A Novel Family of Hairpin Peptides That Inhibit IgE Activity by Binding to the High-Affinity IgE Receptor‡

Gerald R. Nakamura,§ Melissa A. Starovasnik,§ Mark E. Reynolds,|| and Henry B. Lowman*,§

Departments of Protein Engineering and Bioorganic Chemistry, Genentech, Inc., 1 DNA Way, South San Francisco, California 94080

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ABSTRACT: A family of structured peptides that bind to FcεRIα, the α-chain of the high-affinity receptor for IgE, has been identified. Binding selections using FcεRIα and polyvalent peptide-phage libraries yielded a dominant 18-residue peptide-phage clone, as well as related sequences that did not resemble fragments of IgE. Synthetic peptides based on these sequences inhibited IgE binding to its receptor, and were found by NMR analysis to adopt a stable *β*-hairpin structure in solution. Optimized peptides with micromolar receptor affinity exhibited high stability in biological fluids and inhibited cellular histamine release in an in vitro bioassay of IgE activity. The structure−activity relationships of these peptides, which are less than 1% of the size of IgE, suggest an overlap between their binding site and that of IgE on FcεRI. Thus, the peptides demonstrate that blocking a small epitope on this receptor chain is sufficient to block IgE activity. Such structured peptides represent a possible starting point for the design of novel antagonists, and offer the potential for testing in vivo a new approach for treating allergic disease.

Allergic or atopic disease is a widespread and growing health problem; skin test surveys suggest that more than 50 million people in the United States are affected by allergies (*1*). In 1990, an estimated 6 billion dollars was spent on treating allergic asthma alone in the United States (*2*). The amount becomes even more staggering when combined with the cost of treating other forms of allergic disease such as allergic rhinitis, atopic dermatitis, and food allergies. Such numbers stimulate the development of more efficacious therapeutics that treat the disease directly.

The molecular mechanisms underlying allergic responses, which involve IgE, have been elucidated over the past several decades. IgE is produced in response to exposure to foreign substances (allergens) and circulates in the body in the free form and bound to cell-surface receptors (*3*). The high-affinity IgE receptor, known as FcεRI, is expressed as a multimeric complex predominantly on mast cells and basophils (for reviews, see refs *4* and *5*), and usually consists of an α-subunit, a *β*-subunit, and a disulfide-linked homodimeric *γ*-subunit. IgE binding to the receptor occurs only through the α-subunit and does not require the presence of the other subunits (*6*). The structures of free and IgE-bound forms of FcεRIα[1] have recently been revealed by X-ray crystallographic studies (*7*, *8*). Allergen binding to cell-surface, receptor-bound IgE leads to receptor aggregation, triggering a cascade of events in the allergic response (*9*). Ultimately, this activation of cells results in the release of soluble immune mediators such as histamine, and the symptoms associated with allergy.

Historically, therapeutics for allergy have been directed at reducing the degree of inflammation, vasodilation, and congestion. However, recent strategies target the molecules involved in initiating the allergic cascade (*10*−*17*). The premier example is the monoclonal antibody E25, which acts by binding to IgE, thus inhibiting its binding to FcεRI (*13*). E25 (generic name, Omalizumab; trade name, Xolair) has already demonstrated clinical efficacy in reducing the severity of allergic disease (*14*), showing the potential for drugs that prevent interaction of IgE with its receptor. Among other possible inhibitors are protein fragments, such as truncated IgE-Fc, that bind to the receptor, disrupt IgE binding, and prevent activation of cells (*3*, *15*, *16*). In addition, both blocking and nonblocking antibodies have been reported for the IgE receptor (*15*, *17*).

