies, we have now used the yeast *P. pastoris* for recombinant protein expression. Using this system, we obtained expression levels similar to those using NS0 cells, and affinity purification was almost as straightforward as the mammalian system, with no indication of degradation of either product or purifying Ab. Products of WT sFcεRIα produced in NS0 cells (mWT) and in *P. pastoris* (pWT) were compared as a means of testing the suitability of the latter system for our analysis. The mutant K117D, which was generated in NS0 cells and included in a previous study (29), and four new soluble mutants produced in *P. pastoris* (W130A, Y131A, R15A, and F17A) form the basis of the present study.

As shown by SDS gel electrophoresis in Fig. 1A, following affinity purification using the anti-FcεRIα Ab 3B4, WT and mutant sFcεRIα produced in both mammalian and yeast were monodisperse and equivalent in apparent molecular mass. The yeast products exhibited a narrower range, most likely reflecting their modification with carbohydrate chains of a more homogeneous length and/or composition. There are seven N-glycosylation sites within human FcεRIα and, thus, the apparent molecular mass is increased from 21 kDa (core polypeptide) to ~40–60 kDa (mature glycoprotein). The gel staining patterns (Fig. 1A) were confirmed by Western blotting (Fig. 1B) using mAb 15.1 (mWT, K117D, pWT,

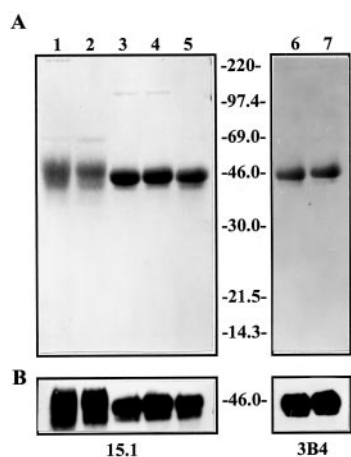


FIGURE 1. SDS-PAGE analysis of purified sFcεRIα. *A*, Five micrograms of each purified sample was loaded per lane and the gel was stained with Coomassie blue. Lane 1, mWT; lane 2, K117D; lane 3, pWT; lane 4, W130A; lane 5, Y131A; lane 6, R15A; lane 7, F17A. The molecular mass markers (Rainbow high-m.w.; Amersham) are shown in kilodaltons. *B*, Corresponding Western blot. Three hundred nanograms of each purified sFcεRIα was electrophoresed and transferred to nitrocellulose, and samples 1–5 were probed with the α2-specific mAb 15.1, and samples 6 and 7 were probed with the α1-specific mAb 3B4.

W130A, and Y131A) and mAb 3B4 (pWT, R15A, and F17A), reinforcing the general absence of aggregated or degraded material and demonstrating that epitopes within the specific domains where the mutations had occurred were not disrupted by the introduction of these single residue changes.

As part of this general quality control analysis, we tested the ability of these mutant receptors to bind Abs that have epitopes located in their unmodified α domain. Using Western blotting, the α2 mutations (K117D, W130A, and Y131A) retained full binding to mAb 3B4 (results not shown). Surprisingly, the α1 mutants (R15A and F17A), while retaining mAb 3B4 binding, demonstrated a dramatic reduction, although not complete loss, of binding to mAb 15.1, which has an epitope located within α2 (Fig. 2). These results provided an explanation for our earlier observation that the R15A and F17A mutants were expressed in lower quantities than the other sFcεRIα products in the initial screening process, which involved an ELISA using mAb 15.1. However, extrapolated yields of these mutants were greatly surpassed following purification (using 3B4) and spectrophotometric quantification (data not shown).

CD spectroscopy

An advantage of the expression of FcεRIα as a soluble protein is that it permits assessment of changes in secondary structure induced by mutagenesis using CD. Spectra obtained for both yeast- and mammalian-derived sFcεRIα (data not shown) exhibited features similar to those previously described (16, 29, 41). The highly characteristic positive dichroism peak at ~230 nm is seen for all

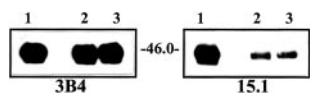


FIGURE 2. Loss of mAb 15.1 binding in sFcεRIα mutants R15A and F17A as determined by Western blot. Duplicate 300-ng samples of sFcεRIα pWT (lane 1), R15A (lane 2), and F17A (lane 3) were analyzed by SDS-PAGE (12%), transferred to nitrocellulose, and probed with either the α1-specific mAb 3B4 or the α2-specific mAb 15.1.

seven species and probably reflects the high content of aromatic residues and the two intrachain disulfide bonds. Equally, the characteristic feature of the β sheet structure of Ig and Ig-like domains, the negative peak ~215 nm, is present in all spectra (data not shown).

Slight differences in the size and, to a lesser extent, position of the positive and negative peaks were observed between the mWT, pWT, and mutant FcεRIα proteins. These differences between the proteins may be due to the short N-terminal peptide (Tyr-Val-Glu-Phe) that is added to the *P. pastoris* material as part of the expression procedure, and/or may reflect glycosylation differences. Moreover, replacement of aromatic residues occurs in several of the mutants and this will be expected to affect the CD spectra. As might be predicted from the FcεRIα crystal structure, we conclude that there is no major unfolding of the β structure induced by any of the point mutations, although we cannot exclude minor perturbations that could influence binding activity.

Binding kinetics using SPR

Following immobilization of sFcεRIα proteins, the binding kinetics of both human and mouse IgE were measured using SPR. Representative sensorgrams of typical experiments are shown in Fig. 3. The traces show the binding of various concentrations of IgE (12.5–250 nM) to immobilized sFcεRIα in real time, where 150–500 s represents the association phase, and 500–800 s represents the dissociation phase.

