

Convergent Recognition of the IgE Binding Site on the High-Affinity IgE Receptor

Jennifer Stamos,¹ Charles Eigenbrot,¹
Gerald R. Nakamura,¹ Mark E. Reynolds,²
JianPing Yin,¹ Henry B. Lowman,¹
Wayne J. Fairbrother,¹ and Melissa A. Starovasnik^{1,*}

¹Department of Protein Engineering

²Department of Medicinal Chemistry

Genentech, Inc.

One DNA Way

South San Francisco, California 94080

Summary

Two structurally distinct classes of peptides were recently identified by phage display that bind the high-affinity IgE receptor, FcεRI, and block IgE binding and subsequent receptor activation. Both classes adopt highly stable structures in solution, one forming a β hairpin, with the other forming a helical “zeta” structure. Despite these differences, the two classes bind competitively to the same site on the receptor. Structural analyses of both peptide-receptor complexes by NMR spectroscopy and/or X-ray crystallography reveal that the unrelated peptide scaffolds have nevertheless converged to present a similar three-dimensional surface to interact with FcεRI and that their modes of interaction share a key feature of the IgE-FcεRI complex, the proline/tryptophan sandwich.

Introduction

Molecules that prevent IgE from binding its high-affinity receptor, FcεRI, have therapeutic potential in the treatment of asthma and allergic disease. The anti-IgE antibody Xolair (Omalizumab), recently approved for the treatment of moderate-to-severe persistent asthma, binds IgE and prevents IgE from binding to its receptors, thereby blocking IgE-mediated signaling on mast cells and basophils (Babu et al., 2001; Owen, 2002). Recently, two classes of peptides have been described that bind the high-affinity IgE receptor (Nakamura et al., 2001, 2002). These peptides were identified by sorting naive peptide phage display libraries for novel agents that would bind the extracellular portion of the α chain of FcεRI (FcεRIα). Although sorted only for binding the receptor, these peptides were also found to block IgE from binding and prevent downstream IgE-mediated signaling events in basophils. The two classes are unrelated in primary sequence and adopt very different, but stable, tertiary structures in solution (Figure 1). Structural analysis of how these peptides bind the high-affinity IgE receptor will be useful in the development of novel IgE antagonists.

The first family of phage-derived peptides described that bind the high-affinity IgE receptor were selected from a naive peptide library of the general form

X₄CX₂GPX₄CX₄ (Nakamura et al., 2001). IgE receptor binding peptides of this class adopt an extended β hairpin conformation with a type I β turn centered at residues 9 and 10 (Figure 1). Key positions required for structure and function were identified from analysis of peptide analogs both by NMR and activity measurements. By far, the most important residue for high-affinity receptor binding was shown to be Pro9 (Figure 1C), despite the fact that having proline at this position is not important for the structure and stability of the peptide.

The second family of peptides described that bind the IgE receptor was selected initially from a naive peptide library of the form X₂CX₃CX₂ (Nakamura et al., 2002). Monomeric 9-mer peptides with the selected sequence motif X₂CPX₂CYX had weak affinity at best; however, one peptide was found to undergo spontaneous conversion to a higher affinity disulfide-linked antiparallel dimer. Subsequent introduction of a 3 residue “linker” sequence to make a 21 residue “single-chain dimer” form of the peptide and further optimization on phage resulted in a nanomolar peptide inhibitor. Peptides of this second class adopt a stable structure composed of two small antiparallel 3₁₀ helices connected by two disulfide bonds (Figure 1B). The backbone structure is nearly symmetric, with residues 1–9 adopting essentially the same structure, resembling the Greek letter zeta (ζ), as residues 13–21, connected by a flexible linker, hence the designation “zeta peptides” based on their three-dimensional structure. As for the hairpins, key positions in the zeta peptide required for structure and function were identified from analysis of peptide analogs both by NMR and activity measurements. Again, the most important residue for high-affinity receptor binding was shown to be a proline, in this case Pro16 (Figure 1B).

The structure of FcεRIα is comprised of two Ig-like domains, D1 and D2 (Garman et al., 1998, 2001). The crystal structure of the Fc portion of IgE in complex with FcεRIα shows an extensive interface involving interactions of both Cε3 domains of IgE with different parts of the receptor surface (Garman et al., 2000); one Cε3 domain binds receptor residues fully contained within D2 (site 1), while the other interacts with residues at the D1-D2 junction (site 2). Interestingly, the site 2 interaction is centered around a proline residue from IgE, Pro426, which is buried between two tryptophan residues on the receptor. FcεRIα undergoes little change in conformation upon IgE binding. The peptides could interfere with IgE binding to its receptor either by direct competition, binding within (or overlapping) one or both IgE binding sites on FcεRI, or through an allosteric mechanism.

Herein, we investigate the molecular mechanism by which peptides of the hairpin and zeta classes bind the high-affinity IgE receptor. Structural analyses of both peptide-receptor complexes show that they indeed bind within IgE binding site 2. Not only do the peptides compete with IgE, but also with one another, utilizing a common interaction motif. Thus, although there is no similarity in sequence or secondary structure between the two

*Correspondence: star@gene.com

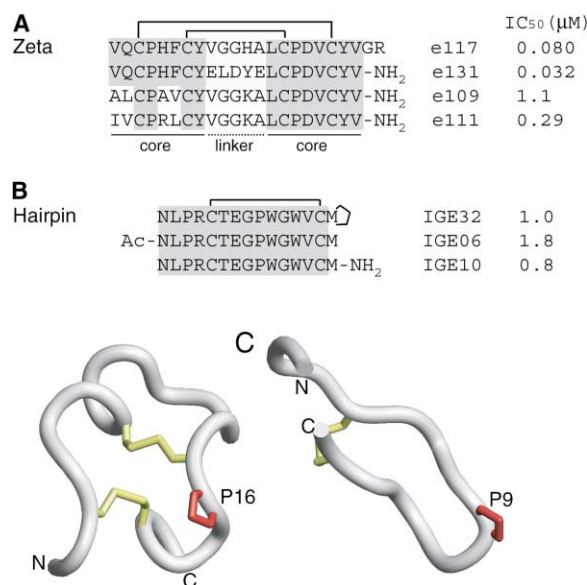


Figure 1. Sequences and Structures of Zeta and Hairpin Peptides Used in This Study

(A) Primary sequences and IgE-receptor binding affinities of the peptides used for structure and function studies. Note that the sequences of the two zeta peptides used for NMR (e117) and X-ray (e131) structural analyses differ only in the flexible “linker” and C-terminal regions; the 3D structures of the core regions of the two peptides are indistinguishable (Nakamura et al., 2002). The IC₅₀ values are from cell-based binding inhibition assays measuring ¹²⁵I-IgE binding to CHO-3D10 cells that express the human FcεRIα subunit as reported previously (Nakamura et al., 2001, 2002).

(B) Solution structure of e131 showing the functionally important proline, Pro16, in red (PDB 1KCO [Nakamura et al., 2002]).

(C) Solution structure of IGE06 showing the functionally important proline, Pro9, in red (PDB code 1JBF [Nakamura et al., 2001]).

peptide classes, they have nevertheless converged to present a similar three-dimensional interaction surface for receptor binding that partially mimics the natural ligand IgE.

Results

Competition between Zeta and Hairpin Peptides for FcεRI Binding

In order to test whether peptides from the two classes would compete with one another for receptor binding, a zeta peptide was examined for whether it could block phage displaying a representative hairpin peptide from binding immobilized FcεRI. Figure 2A shows that not only does a hairpin peptide compete with hairpin-phage for FcεRI binding as expected, but also zeta peptide e109 competes with hairpin-phage for FcεRI binding with an IC₅₀ of 1.4 μM, similar to that measured previously for blocking IgE binding to FcεRI expressed on a cell surface (IC₅₀ = 1.1 μM [Nakamura et al., 2002]). To further test whether the peptides themselves, in the absence of phage, are sufficient to compete with one another for receptor binding, representative peptides with similar affinities were chosen from each structural class; hairpin peptide IGE10 (Mr = 1745 kDa) and zeta peptide e111 (Mr = 2265 kDa) have been shown to bind

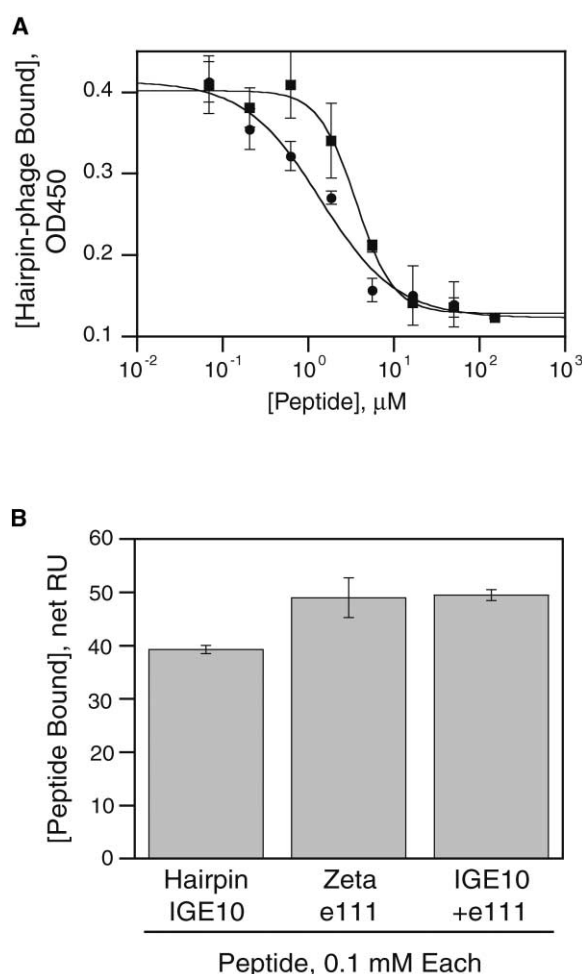


Figure 2. Hairpin and Zeta Peptides Compete for Binding to FcεRIα

(A) Competitive displacement of phage displaying a hairpin peptide from binding immobilized FcεRIα-Fc measured by ELISA (see Experimental Procedures). Data are shown for hairpin peptide IGE06 (squares) and zeta peptide e109 (circles). IC₅₀ values from the fitted curves are 3.6 and 1.4 μM, respectively.