Given new drug delivery technologies, small peptides may also represent drug candidates (*18*); however, linear peptides derived from FcεRIα have shown weak binding affinity at best (*12*). Peptides designed by modeling a loop region of the natural receptor sequence have been reported to inhibit IgE (*11*). Inhibition through binding to the receptor side of the interface might be considered more difficult, especially since structural studies show two sites of interaction on FcεRIα with IgE (*8*). Moreover, linear peptides derived from the IgE sequence have shown only weak inhibition (*19*). To our knowledge, no peptides or small molecules that bind with high affinity and specificity to FcεRIα have been reported.

We have investigated whether structured peptides can be identified for binding to the receptor side of the IgE−receptor interface. Unlike linear peptides, such molecules are poten-

‡ The structure of IGE06 is available from the RCSB Protein Data Bank (1JBF).

* To whom correspondence should be addressed. Telephone: (650) 225-1171. Fax: (650) 225-3734. E-mail: hbl@gene.com.

§ Department of Protein Engineering.

|| Department of Bioorganic Chemistry.

[1] Abbreviations: COSY, two-dimensional correlated spectroscopy; Fc, constant domain region of IgG or IgE; FcεRIα, extracellular domain of the α-chain of the high-affinity IgE receptor; FcεRIα-Ig, FcεRIα fused to the Fc region of human IgG; NOE, nuclear Overhauser effect; NOESY, two-dimensional NOE spectroscopy; PBS, phosphate-buffered saline; ROESY, rotating frame Overhauser effect spectroscopy; SPR, surface plasmon resonance; TOCSY, total coherence spectroscopy.

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tially stable in biological fluids, and therefore could be tested in vivo as IgE antagonists. To identify such molecules, we used phage libraries to select and affinity-mature novel peptides that bind FcεRIα and inhibit IgE binding. The peptides themselves do not activate cells at high concentrations, but do act as functional antagonists in allergen-driven cell-based assays. Remarkably, these peptides form stable β-hairpin structures in solution and are not readily degraded in biological fluids. Analyses of peptide analogues have delineated the binding determinants on these peptides that interact with a site likely within the IgE binding site of FcεRIα. These small peptides (MW < 2000) demonstrate that novel molecules, unrelated in primary sequence to IgE (MW ≈ 200 000), can function as FcεRI antagonists in vitro, and suggest that small-molecule antagonists of the receptor could be developed.

EXPERIMENTAL PROCEDURES

Peptide-Phage Binding Selections and Assays. Ten naïve peptide libraries with a diversity of ~10^8 were displayed polyvalently on the N-terminus of gVIIIp of M13 filamentous phage under transcriptional control of the P_{phoA} promoter (*20*, *21*). The peptides were 18−20 residues in length, containing random sequences with fixed cysteines for possible disulfide formation as described previously (*20*). Libraries were pooled according to their peptide motifs as follows: group 1, $SGTACX_2GPX_4CX_4$; group 2, $X_4CX_2GPX_4CX_4$; group 3, X_{20}; and group 4, $X_7CX_4CX_7$, $X_7CX_5CX_6$, $X_6CX_6CX_6$, $X_6CX_7CX_5$, $X_5CX_8CX_5$, $X_5CX_9CX_4$, and $X_4CX_{10}CX_4$. Phagemids were transformed into XL-1 Blue *Escherichia coli* cells for propagation using VCSM13 helper phage (Stratagene, La Jolla, CA) according to standard methods (*21*). Binding selections were performed in Maxisorp immunoplates (Nalge, Rochester, NY) coated with FcεRIα-Ig [described elsewhere (*6*)] and blocked with assay blocking buffer (PBS containing 0.5% bovine serum albumin). Phage (10^{10}) in 100 μL of binding buffer [PBS (pH 7.4) containing 0.5% bovine serum albumin, 0.05% Tween-20, and 0.005% Triton X-100] were added to eight wells for 2−3 h at 24 °C. Some selections also included a peptide (10 μM) corresponding to g8a.37 (see Figure 1) as a competitor. Wells were rinsed 10 times with wash buffer (PBS containing 0.05% Tween-20). Bound phage were eluted by a 7 min incubation with 0.2 M glycine (pH 2.0), then neutralized with 1 M Tris, and used to re-infect XL-1 Blue *E. coli* cells for propagation and subsequent rounds of binding selection. DNA from selected peptide-phage clones was sequenced using standard Sequenase (Amersham, Piscataway, NJ) procedures.