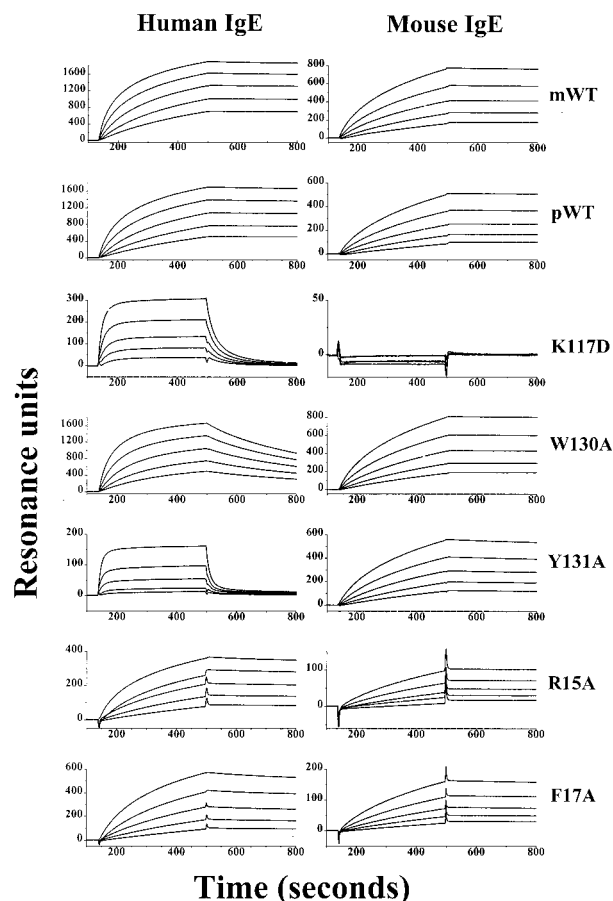


FIGURE 3. Binding of human and mouse IgE to WT and mutant sFcεRIα as determined by SPR. Representative sensorgrams are displayed from five concentrations of IgE (15–250 nM), and global monophasic fitting was used to generate the kinetic values shown in Table I.

Comparison of WT FcεRIα proteins

Results shown in Fig. 3, rows 1 and 2, demonstrate that both mWT and pWT sFcεRIα interact with human and mouse IgE in a very similar manner. This is confirmed by kinetic analysis of the data, which shows that both mWT and pWT sFcεRIα have closely matched values for both association and dissociation rate constants (k_a and k_d , respectively) and resultant affinity constant (K_a ; Table I). This indicates that neither the addition of the N-terminal leader peptide (Tyr-Val-Glu-Phe) introduced by the yeast expression strategy, the C-terminal truncation of the yeast product by four residues compared with the mammalian protein, nor the differences in glycosylation between *P. pastoris* and NS0 cells has any discernable effect on the affinity for either human or mouse IgE. The lack of any effect of differential glycosylation upon binding is not surprising because bacterially expressed receptor or mammalian sFcεRIα clones grown in the presence of tunicamycin still bind IgE with high affinity, demonstrating that sugar moieties within sFcεRIα are not directly involved in IgE binding (22, 42, 43).

The 3-fold lower affinity of mWT sFcεRIα for mouse IgE compared with human IgE (Table I) is less marked than, but still consistent with, previous affinity measurements for human receptor binding to mouse IgE for which a 7-fold reduction compared with human IgE was observed (16). This difference between murine and human IgE binding affinity was not seen for pWT sFcεRIα (Table I). One notable feature of binding to both mWT and pWT sFcεRIα in the present study was the lower total response with mouse compared with human IgE (Fig. 3, compare *left* and *right* columns). This most probably reflects a lower fraction of bindable IgE in the commercial mouse IgE preparation.

Effect of mutations within the α2 domain of FcεRIα

Previous mutagenesis studies, largely in cell-based assays, and the structure determination of the FcεRIα/IgE complex (17) have demonstrated the importance of the K117, W130, and Y131 residues in the interaction with human IgE. Using sFcεRIα mutants with native-like structures as assessed by CD, we wished to determine the individual contribution of each of these residues to the affinity of interaction with both human and mouse IgE. In our previous work, we identified K117D as a mutation that dramatically reduced the binding of sFcεRI to human IgE (29). In the present study, we have confirmed this result, demonstrating that this mutation reduces the affinity for human IgE ~200-fold, principally due to an enhanced dissociation rate (Fig. 3 and Table I). Moreover, it appears that this mutation has an even greater effect on the binding of mouse IgE, as the K117D/mouse IgE interaction was so weak that no meaningful kinetic parameters could be de-

termined (Fig. 3, row 3, and Table I). The other two mutations within α2 behave quite differently. W130A decreased the affinity for human IgE ~25-fold but actually increased, by 5-fold, the affinity for mouse IgE (Fig. 3, row 4, and Table I). Y131A had an even greater effect than W130A, reducing the binding affinity for human IgE 300-fold, with the dramatically increased dissociation rate (>500-fold) as the principal factor. But again, this decreased affinity was not reflected in the binding of mouse IgE, for which only a comparatively small (6-fold) increase in dissociation rate constant, and similar decrease in affinity, was measured (Fig. 3, row 5, and Table I). Thus, we conclude that mutations in human sFcεRIα do not have similar effects on binding to human and mouse IgE.

Effect of mutations within the α1 domain of FcεRIα

Both R15 and F17 have been highlighted as residues that make interdomain contacts important for receptor stability (18, 32). Mutants R15A and F17A produced ~4- and 2-fold reductions, respectively, in the total amount of both human and mouse IgE that was able to bind within the association period compared with pWT sFcεRIα (Fig. 3, rows 5 and 6). However, neither the rates of association nor dissociation were greatly affected, leading to K_a values not very different from pWT sFcεRIα (Table I). These results suggest that a different mechanism is responsible for the ablation of binding in the α1 mutants compared with the α2 mutants, and for this reason we do not include the R15A and F17A mutants in the affinity ranking described below.

Inhibition of passive sensitization of IgE effector cells

sFcεRIα- and receptor-derived peptides, from a therapeutic perspective, would have to block IgE binding to cell-bound FcεRIα, and so we have measured the ability of the mutants to do this using human basophils and human IgE, and RBL cells and mouse IgE, to represent the human system and animal model, respectively. Mouse IgE is able to bind mouse, rat, and human FcεRIα, whereas human IgE can only interact with human FcεRIα (4). We have used the rat cell line primarily for practical reasons due to its relative ease of culture and adherence properties that facilitate multiwell plate assay. However, the FcεRIα extracellular domain sequence identity is much stronger between rat and mouse (71%) compared with human and mouse (51%; Ref. 44). Hence, mouse IgE/mouse FcεRIα binding, as it occurs in a mouse *in vivo* allergy model, is likely to be more closely modeled by the mouse IgE/rat FcεRIα system. Efficacy of the sFcεRIα proteins was assessed by their ability to inhibit IgE-dependent, Ag-induced cellular degranulation.