(B) Competitive binding of peptides in a SPR assay. Individual peptides were first injected at a predetermined saturating concentration for comparison with a mixture of the two peptides at equal concentration. The relative molar amounts (RMA) of peptides bound can be compared by normalizing the SPR signal (RU) by the respective molecular weights of the peptides: RMA(e111) = 0.0216 versus RMA(IGE10) = 0.0223.

receptor with affinities of 0.8 and 0.3 μM, respectively (Nakamura et al., 2001, 2002). In surface plasmon resonance (SPR) binding experiments using saturating peptide concentrations, a similar molar amount of each peptide, based on normalized response units, was found to bind to immobilized FcεRIα. A mixture of the two peptides, each at saturating concentrations, did not yield significantly higher signal than the zeta peptide alone, indicating that the two classes of peptide compete for overlapping sites on the receptor (Figure 2B).

Receptor Mutagenesis Localizes the Zeta Peptide Binding Site

Alanine mutants of FcεRIα were expressed on phage and tested for binding to zeta peptide e131. The assay

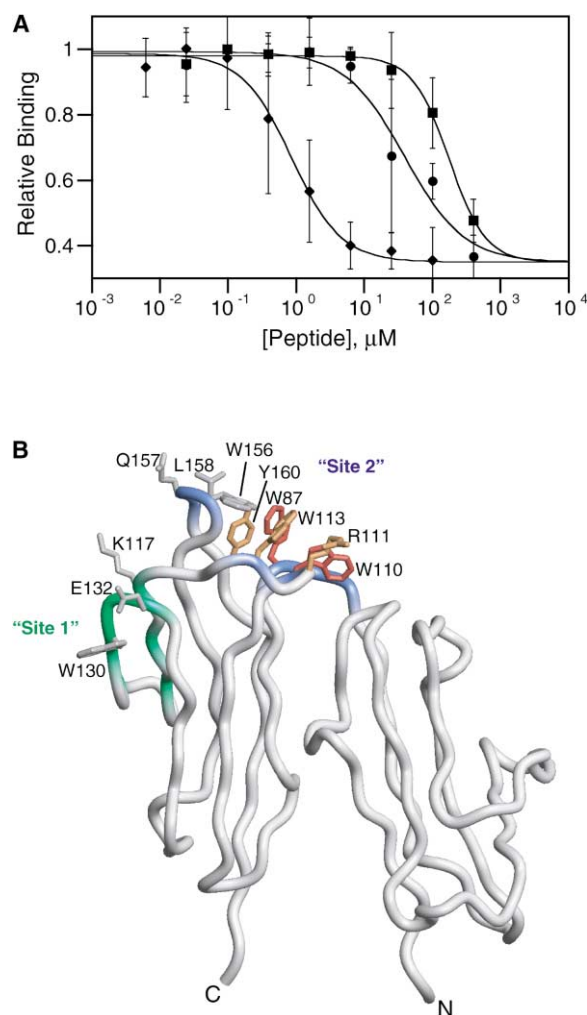


Figure 3. Alanine Mutation of FcεRIα Localizes the Peptide Binding Region

(A) ELISA performed as described in the Experimental Procedures. Data for the wild-type receptor (diamonds) and the two mutants W87A (squares) and W110A (circles) are shown with IC₅₀ values of 0.86, 180, and 39 μM, respectively.

(B) Ribbon diagram of FcεRIα from the structure of the IgE–receptor complex (PDB code 1F6A [Garman et al., 2000]). The backbone of receptor residues involved in IgE binding Site 1 is colored green, while that outlining Site 2 is colored blue. Side chains are shown only for those residues that were tested as individual alanine mutants. Residues that show a >6- or >40-fold decrease in peptide binding when substituted by alanine are colored orange or red, respectively.

used for evaluating the effects of these mutations requires the mutant receptor to be capable of binding IgE; the ability of the peptide to inhibit this binding is then measured to establish the extent to which the receptor mutation affects peptide binding. Substitution of either Trp87 or Trp110 by alanine was found to reduce zeta peptide binding to FcεRI by 210- and 45-fold, respectively (Figure 3A). Individual mutation of Arg111, Trp113, or Tyr160 to alanine also showed a significant decrease in binding (13-, 7-, and 25-fold, respectively; data not shown). However, alanine mutation of Lys117, Trp130, Leu132, Trp156, Gln157, and Leu158 resulted in only a small or insignificant change in the ability of the peptide

to interact with the receptor. Thus, the mutations that disrupt peptide binding map to the tryptophan patch in IgE binding Site 2 (Figure 3B).

NMR Analysis of a Zeta Peptide-Receptor Complex

¹³C/¹⁵N-labeled zeta peptide was obtained by expression of an e117/Z-domain fusion protein in *E. coli* followed by trypsin cleavage and purification of the 23 residue zeta peptide (Nakamura et al., 2002). This peptide was shown previously to bind FcεRI and block IgE binding (80 nM IC₅₀ [Nakamura et al., 2002]). Complete resonance assignments were obtained for the free peptide, but assignment of the FcεRI-bound peptide was complicated by severe line-broadening of peaks from residues buried at the protein-peptide interface. Nevertheless, unambiguous assignments could be obtained for a majority of the peptide resonances based on analysis of a 3D ¹H/¹H/¹³C-edited NOESY (Table 1).

A large variation in linewidths was observed for the peptide resonances in the receptor-bound complex, with those from residues at the termini and in the GGH “linker” being sharper and significantly more intense than those from the rest of the peptide. These sharper peaks also underwent minimal changes in chemical shift, consistent with this group of residues being of minimal importance for receptor binding (as determined from previous analysis of peptide analogs [Nakamura et al., 2002]). In contrast, significant chemical shift perturbations were observed for many of the peaks from residues contained within the “disulfide-bonded core” of the peptide; the most dramatic changes in chemical shift upon receptor binding are seen for the ¹H resonances of Pro16 (Hα, Hβ, Hγ, and Hδ all shift upfield 1.7–3.1 ppm; Table 1). Shifts of this magnitude experienced by all protons within a proline ring can only be explained if the proline lies packed between two aromatic rings in the protein-peptide complex (Case, 1995). Coincidentally, in the crystal structure of the Fc fragment of IgE in complex with FcεRIα, Pro426 of IgE is found “sandwiched” between two tryptophans, Trp87 and Trp110, on the receptor (Garman et al., 2000).

Inspection of a ¹³C-edited, ¹²C-filtered NOESY spectrum showed intermolecular NOEs between isotopically labeled peptide and unlabeled protein. Thirty-eight intermolecular NOEs were assigned involving protons from residues Val1, Gln2, Pro4, Leu14, and Val18 on the peptide. Peptide residues Val1, Leu14, and Val18 showed NOE crosspeaks to at least two distinct aromatic groups on the receptor. Furthermore, peptide residues Val1, Gln2, and Pro4 showed NOEs to a pair of peaks resonating at 0.75 and 0.73 ppm, which were assigned to a pair of methyl groups from the receptor based on crosspeak intensity and resonance frequency. Receptor contacts involving aromatic and methyl-containing side chains are consistent with binding near IgE binding site 2 as suggested by the mutagenesis data discussed above. The only methyl groups in the vicinity of site 2 on the receptor are those of Leu158, allowing tentative assignment of the intermolecular methyl NOEs mentioned above. The pattern of intramolecular peptide NOEs observed indicates that the zeta peptide does not undergo

Table 1. Resonance Assignments for Free and Receptor-Bound Zeta Peptide e117

Residue	H^N , ^{15}N	H^α , $^{13}C^\alpha$	H^β , $^{13}C^\beta$	H^γ , $^{13}C^\gamma$	H^δ , $^{13}C^\delta$
1 Val		3.79; 61.1 3.94 ; 60.7	2.10; 32.7 2.31 ; 33.1	0.90; 20.3 0.92; 19.9 1.15; 20.1 1.24 ; 20.6	
2 Gln	8.61; 124.3 8.75; 125.0	4.41; 55.7 4.55; 55.3	1.94, 2.01; 29.5 1.97*; 29.5	2.30*; 33.7 2.27, 2.33; 33.6	ϵ : 6.77, 7.43; 112.1 6.79, 7.39; 111.9
3 Cys	8.38; 121.8 8.89 ; 122.2	4.88; 53.2 4.33 ; 54.5^{tent}	3.10*; 40.7 na		
4 Pro		4.33; 62.4 4.35; 62.5	1.28, 2.24; 32.7 1.22, 2.21; 32.6	1.69, 1.83; 27.8 1.71, 1.93; 27.5	3.33, 3.57; 50.6 na
5 His	8.69; 118.7 8.78; 120.4	4.40; 59.4 4.44; 59.5	3.29, 3.37; 28.3 3.33, 3.42; 28.2		
6 Phe	7.84; 113.9 8.02 ; 114.5	4.34; 59.0 4.28; 59.3	2.97, 3.27; 37.1 2.81 , 3.19; 36.4		
7 Cys	7.50; 117.8 7.23 ; 118.1	4.19; 55.0 4.25; 54.9	1.86, 2.10; 36.2 na		
8 Tyr	7.91; 118.2 8.00; 118.9	4.85; 57.6 4.87; 57.8	2.54, 3.46; 38.6 2.60, 3.53; 38.5		
9 Val	7.09; 117.0 7.10; 118.3	4.32; 61.6 4.33; 61.7	2.13; 33.2 2.13; 33.2	0.87; 19.8, 0.91; 21.2 0.87; 20.1, 0.93; 21.2	
10 Gly	8.47; 111.7 8.52; 112.1	3.89, 4.07; 45.6 3.92, 4.11; 45.4			
11 Gly	8.31; 109.0 8.27; 108.7	3.84, 3.89; 45.5 3.88*; 45.5			
12 His	8.00; 117.2 8.01; 117.2	4.61; 54.8 4.60; 54.4	3.07, 3.19; 29.4 3.02*; 29.2		
13 Ala	8.29; 124.5 8.36; 124.7	4.28; 52.5 4.31; 52.2	1.24; 18.8 1.13; 18.2		
14 Leu	8.17; 120.5 8.27; 119.7	4.38; 55.5 4.40; 56.4	1.66, 1.71; 42.6 1.82, 1.94; 43.9	1.62; 27.0 1.84 ; 27.2	0.83; 23.4, 0.89; 25.2 0.98; 23.8, 1.06; 24.8
15 Cys	7.80; 118.5 7.66 ; 116.7	4.87; 53.8 5.10 ; 53.8^{tent}	3.21*; 40.9 na		
16 Pro		4.43; 63.2 2.66 ; 62.1	1.85, 2.45; 32.7 0.17 , 0.43 ; 30.9	2.00, 2.05; 27.9 -1.09 , -0.66 ; 24.8	3.56, 3.78; 50.6 1.07 , 1.87 ; 48.7
17 Asp	8.50; 121.5 na	4.43; 57.9 4.24 ; 57.8	2.69, 2.74; 40.1 2.76*; 40.6		
18 Val	8.08; 117.1 na	4.05; 63.7 4.08; 63.0	2.21; 31.3 2.35; 31.1	0.91; 20.1, 0.91; 20.5 0.88; 20.0, 1.05; 20.8	
19 Cys	7.99; 115.5 7.50 ; 115.7	4.49; 54.7 4.46; 54.8	2.10, 2.28; 36.2 2.22, na; 35.9		
20 Tyr	8.04; 119.0 7.84 ; 118.9	4.84; 57.7 4.96; 57.1	2.76, 3.39; 38.9 2.79, 3.40; 38.9		
21 Val	7.36; 119.0 7.33; 119.0	4.15; 62.7 4.18; 62.6	2.10; 32.7 2.13; 32.7	0.97; 21.0, 1.01; 20.7 1.00; 21.0, 1.04; 20.6	
22 Gly	8.61; 113.4 8.68; 113.8	3.90, 3.95; 45.6 3.93, 3.98; 45.5			
23 Arg	7.65; 125.1 7.66 ; 125.2	4.19; 57.0 4.23; 56.9	1.70, 1.88; 31.5 1.73, 1.92; 31.5	1.54*; 27.2 1.58*; 27.3	3.12*; 43.5 3.17*; 43.4