In phage ELISAs (*21*) for binding to FcεRIα-Ig as well as in those testing for inhibition of binding by IgE, phage were diluted 1/100 in binding buffer and added to FcεRIα-Ig-coated Maxisorp plates for 1 h at 24 °C in the presence and absence of 2 μg/mL human myeloma-derived IgE (*22*). Plates were rinsed with wash buffer and developed with horseradish peroxidase-conjugated anti-M13 antibody (Pharmacia, Uppsala, Sweden) and *o*-phenylenediamine substrate. After addition of 2.5 M H_2SO_4, the absorbance was determined at 492 nm.

Peptide sequences from selected gVIIIp phage clones were transferred to a monovalent display format using site-directed

Clone	Sequence	n=	No. at round n: 2	3	4	5
g8a.37	MGTLCLEGPEGWFCIESA		-	12	-	8
g8a.20	TGEACVEGPGAWVCCLEP		-	1	-	8
g8a.18	QEWTCVEGPRGWECIAVL		-	5	-	1
g8a.19	DGSLCFEGPWGDICQSDG		-	4	-	0
g8b.11	EIQECTEGPWGWFCVGSG		2	15	18	-
g8b.35	AEATCTEGPWGWVCMAAD		2	3	15	-
g8b.3	GTDVCVEGPWGEVCYALK		1	0	0	-
g8b.22	NYEECVMGPDGVWCLIPT		1	1	0	-
g8b.23	GRPSCIEGPSGLWCLIES		1	1	0	-
g3a.1	NLPRCTEGPWGWVCMAAD		-	1	-	-
g3a.2	HLPECTEGPWGWVCMAAD		-	1	-	-
g3a.3	NLPTCTEGPWGWVCMAAD		-	1	-	-
g3a.4	VMPTCTEGPWGWVCMAAD		-	1	-	-
g3a.5	AMAQCTEGPWGWVCMAAD		-	1	-	-
g3a.6	GRAQCTEGPWGWVCMAAD		-	1	-	-
g3a.7	DLPACTEGPWGWVCMAAD		-	1	-	-
g3a.8	GRTECTEGPWGWVCMAAD		-	1	-	-

FIGURE 1: Peptide-phage clones. Fixed residues are shown in boxes. Identical or similar residues conserved in multiple clones with the highest frequency are shaded. The number of clones found with identical DNA sequences is shown at specified rounds (*n*) of selection. The first set of sequences (g8a series) refers to clones obtained from initial selections from the $X_4CX_2GPX_4CX_4$ library, the second (g8b series) to those obtained from a second series of selections from the $X_4CX_2GPX_4CX_4$ library, and the third (g3a series) to those obtained from a monovalent phage-display library based upon clone g8b.35.

mutagenesis (*23*) with oligonucleotides encoding the desired peptides and a previously described gIIIp phagemid template (*20*, *21*). Individual peptide-phage clones were propagated, purified, and sequenced as described above. Competitive peptide-phage ELISAs for relative affinity determinations were carried out as described previously (*21*).