Table I. Summary of kinetic data for the interactions of WT and mutant sFcεRIα with human or mouse IgE as determined by SPR^a

sFcεRIα Assayed	k_a ($M^{-1} s^{-1}$)		k_d (s^{-1})		K_a (M^{-1}) ^b	
	Human IgE	Mouse IgE	Human IgE	Mouse IgE	Human IgE	Mouse IgE
mWT	$6.5 \pm 1.3 \times 10^4$	$2.6 \pm 1.6 \times 10^4$	$4.6 \pm 1.9 \times 10^{-5}$	$5.8 \pm 5.4 \times 10^{-5}$	1.4×10^9 (9)	4.4×10^8 (3)
pWT	$5.3 \pm 1.0 \times 10^4$	$2.9 \pm 0.1 \times 10^4$	$5.5 \pm 1.8 \times 10^{-5}$	$1.8 \pm 1.1 \times 10^{-5}$	9.6×10^8 (10)	1.6×10^9 (4)
K117D	$1.0 \pm 0.2 \times 10^5$	— ^c	$1.5 \pm 0.2 \times 10^{-2}$	— ^c	6.7×10^6 (4)	— ^c
W130A	$6.1 \pm 1.1 \times 10^4$	3.4×10^4	$1.6 \pm 0.1 \times 10^{-3}$	4.4×10^{-6}	3.8×10^7 (4)	7.7×10^9 (1)
Y131A	$9.8 \pm 1.7 \times 10^4$	3.3×10^4	$3.1 \pm 0.2 \times 10^{-2}$	1.2×10^{-4}	3.2×10^6 (2)	2.8×10^8 (1)
R15A	$4.3 \pm 0.4 \times 10^4$	$2.2 \pm 0.3 \times 10^4$	$6.8 \pm 5.0 \times 10^{-5}$	$4.7 \pm 0.8 \times 10^{-5}$	6.3×10^8 (3)	4.6×10^8 (2)
F17A	$3.1 \pm 0.4 \times 10^4$	2.5×10^4	$2.0 \pm 0.1 \times 10^{-4}$	1.2×10^{-4}	1.6×10^8 (2)	2.1×10^8 (1)

^a Binding kinetics were determined using global monophasic fitting (BiaEvaluation version 3.0). Results are means $\pm$ SD of experiments carried out at five different IgE concentrations and the number of repeat experiments is given in parentheses. Kinetic data obtained for mutant K117D should be compared with mWT while values for all other mutants should be compared with pWT.

^b Calculated as k_a/k_d .

^c Insufficient binding for kinetic analysis.

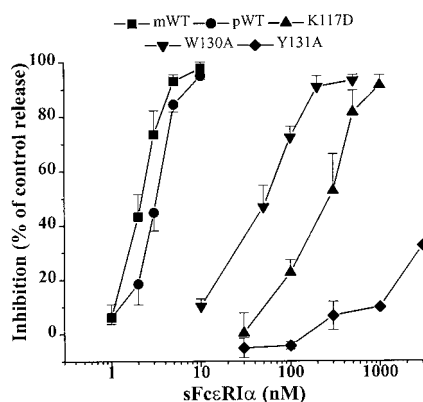


FIGURE 4. Inhibition of passive sensitization of human basophils by WT and $\alpha 2$ -mutated sFcεRIα. Basophils were sensitized with human NIP-IgE (1 μ g/ml) with or without a variable concentration of sFcεRIα for 1 h before activation with NIP-BSA (10 ng/ml) for an additional 40 min. Released histamine was measured and expressed as a percentage of total histamine content. Inhibition of histamine secretion was then expressed as a percentage of the release occurring in the absence of any sFcεRIα (typically ~30–50%).

The results obtained yield the same rank order for the mutants as seen by SPR. mWT and pWT sFcεRIα were equally effective in attenuating the secretion of histamine release from human basophils ($IC_{50} \sim 3$ nM; Fig. 4). Y131A ($IC_{50} > 3000$ nM) was clearly of lower affinity for human IgE in this assay compared with K117D ($IC_{50} = 300$ nM; Fig. 4), which was in turn lower than that of W130A ($IC_{50} = 70$ nM), consistent with data obtained by SPR. The fact that there is not always exact quantitative agreement (for example, the Y131A mutant is much less active in the functional assay than would be predicted from the SPR data) is not unexpected. We have previously shown that K_a values determined by SPR are about 10-fold lower than cell binding assays. This may be due to a number of factors, including the concentration conditions of the experiment and the fact that there are other components of the receptor (β - and γ -chains) on the cell membrane. Furthermore, although the degranulation assay reflects cell binding, additional downstream events are also involved. Nevertheless, we have found a common rank order of affinity/potency for the human sFcεRIα WT and $\alpha 2$ mutant receptor protein interactions with human IgE in the two assays: mWT = pWT > W130A > K117D > Y131A (Fig. 4 and Table I).