Upper values are for the free peptide; lower values represent the FcεRIα-bound peptide; values shown in bold are those that differ by ≥ 0.15 ppm (^1H), 0.6 ppm (^{13}C), or 1.5 ppm (^{15}N) between free and bound states. Asterisks indicate degenerate methylene protons. na indicates that the peak is broadened sufficiently that the resonance cannot be assigned unambiguously. ^{tent} indicates that the nuclei were assigned by amino acid type, but the sequence-specific assignment is tentative, based on calculated chemical shifts in the complex; hence, the $^{13}C_\alpha$ / $^1H_\alpha$ shifts for Cys3 and Cys15 represent the best assignment based on the available data, but are not assigned conclusively.

a significant change in conformation upon receptor binding. Taken together, these data suggest a qualitative model for the zeta peptide-receptor complex, with Pro16 packing between Trp87 and Trp110, and the N-terminal portion of the peptide contacting Leu158.

In order to obtain a more quantitative description of the complex, a model of the peptide based on the free structure (derived from the structure of PDB code 1KCN [Nakamura et al., 2002]) was docked onto the receptor such that the proline face of the peptide would approach the tryptophan patch in site 2. The docked model was refined based on optimizing the agreement between the calculated and observed ^1H chemical shifts of the

peptide in the bound state (see Experimental Procedures). A remarkable correspondence between the calculated and observed ^1H shifts (Figure 4A) is seen for the model of the zeta peptide-receptor complex shown in Figure 4B, suggesting that this model represents a good approximation of the peptide-receptor complex structure.

Crystal Structure of a Zeta Peptide-Receptor Complex

Attempts to obtain suitable crystals of peptide-receptor complex using the same peptide, e117, used for NMR studies described above were unsuccessful. However,

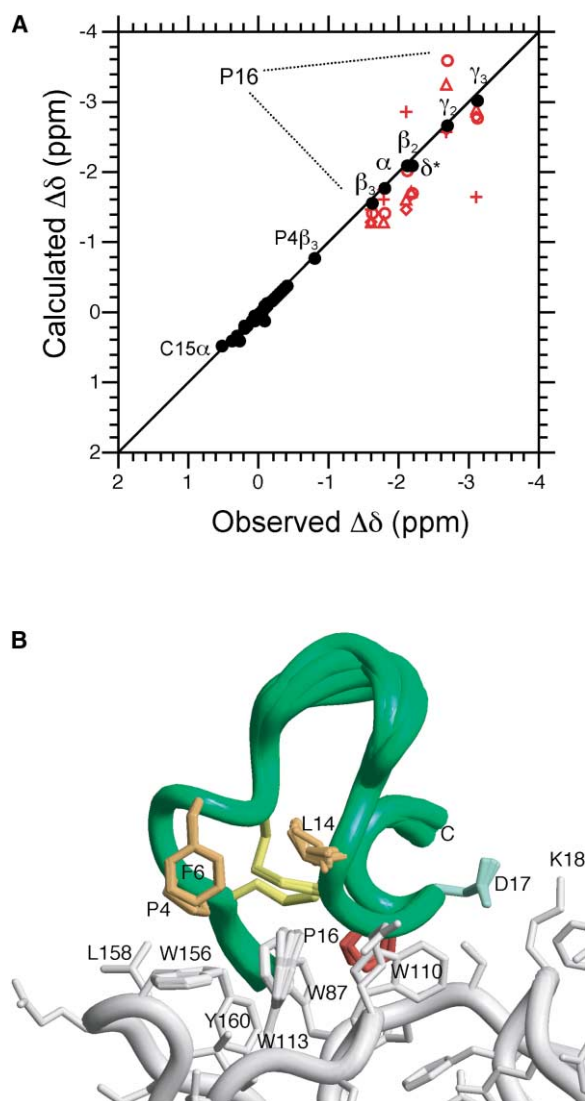


Figure 4. NMR Analysis of e117 Zeta Peptide-Receptor Complex
(A) Agreement between calculated and observed ^1H chemical shifts in the complex (black circles; rms error = 0.04 ppm, $R = 0.9989$). Also plotted are the ^1H chemical shifts calculated for Pro16 from the coordinates of the e131-Fc ϵ RI α crystal structure (red symbols, which differ for each of the four complexes in the asymmetric unit; see text).
(B) Superposition of 20 models of zeta peptide-receptor complex derived from docking and refinement based on ^1H chemical shifts (average rms error = 0.041 ± 0.005 ppm).

the complex of zeta peptide e131 and Fc ϵ RI α (32 nM IC_{50} [Nakamura et al., 2002]) was crystallized with four complexes in the asymmetric unit and diffracted to 3.0 Å resolution (Table 2). The structure was solved by molecular replacement with the coordinates for the free receptor (1F2Q [Garman et al., 1998]) (see Experimental Procedures); clear electron density for the bound peptide was visible after rigid body refinement (Figure 5A). The zeta peptide indeed binds the high-affinity IgE receptor in IgE binding site 2 with Pro16 packed between Trp87 and Trp110 of the receptor (Figure 5). Further contacts

are made by peptide residues Pro4 and Phe6, which lie in a shallow pocket lined by Trp87, Trp113, Trp156, Leu158, and Tyr160 of the receptor (Figure 5C). Peptide and protein bury ~ 430 Å ^{2} each in the interface, which is predominantly hydrophobic in nature. A potential salt bridge is formed between Asp17 on the peptide and Lys18 on the protein, likely accounting for the importance of a negative charge in this vicinity as seen from mutational analysis of e131 (Nakamura et al., 2002).

An overlay of the crystal structure and the NMR-based model for the zeta peptide-receptor complexes, based on superposing only the receptor coordinates, shows that both approaches identified equivalent modes of binding to site 2 for the two related zeta peptides e117 and e131 (Figure 5C; and see Figure 1A). Significant differences are seen only in the conformations of the linker and terminal regions of the peptides, which is not surprising given that these regions differ in primary sequence in the two complexes, were found to be flexible in solution, and participate in crystal packing contacts (see Experimental Procedures). Notably, the correspondence between the calculated shifts for Pro16 of e131 based on the crystallographically defined coordinates and the observed shifts for Pro16 of e117 in the complex in solution is good, with all proton resonances being predicted to be shifted upfield from random coil chemical shift values by >1 ppm (Figure 4A); the differences in the exact values calculated from the different complex structures highlight the exquisite sensitivity of chemical shift and their dependence on precise distance and orientation with respect to aromatic rings. Thus, the nearly rigid-body docking procedure based on ^1H chemical shifts is valid for this complex, since neither component undergoes significant conformational change upon binding.

NMR Analysis of a Hairpin Peptide-Receptor Complex

$^{13}\text{C}/^{15}\text{N}$ -labeled IGE32 hairpin peptide was produced similarly to the zeta peptide as a fusion protein secreted from *E. coli*, except that CNBr cleavage was used to remove the fusion partner since the peptide contained a single methionine residue at the C terminus. Previous studies showed that the C-terminal residue could be modified to other hydrophobic residues without loss of binding (Nakamura et al., 2001), and thus, as expected, IGE32, ending in a lactone following cleavage, shows binding affinity comparable to IGE06 (Nakamura et al., 2001) (~ 1 μM IC_{50} ; Figure 1A). Resonance assignments were obtained for the free and Fc ϵ RI α -bound peptide (Table 3). Striking changes in ^1H chemical shift values were observed for resonances of Pro9 and Thr6, which shift upfield by 1.7–3.2 ppm upon binding the receptor. As discussed above for the zeta peptide, these extreme changes in chemical shift can only be explained if these nuclei pack closely against aromatic rings in the complex.