Peptide Synthesis. Peptides were prepared either manually or by machine, typically on a 0.25 mmol scale using standard solid-phase peptide chemistry (*24*) with FMOC-protected amino acids (*25*) on a *p*-alkoxybenzyl alcohol resin (*26*), or on a Rink amide aminomethyl resin (*27*). Couplings were performed with 2−4 equiv of HBTU-activated amino acid and 4−5 equiv of *N*-methylmorpholine or diisopropylethylamine. FMOC protecting groups were removed with 20% piperdine in DMA. Cleavage and deprotection with TFA containing 5% triisopropylsilane and 1% water afforded crude linear peptides after washing the resin with diethyl ether and extracting the resin with increasing amounts of H_2O in acetic acid and acetonitrile. Disulfide oxidation was carried out at room temperature via dropwise addition of a saturated solution of iodine in acetic acid to the crude linear peptide until a yellow color persisted. The excess iodine was quenched with Zn dust, and the solution was suction-filtered through Celite and lyophilized. The crude cyclized peptides were purified by preparative reverse-phase C18 HPLC (CH_3CN/H_2O gradient, 0.1% TFA) to afford the purified material (generally >98% by analytical HPLC) which was characterized by electrospray ionization mass spectrometry (Sciex API1100) and lyophilized to dryness.

NMR Spectroscopy. Two-dimensional (2D) NMR experiments were carried out on a Bruker AMX-500 spectrometer at 15 °C with a sample containing 2 mM IGE06 in a 92% H_2O/8% D_2O mixture (pH 5.7) and 0.1 mM NaN_3, with 0.1 mM DSS as a chemical shift reference. 2QF-COSY, COSY-35 (with a 35 ms mixing pulse), TOCSY (τ_m = 74 ms), ROESY (τ_m = 150 ms), and NOESY (τ_m = 150 ms) spectra

were acquired as described previously (*28*) using gradient coherence selection (*29*) or excitation sculpting (*30*) for water suppression. After lyophilization and dissolution of the peptide in D_2O, 2D NOESY (τ_m = 150 ms) and COSY-35 spectra were acquired. Complete ^1H resonance assignments (see Table S1 of the Supporting Information) were derived from these data by standard methods (*31*). $^3J_{HN-H\alpha}$ values were obtained by fitting Lorentzian lines to the antiphase doublets of HN−Hα peaks in 2QF-COSY spectra processed to high digital resolution in F_2; $^3J_{HN-H\alpha}$ values for Gly8 and Gly11 were obtained from analysis of the COSY-35 spectrum acquired in H_2O. $^3J_{H\alpha-H\beta}$ values were extracted from COSY-35 spectra acquired on a D_2O solution of the peptide. Distance and dihedral angle restraints were generated as described previously (*32*). Eighty initial structures were calculated using the hybrid distance geometry/simulated annealing program DGII (*33*); 50 of these were further refined by restrained molecular dynamics using the AMBER all-atom force field implemented in DISCOVER as described previously (*32*). Twenty structures having the lowest restraint violation energy and good geometry were chosen to represent the solution conformation of IGE06. The structure with the lowest rmsd from the average coordinates of the ensemble was chosen as the representative structure (model 1 in the Protein Data Bank file).

Peptides were also evaluated to assess the effect of substitutions on their three-dimensional structures using 2D NMR spectroscopy; backbone ^1H resonances were assigned and $^3J_{HN-H\alpha}$ coupling constants measured and compared to those of the reference peptide (IGE06). Peptides were defined to have a structure similar to that of IGE06, provided the chemical shifts, coupling constants, and NOEs were consistent with that structure. Peptides were defined to be less stable when $^3J_{HN-H\alpha}$ coupling constants were less extreme than the reference (e.g., $^3J_{HN-H\alpha}$ changes from $\geq$9.0 to $<$8.5 Hz) and/or when chemical shifts of backbone resonances were closer to random-coil values in the analogue than in IGE06.

Peptide Binding Assays Using Surface Plasmon Resonance (SPR). A BIAcore 2000 SPR instrument was used to compare the relative peptide binding affinities for purified recombinant FcεRIα (residues 1−172) from insect-cell expression (unpublished results). For these experiments, FcεRIα (residues 1−172) was immobilized at densities of 1000−3000 response units (RU) on carboxymethyl dextran biosensor chips (CM-5; BIAcore, Inc., Piscataway, NJ) using the surface-thiol method as described by the manufacturer. Lyophilized peptide preparations were dissolved in 100 mM Hepes buffer (pH 7.2), then diluted serially into PBS containing 0.05% Tween-20 and 0.01% NaN_3 and injected at a rate of 10 μL/min. The amount of peptide bound at steady state was measured as the difference between FcεRIα and blank flow cells at a point when the SPR signal was constant with time (approximately 10 s prior to the end of injection).