Inhibition of mouse IgE binding to RBL cells by the various sFcεRIα proteins again matched the results obtained in the SPR assays (Fig. 5). mWT and pWT sFcεRIα were equally effective in inhibiting passive sensitization ($IC_{50} = 0.2$ nM; Fig. 5A), which argues against there being a significant difference between the mWT and pWT sFcεRIα affinity for mouse IgE, as suggested by the SPR data (Table I). The lower IC_{50} values reported in this assay compared with the human basophil experiments reflect the lower concentration of IgE used for sensitization of RBL cells (0.1 nM compared with 5 nM for human basophils). Introduction of the W130A mutation slightly enhanced the receptor's ability to inhibit sensitization ($IC_{50} = 0.1$ nM) in keeping with the SPR data. Y131A retained much of the WT ability to inhibit mouse IgE binding, with only an ~5-fold reduction in efficacy, in contrast to the results obtained with human basophils. Similar results were obtained for both R15A and F17A, with ~10-fold reduction in efficacy (Fig. 5B). However, as demonstrated in the SPR assays, the K117D mutant displayed a markedly reduced ability to inhibit mouse IgE binding, much more so than for human IgE. In fact, the K117D sFcεRIα mutant showed an ~10,000-fold reduced efficacy

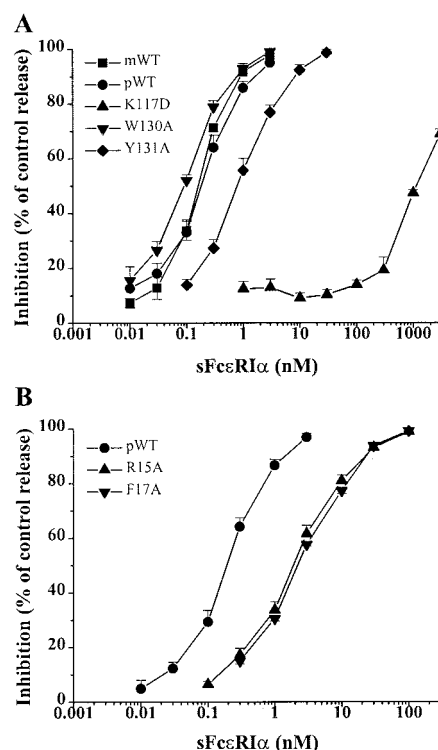


FIGURE 5. Inhibition of passive sensitization of RBL cells by WT and $\alpha 2$ -mutated sFcεRIα (A) and WT and $\alpha 1$ mutated proteins (B). RBL cells were incubated with mouse DNP-IgE (0.1 nM) in the absence or presence of a variable amount of sFcεRIα for 1 h before activation with DNP-HSA (20 ng/ml) for an additional 40 min. Released β -hexosaminidase was quantified and expressed as a percentage of total cellular enzyme. Inhibition of mediator release was then expressed as a percentage of the release occurring in the absence of any sFcεRIα (typically ~20–25%).

in the mouse IgE/RBL assay compared with the WT receptor. The common rank order of affinity/potency for the human WT sFcεRIα and $\alpha 2$ mutant receptor interactions with mouse IgE in both the cell-based and SPR assays is thus: W130A > mWT = pWT > Y131A > K117D (Fig. 5A and Table I).

Discussion

A soluble form of the IgE-binding extracellular domains of FcεRI has been previously expressed in a number of bacterial (22), insect (18, 45), and mammalian (13, 14, 29, 41, 42) systems. We have now produced WT sFcεRIα and a range of mutants using *P. pastoris*. pWT sFcεRIα had near identical IgE binding properties to a previously described mammalian-derived product (29). We have also produced a number of other soluble Fc receptors in *P. pastoris*, including FcγRIIa (M. Powell and P. M. Hogarth, unpublished observations) and FcαR (46). Others have reported expression of sFcεRIα in *P. pastoris*, although no further details were given (18, 47).

We have examined the effects of four new, human sFcεRIα mutant proteins, shown in this study to be structurally native, on the binding to human and mouse IgE, and we have compared these with WT receptor and a previously described mutant K117D (29). IgE from both species was used, as we wished to determine whether certain key residues within human FcεRIα were common to the high-affinity complex formed with both human and mouse IgE. Evaluating the reliability of extrapolation from the mouse to the human system has direct implications for both the screening and in vivo testing of sFcεRIα mutants with elevated affinity, or receptor-derived peptides, as potential therapeutic agents. Point

mutations were initially selected on the basis of our previously published human FcεRIα model (16), but their roles in interdomain contact (α1 mutants) and direct interaction with IgE (α2 mutants) are now definitively demonstrated in the recent crystal structures of sFcεRIα and the sFcεRIα/IgE complex (17, 18).

Both α1 mutants (R15A and F17A) reduced the ability of sFcεRIα to bind both human and mouse IgE, as determined by both SPR and cellular assays. However, unlike the α2 mutants (discussed below), no obvious change in the dissociation rate was observed nor was the association rate greatly affected, yet the total amount of IgE binding was greatly reduced compared with WT. Structural changes induced by mutagenesis at these residues are implied by the reduced binding of mAb 15.1, results paralleled by FACS analysis using cell membrane-expressed forms of R15A and F17A (32). This is significant, as the α2 domain-specific Ab is competitive with IgE for FcεRIα binding, and its epitope has recently been mapped to the C strand region, now clearly identified as a site of IgE interaction (48). Thus, these results support previous work that showed that modifications in the A strand of the α1 domain affect the overall receptor structure (21) and affect IgE binding, although R15 and F17 are not contact residues (32).

Confirmation of this interpretation comes from the sFcεRIα crystal structure (18), which shows that both residues are buried at the α1/α2 interface. Replacement of these residues by alanine would be expected to destabilize the intradomain hydrophobic core, and to account for the observed reduction in mAb binding to α2. It may be that the relative disposition of the two domains is affected, without significantly unfolding either individually, as the CD spectra of these alanine mutants provide no evidence of loss of β structure or unfolding. Thus, destabilization of the α1/α2 interface in the R15A and F17A mutants may result in either the α2 contact residues assuming the correct configuration or the α1 and α2 domains displaying the appropriate relative disposition for IgE binding in only a fraction of the molecules at any one time. This could explain our SPR data, which show that the α1 domain mutants decreased the total amount of IgE binding without greatly changing the kinetics or overall affinity of the interaction. Such an

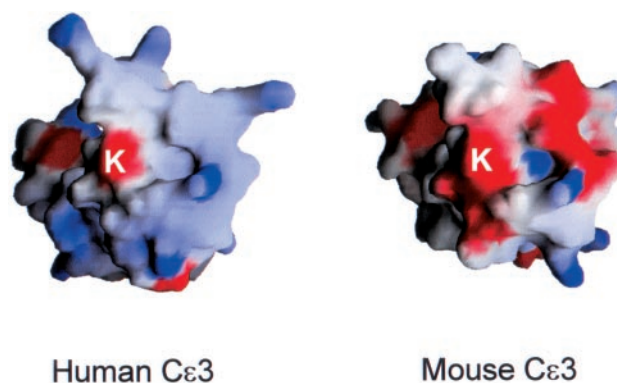


FIGURE 6. The molecular surfaces of homologous regions of human and mouse Cε3 domains. The former is taken from the crystal structure of the complex with human FcεRIα (17) and is centered on the point of interaction with residue K117 of the receptor (indicated as K). The mouse Cε3 surface is calculated from a model based upon the human Cε3 structure. Red indicates negative surface charge and blue indicates positive surface charge. This figure was generated by GRASP.

explanation is also consistent with our finding that these mutations similarly affect binding to both human and mouse IgE.