The pattern of intramolecular NOEs observed indicates that the peptide maintains its hairpin structure when bound to the receptor. A total of 40 intermolecular NOEs were assigned involving resonances from peptide residues Asn1, Leu2, Pro3, Thr6, Val13, and Met15, including NOEs between peptide residues Val13 and

Table 2. Data Reduction and Refinement Statistics for e131-FcεRI Complex

Resolution (Å)	N _{meas} ^a	N _{ref} ^b	Complete ^c	I/σ	R _{merge} ^d	R _{work} ^e	R _{free} ^f
30.0–6.44	16370	3151	100.0	26	0.031	0.31	0.43
6.44–5.12	16879	3143	100.0	26	0.043	0.26	0.28
5.12–4.48	16715	3098	98.0	26	0.049	0.25	0.32
4.48–4.07	16578	3109	98.4	23	0.059	0.26	0.32
4.07–3.78	15543	3078	97.5	16	0.091	0.28	0.32
3.78–3.56	14518	3087	97.8	11	0.150	0.30	0.36
3.56–3.38	13692	3050	96.6	6.2	0.228	0.33	0.35
3.38–3.23	13026	3064	97.0	4.0	0.343	0.35	0.36
3.23–3.11	12732	3049	96.5	2.8	0.499	0.37	0.41
3.11–3.00	12550	2644	83.7	2.0	0.686	0.39	0.44
30.0–3.00	148963	30473	96.5	7.4	0.068	0.29	0.35
Refinement resolution (Å)	30.0–3.0						
Number of reflections	30473						
R value	29.5%						
R _{free}	35.0%						
R value (all)	30.6%						
Rmsd bond distances (Å)	0.008						
Rmsd bond angles (°)	1.4						
Rmsd dihedral angles (°)	26						
Rmsd improper dihedral angles (°)	1.0						
Number of atoms	6744						
Number of atoms occupancy zero	156						
Number of amino acid residues	746						
Number of sugar residues	46						
Number of waters	0						
Number of sulfate ions	2						
Number of citrate ions	2						
Rmsd B factors (Å ²)							
Bond	2.5						
Angle	4.4						
Rmsds versus reference receptor (Å) (NCS)	0.01, 0.01, 0.01						
Rmsds versus reference receptor (Å) (non-NCS)	1.1, 1.6, 2.1						
Rmsds versus reference e131 (Å) (non-NCS)	1.1, 1.6, 1.6						

^aN_{meas} is the total number of observations measured.^bN_{ref} is the number of unique reflections measured at least once.^cComplete is the percentage of possible reflections actually measured at least once.^dR_{merge} = $\sum ||I| - \langle I \rangle| / \sum \langle I \rangle$, where I is the intensity of a single observation and $\langle I \rangle$ the average intensity for symmetry equivalent observations.^eR_{work} = $\sum |F_o - F_c| / \sum |F_o|$, where F_o and F_c are observed and calculated structure factor amplitudes, respectively.^fR_{free} = R_{work} for 968 reflections sequestered from refinement, selected at random.

Met15 and a pair of methyl groups from the protein. As before, these were tentatively assigned to Leu158 of the receptor, giving an orientation of peptide on protein such that Pro9 would pack between Trp87 and Trp110, while the ends of the hairpin would lie near Leu158. A docking protocol similar to that used for the zeta complex was employed to orient the hairpin peptide onto site 2 of the receptor (see Experimental Procedures). Refinement of this model based on optimizing the agreement between calculated and observed ¹H chemical shifts of the peptide resulted in the model shown in Figure 6. The excellent correspondence between the calculated and observed chemical shifts indicates that this model represents a close approximation of the structure of the hairpin-receptor complex (Figure 6A). Indeed, as was found for the zeta peptide complex, a proline on the peptide, Pro9, is sandwiched by Trp87 and Trp110 of the receptor. Thr6 and Val13 lie in the adjacent shallow pocket, which is capped by additional hydrophobic interactions provided by Leu2 of the peptide.

Discussion

Peptides Utilize a Proline Sandwich as Typified by the IgE-FcεRI Complex

Herein we have shown that FcεRI binding peptides of the zeta and hairpin classes compete with one another for receptor binding. Mutagenesis experiments map the peptide binding site to the tryptophan patch in IgE binding site 2. Previous analysis of peptide variants showed that the most important residue for receptor binding by both classes was a proline, Pro16 and Pro9 for zeta (Nakamura et al., 2002) and hairpin (Nakamura et al., 2001) peptides, respectively. Substitutions of these residues by alanine results in complete loss of detectable binding, despite the fact that the structures of the peptides were not compromised by this modification. NMR analysis of the receptor-bound peptides shows that the important proline residues from each peptide class experience remarkably large chemical shift perturbations upon receptor binding, indicating that in both peptide-receptor complexes a proline ring packs against aro-

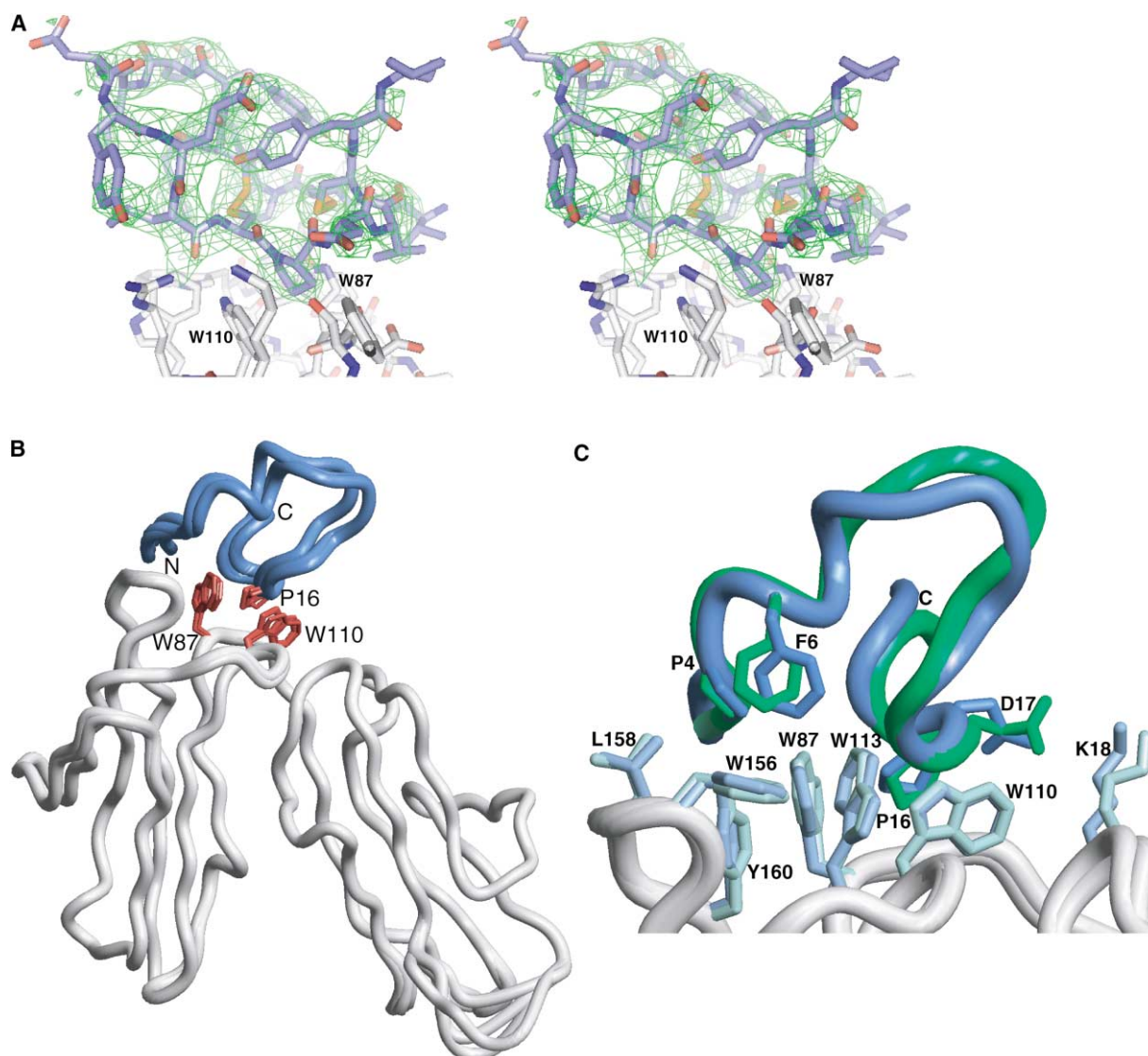


Figure 5. Crystal Structure of e131 Zeta Peptide-Receptor Complex

(A) Stereo view of the Σ_a -weighted, simulated annealing omit map ($2mF_o - DF_o$) electron density within 2 Å of a peptide atom, contoured at 1.0 rmsd (green). To create this map, all four zeta peptides in the asymmetric unit were omitted during annealing and phase calculation. Carbon atoms of the peptide (chain W) are blue, and carbon atoms of the receptor (chain A) are white. Note that Pro16 from the peptide lies between side chains of two tryptophan residues on the receptor (Trp87 and Trp 110).

(B) Overlay of the four peptide-receptor complexes in the asymmetric unit superimposed on the backbone coordinates of each receptor.

(C) Overlay of representative models of the zeta e131 and e117 peptide-receptor complexes evaluated by crystallography (blue) and NMR (green), respectively. The structures were superimposed based only on receptor coordinates. The receptor backbone ribbon is colored white for both the crystallography and NMR models. Selected side chains from the peptide and receptor that are mentioned in the text are labeled.

matic rings. NMR and crystallographic studies reveal that, indeed, Pro16 of the zeta class and Pro9 of the hairpin class intercalate between Trp87 and Trp110 of the receptor, similar to the arrangement seen for Pro426 of IgE in the IgE-FcεRIα complex (Figure 7A) (Garman et al., 2000). Such a proline/tryptophan sandwich appears to be a conserved feature of all immunoglobulin Fc receptor complexes (Radaev et al., 2001; Sondermann et al., 2000; Sondermann and Oosthuizen, 2002); here we show that peptides that share no similarity to an immunoglobulin fold share a common feature in their respective Fc receptor binding interfaces.