Cell-Based Binding and Activity Assays. For binding inhibition assays, CHO-3D10 cells, which express the human FcεRIα subunit (*6*), were cultured overnight in Falcon 96 microwell plates (5×10^4 cells per well). Peptides were diluted in assay buffer, F12/DMEM (Life Technologies, Rockville, MD), 1% bovine serum albumin, 0.05% NaN_3, and 100 mM Hepes (pH 7.2), at initial concentrations of 50−500 μM. This was followed by the addition of 50 μL of Iodogen-labeled [^{125}I]IgE (specific activity, 1600−2300 Ci/mmol) to give a final concentration of 0.75 nM, and incubation for 45−60 min at 4 °C. Cells were washed with assay buffer to remove unbound [^{125}I]IgE. The cells were solubilized in 200 μL of 1 N NaOH for 10 min, and then transferred to a microtube for γ-counting.

Peptides were tested in a modified histamine-release assay (*34*) to determine whether they stimulated histamine release themselves and whether they prevented histamine release in response to ragweed and ragweed-specific IgE. Briefly, 4×10^4 RBL-48 cells (*35*), which express human FcεRIα, were cultured overnight in a Falcon 96 flat microwell dish. For testing peptide stimulation, 0.5 mM peptide was added with incubation for 1 h at 37 °C in a 5% CO_2 humidified incubator. The medium was removed and assayed for histamine as described below. For testing peptide inhibition, peptides were diluted (generally in triplicate) and added to cells for 15 min at 24 °C, followed by addition of 5 μL (420 ng/mL) of ragweed-specific IgE plasma (North American Biologicals, Boca Raton, FL) and incubation for 2 h as described above. To this mixture was added 10 μL of 1.0 μg/mL ragweed allergen (Hollister Stier, Spokane, WA), and the mixture was incubated for 30 min. The medium was assayed for histamine levels using a commercial histamine ELISA kit (IM2015, Coulter/Immunotech, Marseille, France).

Concentrations of released histamine as well as concentrations (IC_{50}) of peptides required to inhibit [^{125}I]IgE binding or histamine release were determined by fitting the data with four-parameter curves using Kaleidagraph software.

RESULTS

Selection of FcεRIα-Binding Peptides from Peptide-Phage Libraries. Peptide libraries displayed in a polyvalent phage format were used to search for antagonists of the high-affinity IgE receptor. Such libraries have often yielded relatively small, structured inhibitors of enzyme activity or protein−protein interactions (*20*, *36*, *37*; reviewed in ref *38*). Following each round of selection, an enrichment factor, the ratio of phage specifically binding FcεRIα-Ig versus those binding a control, was determined. Only one library, encoding the degenerate peptide $X_4CX_2GPX_4CX_4$, exhibited an increase in the level of enrichment: 15-, 670-, and 320-fold in rounds 3−5, respectively. Screening of individual clones from rounds 3 and 4 indicated that more than 95% of the clones specifically bound FcεRIα-Ig, with little or no detectable binding to plates coated with albumin or an IgG antibody. In addition, 95% of the positive clones were inhibited from binding the receptor in the presence of IgE, suggesting that the peptide phage bound at or near the IgE binding site on the receptor.

Interestingly, by round 3 of our initial selections, only four different peptide sequences were found (see Figure 1, g8a series); more than half the sequenced clones had the sequence (g8a.37) MGTLCLEGPEGWFCIESA. The remaining clones were related in sequence, having several residues conserved: Glu7, Gly11, Trp12, and a hydrophobic residue (Leu, Val, or Phe) at position 6. All contained the fixed residues Gly8 and Pro9, as well as Cys5 and Cys14, suggesting a possible disulfide-loop structure. A peptide, IGE01, based on clone g8a.37 was synthesized and found to inhibit [^{125}I]-IgE binding to FcεRI-expressing CHO-3D10 cells, although weakly, with an estimated IC_{50} of $>$160 μM (Figure 2).