The α2 mutant K117D has been shown to dramatically increase the dissociation rate of human IgE from sFcεRIα as measured by SPR (29). We have confirmed and extended these data using both SPR and cellular assays, and we have also shown that this mutation not only affects human IgE binding, but indeed has an even more pronounced effect on mouse IgE binding. By SPR, binding to human IgE was reduced 200-fold, and this was reflected almost exactly in the human basophil assay (Fig. 4). Binding of this mutant to mouse IgE was virtually undetectable by SPR, placing an upper limit of the order of 10^5 M^{-1} on the K_a value. However, in the RBL/mouse IgE assay, a 10,000-fold reduction in efficacy was recorded (Fig. 5), which is entirely consistent with the SPR result. The crystal structure of the human sFcεRIα/human IgE complex

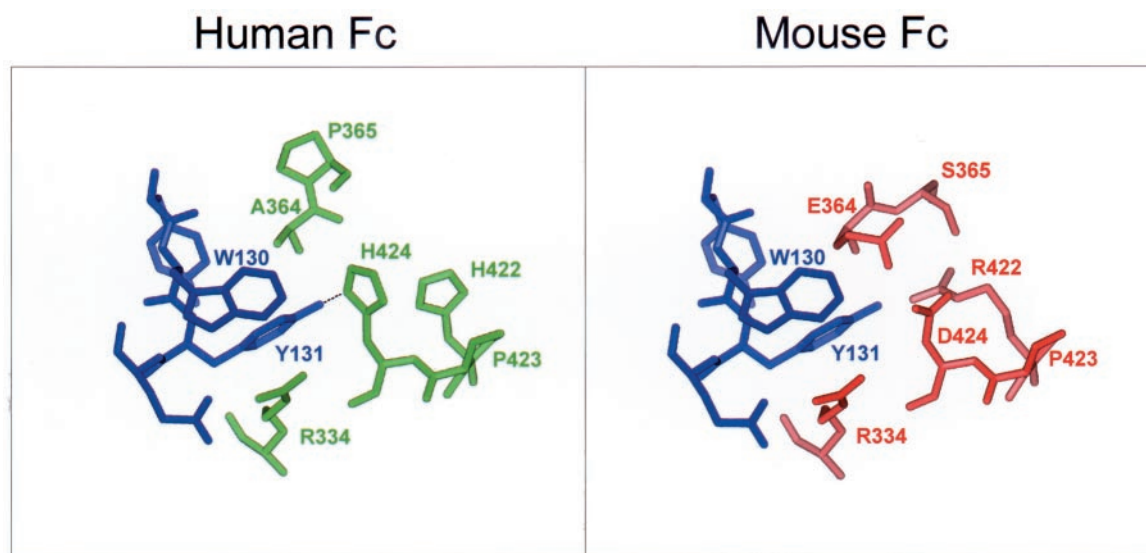


FIGURE 7. A, Details of the interaction between human FcεRIα (blue, indicating residues Trp¹³⁰ and Tyr¹³¹) and human IgE Fc Cε3 domain (green) taken from the crystal structure of the complex (17). The hydrogen bond between Tyr¹³¹ and His⁴²⁴ is indicated. B, The homologous interaction between human FcεRIα (blue) and a model of mouse IgE Fc Cε3 domain (red, with residue numbers following the human sequence for convenient comparison) modeled upon the structure of the human complex. The significance of each of the residues that differ between human and mouse IgE is discussed in the text.

confirms that K117 plays a critical role in the interface (contributing to one of the two subsites, termed “site 1”; Ref. 17), forming a salt bridge with D362 of the Cε3 domain. Clearly, the reversal of charge in the K117D mutant would actively disrupt this interaction. D362 is conserved in mouse IgE, as are residues in its immediate vicinity, but at positions 364 and 365, the human sequence is Ala-Pro, whereas it is Glu-Ser in mouse. This must alter the polypeptide chain backbone conformation (at least at position 365, as shown by modeling), and mean that the position of D362 is slightly different in mouse relative to human IgE. This could account for the differential effect of the K117D mutation upon binding to human and mouse IgE. More significantly, the negatively charged residue, E364, in mouse IgE (in place of the neutral A364 of human IgE), and a similar substitution of the negatively charged E332 in mouse IgE (in place of the neutral N332 of human IgE), both lie within 6 Å of the side chain of residue 117. The considerably enhanced negative charge distribution at the surface of mouse Cε3 in the vicinity of K117D is shown in Fig. 6, where “K” indicates the location of the lysine side chain from the receptor. Thus, the conserved aspartic acid residue 362 (red patch underneath the K in each panel of Fig. 6) is flanked by the negative charges of glutamic acid residues 332 (below) and 364 (to the right) in mouse Cε3. This may well account for the fact that binding of the K117D (positive to negatively charged) mutant to mouse IgE is reduced by four orders of magnitude and is virtually undetectable by SPR.

By SPR and in the cell-based assays, both the W130A and Y131A mutations also significantly reduced FcεRIα affinity for human IgE, with Y131A having a particularly marked effect (300-fold reduction in K_a by SPR) compared with W130A (25-fold reduction). Because both side chains are effectively removed by the alanine substitutions, we may conclude that Y131 contributes more to the binding energy of the FcεRIα/IgE complex than W130. In a previous study using membrane-expressed FcεRIα, we found that although the Y131A mutant dramatically reduced human IgE binding affinity, W130A increased the affinity for human IgE, though only 2-fold (30). These affinity differences observed between the soluble and membrane-spanning versions of W130A might be due to the membrane form interacting with other cellular proteins that may change receptor affinity in a manner previously described for other Fc receptors (6, 7).