Proline/Tryptophan Interactions Are Common in Protein-Protein Interfaces

While phage-derived peptides could have been selected for binding anywhere on the surface of the IgE receptor, two distinct classes of peptides were identified that both bind the tryptophan-rich patch at the D1/D2 junction. Interestingly, conserved surface-exposed tryptophan residues can be predictive of protein binding sites (Ma et al., 2003), where frequently tryptophan is found in contact with proline residues (Glaser et al., 2001). In particular, SH3 domains, EVH1 domains, WW domains, and profilin all take advantage of proline/tryptophan in-

Table 3. Resonance Assignments for Free and Receptor-Bound Hairpin Peptide IGE32

Residue	H ^α , ¹³ C ^α	H ^β , ¹³ C ^β	H ^γ , ¹³ C ^γ	H ^δ , ¹³ C ^δ
1 Asn	4.34; 52.6 4.23; 52.7	2.86, 2.97; 38.1 2.82, 2.92; 38.9		
2 Leu	4.68; 53.2 4.89; 52.5	1.61*; 41.6 1.24, 1.56; 42.3	1.71; 27.0 1.73; 26.8	0.95; 23.3, 0.96; 25.1 0.72; 25.7, 1.09; 24.0
3 Pro	4.47; 63.0 4.71; 62.7	1.89, 2.25; 32.2 2.39, 2.58; 27.7	2.05*; 27.3 2.05*; 27.3	3.66, 3.86; 50.5 na
4 Arg	4.46; 56.1 4.17; 57.6	1.86, 1.93; 31.0 1.97, 2.09; 30.5	1.65, 1.73; 27.2 1.85*; 27.5	3.25*; 43.3 3.24*; 42.7
5 Cys	5.66; 55.5 (5.99; 54.3**)	2.69, 3.11; 47.6 (2.34; 3.11; 49.7**)		
6 Thr	4.56; 60.8 3.89; 60.8	4.05; 71.6 3.50; 69.7	1.19; 21.2 -0.54; 15.2	
7 Glu	3.88; 56.3 4.12; 57.3	0.94, 1.61; 29.5 1.97, 2.09; 31.9	1.43, 1.77; 29.5 2.22, 2.39; 35.4	
8 Gly	3.75, 4.37; 44.5 (4.00, 4.80; 45.1**)			
9 Pro	4.04; 64.2 1.90; 62.0	0.80, 1.87; 31.0 -1.32, -0.42; 28.8	1.49, 1.73; 26.7 -1.25, -0.80; 24.3	3.33, 3.42; 49.3 0.12, 1.71; 47.3
10 Trp	4.90; 55.8 4.74; 56.0	3.13, 3.52; 28.3 2.94, 3.52; 27.2		
11 Gly	3.81, 4.59; 44.1 (3.92, 4.78; 42.9**)			
12 Trp	4.85; 57.7 4.73; 58.6	3.15*; 30.0 3.18, 3.26; 29.5		
13 Val	4.43; 61.6 4.73; 59.6	2.09; 34.2 2.22; 35.3	0.96; 20.9, 0.97; 20.7 1.07; 19.7, 1.22; 21.0	
14 Cys	5.42; 55.0 (5.89; 54.6**)	2.91, 3.03; 46.2 (2.94, 3.07; 47.4**)		
15 Met'	4.33; 55.2 4.36; 54.5	1.94, 2.12; 37.6 1.41, 1.81; 37.8	3.67*; 61.2 3.30, 3.42; 60.7	

Upper values are for the free peptide; lower values represent the FcεRIα-bound peptide; values shown in bold are those that differ by ≥ 0.15 ppm (¹H), 0.6 ppm (¹³C), or 1.5 ppm (¹⁵N) between free and bound states. * indicates degenerate methylene protons. na indicates that the peak is broadened sufficiently that the resonance cannot be assigned unambiguously. **Peaks for Cys5/Cys14 and Gly8/Gly11 could be assigned by amino acid-type, but not sequence-specifically in the FcεRI-bound state, thus the frequencies given for Cys5, for example, might actually be switched with those for Cys14, and similarly for Gly8 and Gly11. HN resonances were not assigned in the bound state, as most were too broad to obtain adequate correlations.

teractions at the core of their respective protein-protein interfaces (Huang et al., 2000; Kay et al., 2000; Zarrinpar and Lim, 2000). In all of these cases, not only do the proline and tryptophan rings show good van der Waals packing, but also the backbone carbonyl group of the amino acid preceding the proline by two residues forms a hydrogen bond with the tryptophan indole nitrogen (Huang et al., 2000). An analogous interaction is seen in the case of the zeta peptide-IgE receptor complex, with the carbonyl of Leu14 forming a hydrogen bond with Trp110. Such an interaction is not apparent however in the hairpin peptide complex, although the side chain of peptide residue Glu7 may provide additional favorable contacts, either through hydrogen bonding with the indole of Trp110 or by forming a salt bridge with Arg111 of the receptor.

Structurally Distinct Peptides Present a Common Binding Motif

The proline/tryptophan sandwich is not the only interaction in common between the zeta and hairpin peptides. In both cases, they also contact an adjacent shallow hydrophobic pocket on the receptor surface. Three-dimensional superposition of the receptor in the two peptide-receptor complexes shows that the position of residues Pro4 and Phe6 of the zeta peptide occupy the

same space on the receptor as those of Val13 and Thr6 of the hairpin in their respective receptor complexes (Figure 7). This results in Trp87 of FcεRI being fully buried within each peptide-receptor complex, being packed by a proline on one side and hydrophobic side chains from the peptide on the other. Indeed the overall shapes of the zeta and hairpin peptide's receptor interaction surfaces are surprisingly similar (Figure 7C). Each peptide provides a hydrophobic groove into which Trp87 can pack, lined by either disulfide bonds of the zeta peptide or the conserved glycine residues (Gly8 and Gly11) of the hairpin peptide where, for example, the Gly8Ala mutation leads to a 160-fold loss in receptor binding (Nakamura et al., 2001). Thus, while the peptide scaffolds are entirely different (one β hairpin, one helical), they present similar three-dimensional surfaces for binding receptor, not unlike that seen for the multiple diverse natural proteins that bind a common site on the Fc region of IgG (DeLano et al., 2000).

In contrast, IgE does not make contacts in the hydrophobic pocket neighboring the proline/tryptophan sandwich on the receptor (Figure 7A). Instead, IgE recruits high-affinity binding for its receptor by making additional contacts remote from this area in IgE binding "site 1" located within the D2 domain of the receptor. Restricting the size of the peptides to 100-fold smaller

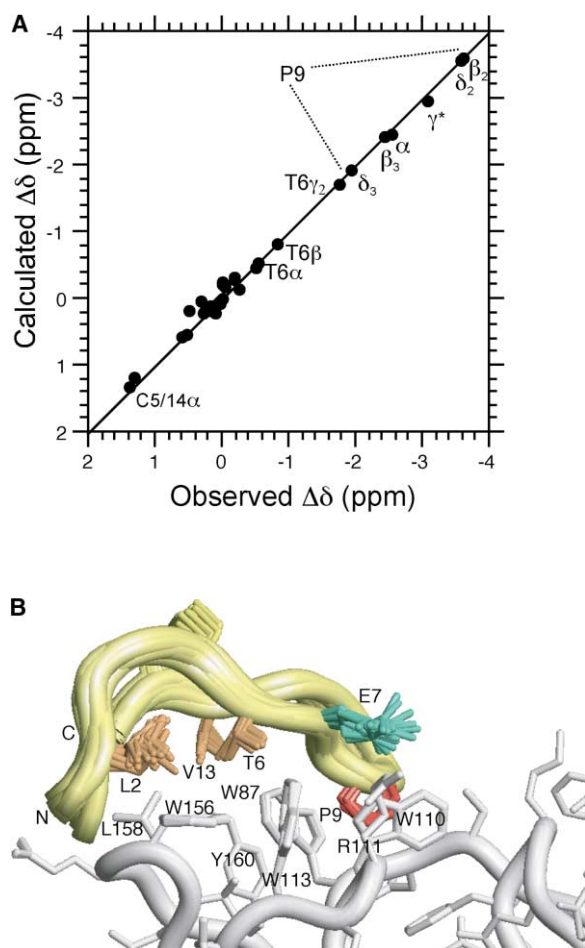


Figure 6. NMR Analysis of IGE32 Hairpin Peptide-Receptor Complex

(A) Agreement between calculated and observed ^1H chemical shifts in the complex (rms error = 0.11 ppm; $R = 0.9967$).
(B) Superposition of 16 models of hairpin peptide-receptor complex derived from docking and refinement based on ^1H chemical shifts (average rms error = 0.11 ± 0.01 ppm).

than IgE requires that they exploit local interactions to achieve high-affinity binding. The selection of these very structurally diverse peptides that nevertheless bind to the same receptor site using very similar types of interactions represents an elegant example of convergent evolution in vitro and identifies an additional contact area not exploited by the natural ligand IgE.

Experimental Procedures

IgE Receptor Expression and Purification

DNA encoding the extracellular domain, residues 1–176, of human FcεRIα (Hakimi et al., 1990) was subcloned into the baculovirus transfer vector pAcGP67B (PharMingen), which was then cotransfected with BaculoGold DNA (PharMingen) into Sf9 cells; recombinant virus was isolated and amplified in Sf9 cells. For protein production, Hi-5 (*T. ni*) cells were infected with recombinant viral stock and grown in shaker flasks for 72 hr at 27°C in ESF 921 media (Expression Systems LLC). Supernatants were collected and filtered through 0.2 μm filters and then loaded onto a CNBr-Sepharose-linked (Pharmacia) e117 peptide/Z-domain fusion column (see below). The column was washed with buffer (50 mM sodium phosphate [pH 6.0],

450 mM NaCl) until UV absorbance at 280 nm was reduced to zero. IgE receptor was eluted by washing with five column volumes of elution buffer (50 mM sodium phosphate [pH 6.0], 500 mM NaCl, 5 M urea). Fractions containing IgE receptor as analyzed by SDS-PAGE were pooled and concentrated. Protein was then loaded onto a Sephadex-75 size exclusion column (Amersham Pharmacia Biotech) preequilibrated with 50 mM sodium phosphate (pH 6), 450 mM NaCl. Protein purity after size exclusion was >95% as analyzed by SDS-PAGE. Typical yield of IgE receptor was 1–2 mg/l of infected cells. The IgE receptor is heavily glycosylated, with an average molecular mass of ~30 kDa, while the polypeptide molecular weight alone is 20 kDa.

Phage ELISA Competition

Oligonucleotide-directed mutagenesis was used to modify a phagemid vector, pH0753, for display of the hairpin peptide NLP RCTEGPWGWVCMAD on the N terminus of the C-terminal domain of the M13 gIII protein; peptide-phage were grown as described previously (Lowman, 1998). FcεRIα-Fc (2 μg/ml) was coated onto Nunc Maxisorp plates (Nakamura et al., 2001). Three-fold serial dilutions of peptides IGE06 or e109 were mixed with an equivalent volume of phage (diluted to yield <40% saturation) and transferred to the immobilized receptor plate. After 1 hr at room temperature, the plates were washed and developed as described (Lowman, 1998). IC₅₀ values were determined from a four-parameter curve fit of the ELISA signal using Kaleidagraph.