Peptide	Sequence	IC_{50}(μM)	S.S.
IGE01	MGTLCLEGPEGWFCIESA-nh_2	>160	
IGE02	Ac-EIQECTEGPWGWFCVGSG-nh_2	37	
IGE03	Ac-EIQECTEGPWGWVCM-nh_2	7.6	
IGE04	Ac-NLPRCTEGPWGWVCMAAD-nh_2	1.5	++
IGE05	Ac-NLPRCTEGPWGWVCM-nh_2	1.8	
IGE06	Ac-NLPRCTEGPWGWVCM	1.8	++
IGE07	NLPRCTEGPWGWVCM	1.0	++
IGE08	NLPRCTEGPWGWVCI	1.4	
IGE09	NLPRCTEGPWGWVC-nh_2	13	++
IGE10	NLPRCTEGPWGWVCM-nh_2	0.8	
IGE11	LPRCTEGPWGWVCM-nh_2	3.3	
IGE12	PRCTEGPWGWVCM-nh_2	34	
IGE13	CTEGPWGWVCI-nh_2	>500	
IGE14	Ac-NLPRCAEGPWGWVCM	6.9	
IGE15	Ac-NLPRCTAGPWGWVCMAAD-nh_2	10	
IGE16	NLPRCTEAPWGWVCM	160	++
IGE17	NLPRCTEGAWGWVCM	>500	++
IGE18	NLPRCTEGpWGWVCM	>500	-
IGE19	Ac-NLPRCTEGx_1WGWVCM	23	++
IGE20	Ac-NLPRCTEGx_2WGWVCM	44	++
IGE21	Ac-NLPRCTEGx_3WGWVCM	84	-
IGE22	Ac-NLPRCTEGx_4WGWVCM	360	
IGE23	Ac-NLPRCTEGx_5WGWVCM	>500	++
IGE24	NLPRCTEGPAGWVCM	82	++
IGE25	NLPRCTEGPWAWVCM	43	+
IGE26	Ac-NLPRCTEGPWaWVCM	8.6	++
IGE27	Ac-NLPRCTEGPWeWVCM	1.7	++
IGE28	NLPRCTEGPWGAVCM	120	+
IGE29	Ac-NLPRCTEGPWGx_6VCM	1.2	++
IGE30	Ac-NLPRCTEGPWGWACM	14	
IGE31	NLPRCTEGPWGWYCM	130	++

FIGURE 2: Synthetic peptide analogues. In each peptide, a disulfide bond connects the two Cys residues. Shaded positions indicate changes vs the IGE06 sequence. Non-natural amino acid substitutions are as follows: a, D-Ala; e, D-Glu; p, D-Pro; x_1, L-pipecolic acid; x_2, *N*-methylalanine; x_3, *N*-methylglycine; x_4, L-octahydroindole-2-carboxylic acid; x_5, hydroxyproline; and x_6, 2-naphthylalanine. Ac- and -nh_2 indicate N-terminal acetyl and C-terminal amide groups, respectively. The activity of peptides in cell-based IgE binding assays (see Figure 3B) is shown as the mean IC_{50} (micromolar). Errors (standard deviation) in IC_{50} were generally ±30−50%. All peptides that have a structural score (SS) have been evaluated by NMR (see Experimental Procedures) and found to have (++) structure similar to that of IGE06, (+) structure similar to that of IGE06, but less stable, or (−) structure different from that of IGE06.