The human sFcεRIα/human IgE crystal structure rationalizes the role of these two residues, which contribute to the same subsite as K117 (“site 1”; Ref. 17). Y131 projects into a pocket in Cε3, hydrogen bonding to H424, and packing between A364 and R334 (Fig. 7). W130 lies alongside Y131 and makes tenuous contact with the same residues H424, A364, and R334; it is peripheral to the interaction and predominantly exposed, even in the complex. It is easy to see why the Y131A mutation has a more profound effect upon binding to human IgE than W130A because the former removes a hydrogen bond and leaves a cavity at the interface, whereas the latter merely results in the loss of a small surface area of hydrophobic interaction. However, Y131A has a much smaller effect upon binding to mouse IgE than to human IgE—a 6-fold reduction in K_a compared with a 300-fold reduction. In mouse IgE, critical contact residues for Y131, namely H424 and A364, are Asp and Glu, respectively, changing the nature of the interacting surface of Cε3 considerably (Fig. 7), and in particular, the surface charge (Fig. 6). These differences may account in part for the (~7-fold) lower binding affinity of human FcεRIα for mouse IgE than human IgE (16). Certainly they can account for the fact that the Y131A mutation has a greater effect upon human IgE binding than mouse IgE binding, because for the latter, there is no hydrogen bond to lose, and the Cε3 pocket is less hydrophobic (Fig. 7).

Although the mutation W130A reduces binding to human IgE, it actually enhances binding to mouse IgE (5-fold as judged by SPR). This can also be rationalized because replacement of the bulky, hydrophobic tryptophan side chain with the methyl group of alanine removes only a weak (but favorable) hydrophobic interaction with human IgE. However, in mouse IgE, this substitution removes unfavorable interactions with the nearest contact partners on Cε3, namely D424 and E364 (Fig. 7). It is interesting to note that although K117 and Y131 are conserved between human, mouse, and rat FcεRIα, W130 in the human receptor is replaced by a serine in the rodent sequences (49). It is perhaps not surprising then that replacement of the bulky hydrophobic tryptophan with an alanine residue, more similar to the native mouse FcεRIα sequence, elevates the affinity of this mutant form of human sFcεRIα for mouse IgE compared with the WT sequence.

Inspection of the mouse, rat, and human receptor sequences, together with the crystal structure of the complex, offers an explanation for the well-known observation that although both rodent IgEs bind to human receptor (albeit with reduced affinity as discussed above), the rodent receptors do not bind to human IgE (49, 50). However, this lack of cross-reactivity appears to have a different explanation for the two species. Mouse receptor, uniquely among the known sequences, has a deletion at position 135 and a proline at 134; this must substantially alter the main chain conformation in this region immediately adjacent to contact residues such as Tyr¹³¹. Although this could clearly account for the nonreactivity of mouse receptor for human IgE, the rat sequence is highly homologous to human receptor in this region. (Trp¹³⁰ is replaced by serine in rat receptor, but this is unlikely to account for the lack of binding because our W130A mutation only reduces 25-fold the affinity for human IgE, as discussed above.) However, rat receptor has a lysine at position 157, where the human and mouse sequences have the neutral glutamine. In the crystal structure of the human complex, Gln¹⁵⁷ packs between Asn³³² and Arg³³⁴ on the Cε3 domain of IgE. Arg³³⁴ is conserved across all known IgE sequences, and although Lys¹⁵⁷ in the rat receptor would interact unfavorably with Arg³³⁴, mouse and rat IgE have a glutamic acid at position 332 with which a salt bridge could be formed. Human IgE does not have this neutralizing negative charge, which may account for the nonreactivity.

In conclusion, we have found that mutagenesis of residues in the α1 domain of FcεRIα at the interface with α2 can significantly reduce the fraction of active receptor molecules. Thus, targeting of this region may be a means to inhibit IgE binding. We have also found that mutagenesis of three contact residues in the IgE binding site in the α2 domain (K117, W130, and Y131) affects human IgE binding as expected, and we show quantitatively that Y131 contributes more to the binding energy of the FcεRIα/IgE complex than W130. We have previously described a peptide derived from the C-C' region of FcεRIα that was able to inhibit both human IgE binding to immobilized sFcεRIα and the sensitization of RBLs with mouse IgE (16). However, this peptide did not include K117, which lies at the N-terminal end of the C strand, nor W130 or Y131, which lie at the C-terminal end of the C' strand. Thus, an extended, C-C'-cyclized peptide incorporating these contact residues is likely to have an elevated affinity for human IgE and could, therefore, be of greater potential therapeutic utility.

Most significantly, however, we have shown that substitutions in human FcεRIα differentially affect binding to human and mouse IgE. The K117D mutation reduces binding to mouse IgE to a far greater degree than to human IgE, and the W130A mutation even enhances binding to mouse IgE, whereas it reduces binding to human IgE. It is particularly striking that the affinity differences

recorded for these mutants (by SPR) are reflected, at least qualitatively, in the results of the cell-based assays; even the 5-fold increase in affinity of the W130A mutant is detectable in the RBL assay. Thus, unless the binding data indicate comparable affinities, assaying soluble human receptor proteins, or receptor-based peptides in a mouse allergy model, may not be a reliable guide to their performance in the human system.

Acknowledgments

We thank Dr. J.-P. Kinet (Harvard Medical School) for providing anti-FcεRIα mAb 15.1 and Prof. F. L. Pearce (University College London, London, U.K.) for use of the histamine autoanalyzer. We also thank Dr. M. Varsani and S. Yue (University College London) for help with the basophil experiments. We also acknowledge the photographic assistance of M. Simon (Randall Centre) and J. Pfeiffer (University of New Mexico, Albuquerque, NM). The facilities of the Division of Cell Pathology, University of New Mexico, are gratefully acknowledged in the preparation of this manuscript.