Competitive Binding Experiments Using

Surface Plasmon Resonance

Binding of peptides to immobilized FcεRIα (residues 1–176) was carried out as described previously (Nakamura et al., 2001) using a BIAcore-2000 instrument (BIAcore, Inc., Piscataway, NJ). Briefly, FcεRIα was immobilized using surface-thiol chemistry. Peptides were injected in PBS containing 0.05% Tween-20 and 0.01% sodium azide, at 25°C. The amount of each peptide bound at steady state was measured as the difference in surface plasmon resonance (SPR) signal between the FcεRIα-coupled flow cell and a control flow cell containing no immobilized protein.

Display and Mutagenesis of FcεRIα on Phage

DNA encoding human FcεRIα residues 1–176 was inserted into phagemid vector pH0753 for display of the extracellular domain of the α chain of the high-affinity IgE receptor at the N terminus of the C-terminal domain of M13 gIIIp. Alanine substitutions of individual residues were incorporated into this FcεRI phagemid using standard Kunkel mutagenesis (Kunkel et al., 1987), and phage displaying the IgE receptor were prepared as described (Sidhu et al., 2000). The display of wild-type and mutant FcεRI phage was confirmed by phage ELISA binding to immobilized IgE as described above.

Peptide Binding to FcεRI Alanine Mutants

100–400 μM soluble peptide was serially diluted and incubated with a previously titrated amount of wild-type or mutant FcεRI-displaying phage. After a 30 min incubation period at room temperature, the mixture was transferred to an IgE-coated 96-well immunoassay plate for 60 min at room temperature. Assay plates were washed, incubated with horseradish peroxidase-conjugated anti-M13-phage, and developed as described above. IC₅₀ values for each peptide were determined using a four-parameter curve fit in Kaleidagraph. Each value was normalized for total binding. The mean of three separate assays is shown with error bars in Figure 3A. The necessity of binding IgE for readout from this assay ensures that the protein is expressed and folded properly on the phage surface. All mutant proteins that were evaluated for peptide binding showed IgE binding affinities comparable to or within 10-fold that of the wild-type receptor. Nonetheless, the observed impact of a given mutation on peptide binding will be independent of any effects the mutation has on IgE binding, since the IgE in this experiment is used only as a capture reagent.

Generation of Isotopically Labeled Peptides

Hairpin and zeta peptides were produced in *E. coli* as fusion proteins to the Z-domain of protein A as described previously (Dennis et al.,

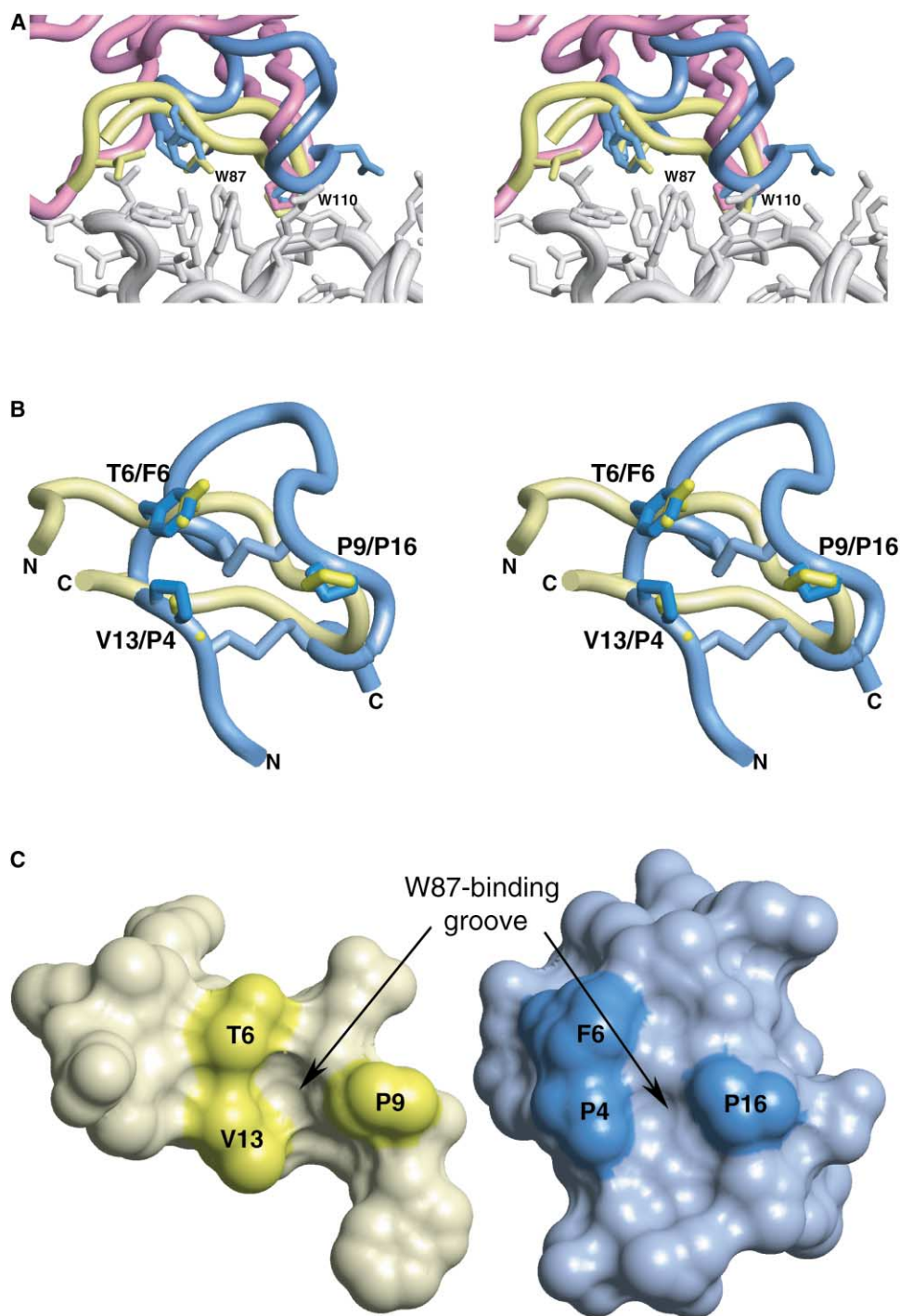


Figure 7. Comparison of Three Fc ϵ RI Complexes

(A) Stereoview of an overlay of the IgE complex crystal structure (purple) (1F6A [Garman et al., 2000]), e131 zeta peptide complex crystal structure (blue), and IGE32 hairpin peptide complex model based on NMR analysis (yellow), superposed on receptor coordinates only. The Fc ϵ RI α receptor structure is shown in white ribbon for all three complexes; side chains are shown for the receptor in the zeta peptide complex only. The view shown is essentially the same as that in Figures 4B and 6B.

(B) Stereoview of an overlay of the backbone structures of zeta (e131) and hairpin (IGE32) peptides taken from their respective receptor complex structures, rotated $\sim 90^\circ$ relative to the view in (A), showing the receptor binding face of each peptide.

(C) Surface representation of hairpin (left) and zeta (right) peptide oriented as in (B) but not superposed for clarity.

2001) to facilitate isotopic labeling. Peptides were labeled using a protocol and media described previously for the alkaline phosphatase induction system in *E. coli* (Reilly and Fairbrother, 1994). Briefly,

growth media supplemented with 50 μ g/ml carbenicillin was inoculated with *E. coli* strain 27C7 harboring the peptide/Z-domain expression plasmid. Cultures were grown at 30°C in shaker flasks for

24 hr. Cells were harvested and then washed with 300 ml wash media for every 1 liter of cells to remove excess phosphate. Cells were then resuspended in production media supplemented with 50 μ g/ml carbenicillin and incubated in shaker flasks at 30°C for an additional 24 hr. Cells were harvested by centrifugation and placed at -20°C for overnight storage.

Cells were thawed and combined with 40 ml of 20 mM Tris (pH 8.0) for each 10 g of cell paste. Cells were stirred at 4°C for 30 min to release protein from the periplasm. The sample was then centrifuged at 20,000 $\times$ g to remove insoluble material. The supernatant was loaded onto a Q-Sepharose Fast Flow column (Amersham Pharmacia Biotech) and eluted with a 0–500 mM NaCl gradient in the presence of 20 mM Tris (pH 8.0). Fractions containing protein were pooled and dialyzed against H₂O. The samples were then lyophilized and resuspended in 1–2 ml of 20 mM Tris (pH 8), 500 mM NaCl. The protein was loaded onto a Sephadex-75 size exclusion column (Amersham Pharmacia Biotech) preequilibrated with 20 mM Tris (pH 8.0), 500 mM NaCl. Protein purity after size exclusion was >95% as analyzed by SDS-PAGE. The yields of peptide/Z-domain fusion proteins were approximately 20 mg/l and 40 mg/l for hairpin and zeta peptides, respectively.

The zeta peptide was cleaved from the Z-domain by addition of 1 mg of trypsin to 80 mg of fusion protein in cleavage buffer (PBS + 1.1 mM CaCl₂) and allowed to react for 20 min. The peptide was then separated from the Z-domain using C₁₈ HPLC. Fractions containing the peptide (as assayed by mass spectrometry) were pooled and lyophilized.

For the hairpin peptide, 80 mg of hairpin/Z-domain fusion protein was dissolved in 5 ml 96% formic acid and 5 drops of water. A spatula tip of CNBr was added; the reaction was stirred for 3 hr and quenched with 25 ml water and 2 ml TFA, stirred for 5 min, and then rinsed into a lyophilization jar with 1:1 acetonitrile/water (25 ml) and lyophilized. The sample was then put on a 10 g Sep-Pak Cartridge (Waters), washed with 10% aqueous acetic acid, and eluted with 80% acetonitrile/10% acetic acid in water. The sample was then diluted with water and lyophilized to give 30 mg white solid. The Z-domain fusion partner was removed by passage over an IgG sepharose column (Pharmacia). The flow-through was then lyophilized, desalted over a Sep-Pak Cartridge, and further purified by reverse phase HPLC to yield 1.5 mg pure peptide.

NMR Spectroscopy

All NMR spectra were acquired on a Bruker DRX-800 spectrometer equipped with a 5 mm inverse triple-resonance (¹H/¹³C/¹⁵N) probe with three-axis gradient coils. Proton chemical shifts were referenced to internal 3-(trimethyl-silyl)propane-1,1,2,2,3,3-*d*₆-sulfonic acid (DSS; Isotec); ¹³C and ¹⁵N chemical shifts were referenced indirectly to DSS and liquid ammonia, respectively (Wishart et al., 1995). Acquisition temperatures were evaluated to identify optimal conditions to minimize peak linewidths and improve sensitivity. NMR spectra for the zeta peptide e117-receptor complex were obtained at 35°C with a sample containing 300 μ M complex in PBS (pH 5.3), either in 92% H₂O/8% D₂O or, after lyophilization and resuspension, in "100%" D₂O. NMR spectra for the hairpin peptide IGE32-receptor complex were obtained at 20°C with a sample containing 500 μ M complex (in PBS [pH 5.3], lyophilized, and resuspended in "100%" D₂O). Slow exchange behavior was observed for most resonances of the zeta peptide complex, with significant broadening seen only for residues at the interface. The hairpin peptide complex showed slow-to-intermediate exchange behavior throughout the molecule, necessitating the lower acquisition temperature and high field spectrometer for adequate sensitivity.

The resonances of free e117 peptide were assigned by standard analysis of 2D ¹H/¹⁵N-HSQC, 3D ¹H/¹⁵N-TOCSY-HSQC (mixing time, 90 ms), 2D ¹H/¹³C-HSQC, 2D CBA(CO)NH, and 2D ¹H/¹³C-HCCH-TOCSY (Cavanagh et al., 1995). Resonances in the receptor-bound state were assigned by analysis of 2D ¹H/¹⁵N-HSQC, 3D ¹H/¹⁵N-NOESY-HSQC, 2D ¹H/¹³C HSQC, and 3D ¹³C-edited NOESY (mixing time, 110 ms) (Cavanagh et al., 1995) due to the lower sensitivity of J-correlated spectra for the ~32 kDa complex. Intermolecular NOEs were identified in a ¹³C-*F*₂-edited, ¹²C-*F*₃-filtered NOESY spectrum (mixing time, 110 ms) (Zwahlen et al., 1997).

The resonances of free IGE32 peptide were assigned by analysis

of 2D ¹H/¹³C-HSQC, 2D CBA(CO)NH, 2D ¹H/¹³C-HCCH-TOCSY. Resonances in the receptor-bound state were assigned by analysis of 2D ¹H/¹³C-HSQC and 3D ¹³C-edited NOESY (mixing time, 110 ms). The resonances for the important Pro9 were extremely broad and hence did not show cross peaks in the ¹³C-edited NOESY spectrum, and thus, assignment of these resonances was confirmed by analysis of a 2D ¹H/¹³C-HSQC spectrum acquired on a sample prepared with ¹³C-Pro9 specifically labeled hairpin peptide (IGE06) generated by chemical synthesis (Nakamura et al., 2001). For the hairpin-receptor complex, the ¹³C-*F*₂-edited, ¹²C-*F*₃-filtered NOESY spectrum was significantly less sensitive than the ¹³C-edited NOESY in detecting the same NOE crosspeaks, and thus, intermolecular NOEs were assigned from the ¹³C-edited NOESY spectrum as those peaks between known peptide resonances and a ¹H frequency not coincident with any peptide frequency.

Chemical Shift Refinement

The zeta and hairpin peptides were docked manually into IgE binding site 2 of FcεRIα using coordinates derived from the free structures of related peptides (PDB codes 1KCN [Nakamura et al., 2002] and 1JBF [Nakamura et al., 2001] for zeta and hairpin peptides, respectively) and from the crystal structure of the IgE/FcεRIα complex (PDB code 1F6A [Garman et al., 2000]). The initial peptide placements were based on the assumption that the functionally important proline residues of the peptides, which experienced the most significant ¹H chemical shift perturbations upon receptor binding, would bind between Trp87 and Trp110 of the receptor, in approximately the same way as observed for Pro426 of IgE. The resulting peptide orientations appeared consistent with intermolecular NOE correlations observed between the ¹³C-labeled peptides and the unlabeled receptor; in particular, intermolecular NOEs between zeta peptide residues Val1, Gln2, and Pro4 or hairpin peptide residues Val13 and Met15, and an unidentified pair of receptor methyl groups could be assigned tentatively to Leu158 of the receptor since these are the only methyl groups in proximity to site 2. Calculation of the docked peptide ¹H chemical shifts (Ösapay and Case, 1991) using the program SHIFTS 4.1 (X.P. Xu and D.A. Case, The Scripps Research Institute, La Jolla, CA; <http://www.Scripps.edu/case/qshifts/qshifts.htm>) showed good general agreement with the experimentally observed chemical shifts, but also suggested that the manually docked models could be refined further.

All the assigned aliphatic ¹H chemical shift data were therefore incorporated as restraints (force constant = 25 kcal·mol⁻¹·ppm⁻¹) in a simple simulated annealing protocol, using the program AMBER 6.0 (<http://amber.Scripps.edu/doc6/>). For initial rounds of refinement, the chemical shifts of prochiral methylene and methyl protons were averaged. The chemical shifts of all peptide protons were back-calculated from the resulting models using SHIFTS 4.1 and compared to the experimentally observed chemical shifts. In cases where the chemical shifts of the prochiral protons were sufficiently different and the agreement between the calculated and observed values was close, it was possible to make stereospecific assignments that were incorporated into subsequent rounds of refinement. In this way, stereospecific assignments were possible for Pro4 β, Leu14 β, and Pro16 β and γ protons of the zeta peptide, and Pro9 β and δ and Val13 γ protons of the hairpin peptide.

Additional distance and dihedral angle restraints, derived from those used to calculate the solution structures of the free peptides, were incorporated into the refinement to ensure that the peptide structures were maintained. In the case of the zeta peptide, the intrapeptide distance restraints corresponded to those used for calculation of the free solution structure plus an additional 0.5 Å, and were applied with a force constant of 32 kcal·mol⁻¹·Å⁻². In the case of the hairpin peptide, intrapeptide distance restraints corresponding to those used for calculation of the free peptide structure were applied without modification using a force constant of 20 kcal·mol⁻¹·Å⁻². In both cases, the dihedral angle restraints were equivalent to those used for calculation of the free peptide structures and were applied with a force constant of 80 kcal·mol⁻¹·rad⁻².

The coordinates of the receptor were restrained to be nearly fixed during the simulated annealing and subsequent restrained minimization calculations using a harmonic potential with a force constant of 5 kcal·mol⁻¹. For calculations with the zeta peptide, a weak har-

monic potential (force constant = 0.5 kcal·mol⁻¹) was also used to restrain the disulfide-bonded core of the peptide (residues Cys3–Tyr8 and Ala13–Tyr20). In the case of the hairpin peptide calculations, a similar weak harmonic potential (force constant = 0.25 kcal·mol⁻¹) was used in the initial rounds of refinement to restrain the entire peptide except residues Asn1, Leu2, Thr6, Trp10, and Trp12; during the final rounds of refinement, these restraints were removed.

Intermolecular NOEs between unambiguously assigned peptide protons and unassigned receptor methyl or aromatic protons were assigned tentatively based on the docked and refined models. The tentatively assigned intermolecular NOEs were incorporated iteratively into the refinement calculations as upper-bound distance restraints of 5.8 and 5.0 Å (plus appropriate pseudo-atom corrections) for the zeta and hairpin peptides, respectively, only if they were clearly satisfied in the current models. In total, 17 and 22 intermolecular distance restraints were included in the final simulated annealing calculations for the zeta and hairpin peptides, respectively.

Purification and Crystallization of the e131-FcεRIα Complex

Synthesized e131 peptide (Nakamura et al., 2002) was added to purified IgE receptor at a ratio of 1.5:1. The complex was then purified using a Sephadex-75 size exclusion column (Amersham Pharmacia Biotech) preequilibrated with 10 mM MES (pH 6.0), 250 mM NaCl. Fractions containing complex, as assayed by SDS-PAGE, were pooled and concentrated to 12 mg/ml. Crystals were obtained using the hanging drop vapor diffusion method with a precipitant composed of 30% PEG MME 2000, 0.2 M ammonium sulfate, and 0.1 M sodium citrate (pH 4.2). Small hexagonal plates appeared after 2 weeks, redissolved after 1–2 months, and then reappeared as much larger (0.4 mm on each edge), block-shaped crystals after 5 months. The larger crystals were flash frozen directly from the mother liquor in liquid nitrogen and used for data collection.

X-Ray Crystallography

Data were collected from a frozen (100 K) crystal at SSRL beamline 9-2 using an ADSC Quantum4 CCD detector 250 mm from the crystal. Ninety images 1.5° wide were collected with 1.033 Å wavelength X-rays. DENZO/SCALEPACK yielded 30,473 unique reflections in a C222₁ cell with *a* = 199.7, *b* = 149.7, and *c* = 104.1 Å, and refined mosaicity of 0.85° (Table 2).