References

- Sutton, B. J., and H. J. Gould. 1993. The human IgE network. *Nature* 366:421.
- Costa, J. J., P. F. Weller, and S. J. Galli. 1997. The cells of the allergic response: mast cells, basophils, and eosinophils. *J. Am. Med. Assoc.* 278:1815.
- Metzger, H. 1992. Fc receptors. *Annu. Rev. Immunol.* 9:457.
- Kinet, J.-P. 1999. The high-affinity IgE receptor (FcεRI): from physiology to pathology. *Annu. Rev. Immunol.* 17:931.
- Metcalfe, D. D., D. Baram, and Y. A. Mekori. 1997. Mast cells. *Physiol. Rev.* 77:1033.
- Suzuki, K., T. Hirose, H. Matsuda, S. Hasegawa, K. Okumura, and C. Ra. 1998. The Fc receptor (FcR) γ subunit is essential for IgE-binding activity of cell-surface expressed chimeric receptor molecules constructed from human high-affinity IgE receptor (FcεRI) α and FcRγ subunits. *Mol. Immunol.* 35:259.
- Miller, K. L., A.-M. Duchemin, and C. L. Anderson. 1996. A novel role for the Fc receptor γ subunit: enhancement of FcRγ ligand affinity. *J. Exp. Med.* 183:2227.
- Heusser, C., and P. Jardieu. 1997. Therapeutic potential of anti-IgE antibodies. *Curr. Opin. Immunol.* 9:805.
- MacGlashan, D. W., Jr., B. S. Bochner, D. C. Adelman, P. M. Jardieu, A. Togias, J. McKenzie-White, S. A. Sterbinsky, R. G. Hamilton, and L. M. Lichtenstein. 1997. Down-regulation of FcεRI expression on human basophils during in vivo treatment of atopic patients with anti-IgE antibody. *J. Immunol.* 158:1438.
- Sutton, B. J., and H. J. Gould. 1999. Regulation of IgE-mediated inflammation by soluble fragments of the high affinity IgE receptor. In *Immunotherapy in Asthma*. J. Bousquet and H. Yssel, eds. Marcel Dekker, New York, p. 411.
- Hide, M., D. M. Francis, C. E. Grattan, J. Hakimi, J. P. Kochan, and M. W. Greaves. 1993. Autoantibodies against the high-affinity IgE receptor as a cause of histamine release in chronic urticaria. *N. Engl. J. Med.* 328:1599.
- Fiebiger, E., F. Hammerschmidt, G. Stingl, and D. Maurer. 1998. Anti-FcεRIα autoantibodies in autoimmune-mediated disorders: identification of a structure-function relationship. *J. Clin. Invest.* 101:243.
- Ra, C., S. Kuromitsu, T. Hirose, S. Yasuda, K. Furuichi, and K. Okumura. 1993. Soluble human high-affinity receptor for IgE abrogates the IgE-mediated allergic reaction. *Int. Immunol.* 5:47.
- Gavin, A. L., J. Snider, M. D. Hulett, I. F. McKenzie, and P. M. Hogarth. 1995. Expression of recombinant soluble FcεRI: function and tissue distribution studies. *Immunology* 86:392.
- Naito, K., M. Hiram, K. Okumura, and C. Ra. 1995. Soluble form of the human high-affinity receptor for IgE inhibits recurrent allergic reaction in a novel mouse model of type I allergy. *Eur. J. Immunol.* 25:1631.
- McDonnell, J. M., A. J. Beavil, G. A. Mackay, B. A. Jameson, R. Korngold, H. J. Gould, and B. J. Sutton. 1996. Structure based design and characterization of peptides that inhibit IgE binding to its high-affinity receptor. *Nat. Struct. Biol.* 3:419.
- Garman, S. C., B. A. Wurzburg, S. S., Tarchevskaya, J.-P. Kinet, and T. S. Jardetsky. 2000. Structure of the Fc fragment of human IgE bound to its high-affinity receptor FcεRIα. *Nature* 406:259.
- Garman, S. C., J.-P. Kinet, and T. S. Jardetsky. 1998. Crystal structure of the human high-affinity IgE receptor. *Cell* 95:951.
- Hogarth, P. M., M. D. Hulett, F. L. Ierino, B. Tate, M. S. Powell, and R. I. Brinkworth. 1992. Identification of the immunoglobulin binding regions (IBR) of FcγRII and FcεRI. *Immunol. Rev.* 125:21.
- Hulett, M. D., I. F. McKenzie, and P. M. Hogarth. 1993. Chimeric Fc receptors identify immunoglobulin-binding regions in human FcγRII and FcεRI. *Eur. J. Immunol.* 23:640.
- Mallamaci, M. A., R. Chizzonite, M. Griffin, M. Nettleton, J. Hakimi, W. H. Tsien, and J. P. Kochan. 1993. Identification of sites on the human FcεRIα subunit which are involved in binding human and rat IgE. *J. Biol. Chem.* 268:22076.
- Robertson, M. W. 1993. Phage and *Escherichia coli* expression of the human high affinity immunoglobulin E receptor α-subunit ectodomain: domain localization of the IgE-binding site. *J. Biol. Chem.* 268:12736.
- Scarselli, E., G. Esposito, and C. Traboni. 1993. Receptor phage: display of functional domains of the human high affinity IgE receptor on the M13 phage surface. *FEBS Lett.* 329:223.
- Vangelista, L., S. Laffer, R. Turek, H. Gronlund, W. R. Sperr, P. Valent, A. Pastore, and R. Valenta. 1999. The immunoglobulin-like modules Cε3 and α2 are the minimal units necessary for human IgE-FcεRI interaction. *J. Clin. Invest.* 103:1571.
- Maxwell, K. F., M. S. Powell, M. D. Hulett, P. A. Barton, I. F. McKenzie, T. P. Garrett, and P. M. Hogarth. 1999. Crystal structure of the human leukocyte Fc receptor, FcγRIIa. *Nat. Struct. Biol.* 6:437.
- Sondermann, P., R. Huber, and U. Jacob. 1999. Crystal structure of the soluble form of the human FcγRIIb: a new member of the immunoglobulin superfamily at 1.7 Å resolution. *EMBO J.* 18:1095.
- Zhang, Y., C. C. Boesen, S. Radaev, A. G. Brooks, W.-H. Fridman, C. Sautes-Fridman, and P. D. Sun. 2000. Crystal structure of the extracellular domain of human FcγRIII. *Immunity* 13:387.
- Danho, W., R. Makofse, J. Swistok, M. Mallamaci, M. Nettleton, V. Madison, D. Greeley, D. Fry, and J. Kochan. 1995. High affinity IgE-receptor α-subunit-derived peptides as antagonists of human IgE binding. In *The Ninth International Congress of Immunology*, July 23–29. San Francisco.
- Cook, J. P. D., A. J. Henry, J. M. McDonnell, R. J. Owens, B. J. Sutton, and H. J. Gould. 1997. Identification of contact residues in the IgE binding site of human FcεRIα. *Biochemistry* 36:15579.
- Hulett, M. D., R. I. Brinkworth, I. F. C. McKenzie, and P. M. Hogarth. 1999. Fine structure analysis of the interaction of FcεRI with IgE. *J. Biol. Chem.* 274:13345.
- McDonnell, J. M., A. J. Beavil, G. A. Mackay, A. J. Henry, J. P. Cook, H. J. Gould, and B. J. Sutton. 1997. Structure-based design of peptides that inhibit IgE binding to its high-affinity receptor FcεRI. *Biochem. Soc. Trans.* 25:387.
- Rigby, L. J., V. C. Epa, G. A. Mackay, M. D. Hulett, B. J. Sutton, H. J. Gould, and P. M. Hogarth. 2000. Domain one of the high affinity IgE receptor, FcεRI, regulates binding to IgE through its interface with domain two. *J. Biol. Chem.* 275:9664.
- Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 77:51.
- Cregg, J. M., T. S. Vedvick, and W. C. Raschke. 1993. Recent advances in the expression of foreign genes in *Pichia pastoris*. *BioTechnology* 11:905.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680.
- Bruggemann, M., G. T. Williams, C. I. Bindon, M. R. Clark, M. R. Walker, R. Jefferis, H. Waldmann, and M. S. Neuberger. 1987. Comparison of the effector functions of human immunoglobulins using a matched set of chimeric antibodies. *J. Exp. Med.* 166:1351.
- Shi, J., R. Ghirlando, R. L. Beavil, A. J. Beavil, M. B. Keown, R. J. Young, R. J. Owens, B. J. Sutton, and H. J. Gould. 1997. Interaction of the low-affinity receptor CD23/FcεRII lectin domain with the Fcε3–4 fragment of human immunoglobulin E. *Biochemistry* 36:2112.
- Henry, A. J., J. P. D. Cook, J. M. McDonnell, G. A. Mackay, J. Shi, B. J. Sutton, and H. J. Gould. 1997. Participation of the N-terminal region of Cε3 in the binding of human IgE to its high-affinity receptor FcεRI. *Biochemistry* 36:15568.
- MacDonald, S. M., L. M. Lichtenstein, D. Proud, M. Plaut, R. M. Naclerio, D. W. MacGlashan, Jr., and A. Kagey-Sobotka. 1987. Studies of IgE-dependent histamine releasing factors: heterogeneity of IgE. *J. Immunol.* 139:506.
- Gueix, N., and Peitsch, M. C. 1997. SWISS-MODEL and the Swiss-Pdb Viewer: an environment for comparative protein modeling. *Electrophoresis* 18:2714.
- Sechi, S., P. P. Roller, J. Willette-Brown, and J.-P. Kinet. 1996. A conformational rearrangement upon binding of IgE to its high affinity receptor. *J. Biol. Chem.* 271:19256.
- Blank, U., C. S. Ra, and J.-P. Kinet. 1991. Characterization of truncated α chain products from human, rat, and mouse high affinity receptor for IgE. *J. Biol. Chem.* 266:2639.
- Letourneur, O., S. Sechi, J. Willette-Brown, M. W. Robertson, and J.-P. Kinet. 1995. Glycosylation of human truncated FcεRI α chain is necessary for efficient folding in the endoplasmic reticulum. *J. Biol. Chem.* 270:8249.
- Ra, C., M.-H. E. Jouvin, and J.-P. Kinet. 1989. Complete structure and expression of the mouse mast cell receptor for IgE (FcεRI) and surface expression of chimeric receptors (rat-mouse-human) on transfected cells. *J. Biol. Chem.* 264:15323.
- Yagi, S., M. Yanagida, I. Tanida, A. Hasegawa, K. Okumura, and C. Ra. 1994. High-level expression of the truncated α chain of human high-affinity receptor for IgE as a soluble form by baculovirus-infected insect cells: biochemical characterization of the recombinant product. *Eur. J. Biochem.* 220:593.
- Wines, B. D., M. D. Hulett, G. P. Jamieson, H. M. Trist, J. M. Spratt, and P. M. Hogarth. 1999. Identification of residues in the first domain of human Fcα receptor essential for interaction with IgA. *J. Immunol.* 162:2146.
- Helm, B. A., A. C. Spivey, and E. A. Padlan. 1997. Peptide blocking of IgE/receptor interaction: possibilities and pitfalls. *Allergy* 52:1155.
- Rigby, L. J., H. Trist, M. D. Hulett, P. M. Hogarth, L. J. Rigby, and V. C. Epa. 2000. Monoclonal antibodies and synthetic peptides define the active site of FcεRI and a potential receptor antagonist. *Allergy* 55:609.
- Padlan, E. A., and B. A. Helm. 1992. A modeling study of the α-subunit of human high-affinity receptor for immunoglobulin-E. *Receptor* 2:129.
- Kulczycki, A., I. Sersky, C., and Metzger, H. 1974. The interaction of IgE with rat basophilic leukemia cells. I. Evidence for specific binding of IgE. *J. Exp. Med.* 139:600.
- Sterk, A. R., and Ishizaka, T. 1982. Binding properties of IgE receptors on normal mouse mast cells. *J. Immunol.* 128:838.