Based on an approximate molecular weight of 32 kDa per peptide-receptor complex, the crystallographic asymmetric unit probably contained between four and eight copies of the complex. Four-fold symmetry was revealed by a peak, 43% as high as the origin peak, in a self-rotation Patterson map performed using REPLACE (Tong, 1993). The rotational and positional placement of four FcεRIα monomers (Garman et al., 1998) was performed using AMoRe (CCP4, 1994; Navaza, 1994), but required the use of normalized structure factors. A total of 968 reflections were sequestered from refinement for calculation of *R*_{free}. Rigid-body refinement against data at 10–4.0 Å resolution yielded *R*_{free} and *R* values of 44.8% and 42.4%, respectively (X-PLOR98 [Brünger, 1992]; Accelrys, San Diego, CA). Several rounds of solvent flattening and averaging using SIGMAA (Read, 1986) and DM (CCP4, 1994) allowed docking (XtalView [McRee, 1993]) of an e131 peptide (Nakamura et al., 2002) on each of the four receptor molecules, as well as addition of carbohydrate residues. Bulk solvent and overall anisotropic corrections were applied to the data. Thermal factor restraints were optimized using *R*_{free}. Refinement of a one-receptor/one-peptide model with strict noncrystallographic symmetry (NCS) constraints was abandoned due to a 1% increase in *R*_{free}. (Experiments applying less tightly coupled NCS thermal factor restraints also increased *R*_{free}.) Final refinements employed tight noncrystallographic restraints among those parts of the receptor molecules not contacting the peptides and tightly restrained individual atomic thermal factors. 85.8% of residues have main chain dihedral angles in the “most favored” (Laskowski et al., 1993) region of Ramachandran space, and one residue (Leu 10_Y) is in a “disallowed” region. The average thermal factors for the four peptides and four receptors are 73 and 92 Å², respectively. The final *R* and *R*_{free} values for data 30–3.0 Å are 29.5% and 35.0%. These *R* values are relatively high, likely due in part to the high degree of receptor glycosylation (~30% by weight). Of the

20 largest features in the final *F*_o – *F*_c electron density map, 10 are tentative water sites and 10 are associated with disordered carbohydrate residues or protein segments that are difficult to model accurately. Addition of 10 water oxygens had a negligible effect on *R*_{free}, and they were not retained.

The crystals are composed from tetramers of peptide-receptor complexes arranged as dimers of dimers. Crystallographic symmetry provides contacts between tetramers mediated mostly via carbohydrate attached to Asn42. Large “solvent” voids approximately 50 × 55 × 90 Å are presumably occupied by poorly ordered carbohydrate. A noncrystallographic dimer is formed by a pseudo 2-fold axis running between peptides bound to receptors “A” and “C,” which provides peptide-peptide contacts of ~130 Å² between peptide “linker” regions. All four peptides contact their receptors across ~430 Å², but the pseudo 2-fold also brings the neighboring receptor in for some contact with the reference peptide. The details of these neighboring receptor contacts differ somewhat between peptides. An additional pseudo 2-fold axis relates the dimers. Trp130, Tyr131, and Glu132 from receptors “A” and “B” (i.e., residues from the “site 1” region; Figure 3B) are involved in an interdimer contact on this axis.

Acknowledgments

We thank Cliff Quan, Martin Struble, and Jeff Tom for peptide synthesis and purification, Jim Bourell for mass spectrometry, David Case for help with chemical shift calculations, and Andrea Cochran, Abraham de Vos, and Nicholas Skelton for encouragement and helpful discussions. Portions of this research were carried out at the Stanford Synchrotron Radiation Laboratory, a national user facility operated by Stanford University on behalf of the U.S. Department of Energy, Office of Basic Energy Sciences. The SSRL Structural Molecular Biology Program is supported by the Department of Energy, Office of Biological and Environmental Research, and by the National Institutes of Health, National Center for Research Resources, Biomedical Technology Program, and the National Institute of General Medical Sciences. All of the authors are or were employees of Genentech and declare competing financial interests.

Received: December 9, 2003

Revised: March 31, 2004

Accepted: April 26, 2004

Published: July 13, 2004

References

- Babu, K.S., Arshad, S.H., and Holgate, S.T. (2001). Omalizumab, a novel anti-IgE therapy in allergic disorders. *Expert Opin. Biol. Ther.* 1, 1049–1058.
- Brünger, A.T. (1992). X-PLOR. A System for X-Ray Crystallography and NMR, 3.1 Edition (New Haven, CT: Yale University Press).
- Case, D.A. (1995). Calibration of ring-current effects in proteins and nucleic acids. *J. Biomol. NMR* 6, 341–346.
- Cavanagh, J., Fairbrother, W.J., Palmer, A.G., III, and Skelton, N.J. (1995). *Protein NMR Spectroscopy: Principles and Practice* (San Diego, CA: Academic Press).
- CCP4 (Collaborative Computational Project 4) (1994). The CCP4 suite: programs for protein crystallography. *Acta Crystallogr. D Biol. Crystallogr.* 50, 760–763.
- DeLano, W.L., Ultsch, M.H., de Vos, A.M., and Wells, J.A. (2000). Convergent solutions to binding at a protein-protein interface. *Science* 287, 1279–1283.
- Dennis, M.S., Roberge, M., Quan, C., and Lazarus, R.A. (2001). Selection and characterization of a new class of peptide exosite inhibitors of coagulation factor VIIa. *Biochemistry* 40, 9513–9521.
- Garman, S.C., Kinet, J.P., and Jardetzky, T.S. (1998). Crystal structure of the human high-affinity IgE receptor. *Cell* 95, 951–961.
- Garman, S.C., Wurzburg, B.A., Tarchevskaya, S.S., Kinet, J.P., and Jardetzky, T.S. (2000). Structure of the Fc fragment of human IgE bound to its high-affinity receptor FcεRIα. *Nature* 406, 259–266.
- Garman, S.C., Sechi, S., Kinet, J.P., and Jardetzky, T.S. (2001). The

analysis of the human high affinity IgE receptor from multiple crystal forms. *J. Mol. Biol.* **311**, 1049–1062.

Glaser, F., Steinberg, D.M., Vakser, I.A., and Ben-Tal, N. (2001). Residue frequencies and pairing preferences at protein-protein interfaces. *Proteins* **43**, 89–102.

Hakimi, J., Seals, C., Kondas, J.A., Pettine, L., Danho, W., and Ko-chan, J. (1990). The α subunit of the human IgE receptor (FcER1) is sufficient for high affinity IgE binding. *J. Biol. Chem.* **265**, 22079–22081.

Huang, X., Poy, F., Zhang, R., Joachimiak, A., Sudol, M., and Eck, M.J. (2000). Structure of a WW domain containing fragment of dystrophin in complex with β -dystroglycan. *Nat. Struct. Biol.* **7**, 634–638.

Kay, B.K., Williamson, M.P., and Sudol, M. (2000). The importance of being proline: the interaction of proline-rich motifs in signaling proteins with their cognate domains. *FASEB J.* **14**, 231–241.

Kunkel, T.A., Roberts, J.D., and Zakour, R.A. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154**, 367–382.

Laskowski, R.A., MacArthur, M.W., Moss, D.S., and Thornton, J.M. (1993). PROCHECK: a program to check the stereochemical quality of protein structures. *J. Appl. Crystallogr.* **26**, 283–291.

Lowman, H.B. (1998). Combinatorial peptide library protocols. In *Methods in Molecular Biology*, S. Cabilly, ed. (Totowa, NJ: Humana Press), pp. 249–264.

Ma, B., Elkayam, T., Wolfson, H., and Nussinov, R. (2003). Protein-protein interactions: structurally conserved residues distinguish between binding sites and exposed protein surfaces. *Proc. Natl. Acad. Sci. USA* **100**, 5772–5777.

McRee, D.E. (1993). *Practical Protein Crystallography*, 2nd Edition (San Diego, CA: Academic Press).

Nakamura, G.R., Starovasnik, M.A., Reynolds, M.E., and Lowman, H.B. (2001). A novel family of hairpin peptides that inhibit IgE activity by binding to the high-affinity IgE receptor. *Biochemistry* **40**, 9828–9835.

Nakamura, G.R., Reynolds, M.E., Chen, Y.M., Starovasnik, M.A., and Lowman, H.B. (2002). Stable “zeta” peptides that act as potent antagonists of the high-affinity IgE receptor. *Proc. Natl. Acad. Sci. USA* **99**, 1303–1308.

Navaza, J. (1994). AMoRe: an automated package for molecular replacement. *Acta Crystallogr. A* **50**, 157–163.

Ösapay, K., and Case, D.A. (1991). A new analysis of proton chemical shifts in proteins. *J. Am. Chem. Soc.* **113**, 9436–9444.

Owen, C.E. (2002). Anti-immunoglobulin E therapy for asthma. *Pulm. Pharmacol. Ther.* **15**, 417–424.

Radaev, S., Motyka, S., Fridman, W.-H., Sautees-Fridman, C., and Sun, P.D. (2001). The structure of a human type III Fc γ Receptor in complex with Fc. *J. Biol. Chem.* **276**, 16469–16477.

Read, R.J. (1986). Improved Fourier coefficients for maps using phases from partial structures with errors. *Acta Crystallogr. A* **42**, 140–149.

Reilly, D., and Fairbrother, W.J. (1994). A novel isotope labeling protocol for bacterially expressed proteins. *J. Biomol. NMR* **4**, 459–462.

Sidhu, S.S., Lowman, H.B., Cunningham, B.C., and Wells, J.A. (2000). Phage display for selection of novel binding peptides. *Methods Enzymol.* **328**, 333–363.

Sondermann, P., and Oosthuizen, V. (2002). Mediation and modulation of antibody function. *Biochem. Soc. Trans.* **30**, 481–486.

Sondermann, P., Huber, R., Oosthuizen, V., and Jacob, U. (2000). The 3.2-Å crystal structure of the human IgG1 Fc fragment-Fc γ R1II complex. *Nature* **406**, 267–273.

Tong, L. (1993). REPLACE, a suite of computer programs for molecular-replacement calculations. *J. Appl. Crystallogr.* **26**, 748–751.

Wishart, D.S., Digam, C.G., Yao, J., Abildgaard, F., Dyson, H.J., Oldfield, E., Markley, J.L., and Sykes, B.D. (1995). ^1H , ^{13}C and ^{15}N chemical shift referencing in biomolecular NMR. *J. Biomol. NMR* **6**, 135–140.

Zarrinpar, A., and Lim, W.A. (2000). Converging on proline: the mechanism of WW domain peptide recognition. *Nat. Struct. Biol.* **7**, 611–613.

Zwahlen, C., Sébastien, P.L., Vincent, J.F., Greenblatt, J., Konrat, R., and Kay, L.E. (1997). Methods for the measurement of intermolecular NOEs by multinuclear NMR spectroscopy: application to a bacteriophage N-peptide/boxB RNA complex. *J. Am. Chem. Soc.* **119**, 6711–6721.

Accession Numbers

Coordinates and structure factors for the $\epsilon 131\text{-Fc}\epsilon\text{R1}\alpha$ complex crystal structure have been deposited with the RCSB Protein Data Bank with accession code 1RPQ.