In a second series of selections on the $X_4CX_2GPX_4CX_4$ library, peptide IGE01 was included at 10 μM as a competitor to promote recovery of higher-affinity variants. At rounds 2 and 3, all sequenced clones appeared to be related to g8a.37, and no new clones were found in round 4 (Figure 1, g8b series). Peptides based on each of these sequences were synthesized. The peptide g8b.35 was not soluble in the cell-based binding assay buffer; however, peptide IGE02 (Figure 2) was fully soluble and inhibited [^{125}I]IgE binding to FcεRI on cells with an IC_{50} value of 37 μM, a >4-fold improvement in activity over the IGE01 peptide.

Optimization of Peptides by Monovalent Phage Display. We used monovalent phage display (see refs *21* and *38*) as a means of selecting higher-affinity peptide variants. Phage clones g8b.11 and g8b.35 (see Figure 1) were used as starting templates ("wild-type"), and new libraries were constructed with randomization in the four N-terminal, the eight loop-region (including the previously fixed Gly-Pro), and the four C-terminal residues. Only the g8b.35 N-terminal library exhibited significant binding enrichment. A small sampling of sequences revealed N-terminal sequences with no overall consensus, except for Pro3, and a hydrophobic residue at position 2 (Figure 1). A synthetic peptide, IGE04, based on clone g3a.1 (Figure 1), inhibited [^{125}I]IgE binding to FcεRI on cells with an IC_{50} value of 1.5 μM, representing a more than 100-fold improvement over the initial IGE01 peptide and a 25-fold improvement over the IGE02 peptide (Figure 2). Comparison of IGE05 with IGE03 shows that changes in the four N-terminal residues improved affinity ~4-fold.

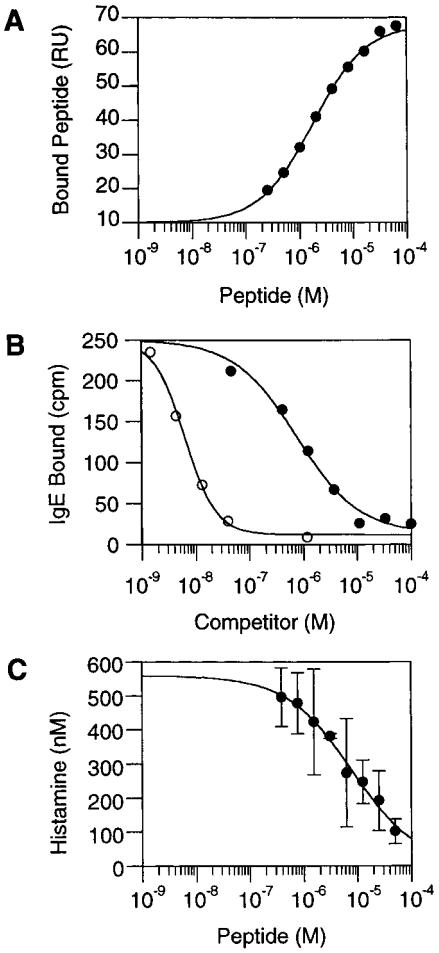

FIGURE 3: Assays of peptide IGE06 binding and activity. Typical experiments are shown. (A) A direct binding assay using SPR for measuring the relative affinities of peptides for FcεRIα. Serial dilutions of peptide were injected over a biosensor chip coupled with FcεRIα. The amount of peptide bound over background at steady state yields an estimate of peptide binding affinity (EC_{50} ± standard error) of 1.1 ± 0.1 μM in the experiment whose results are shown. (B) Binding inhibition using ^{125}I-labeled IgE. Cells expressing human FcεRIα were incubated with cold IgE (○) or serial dilutions of peptide IGE06 (●). The IC_{50} (± standard error) measured for IGE06 inhibition of IgE binding was 0.75 ± 0.17 μM in this experiment. (C) Cell-based activity assay of histamine release (mean ± standard deviation of triplicate samples). IGE06 inhibited IgE-induced histamine release from basophils expressing human FcεRIα with an IC_{50} of 7.9 ± 2.2 μM in the experiment whose results are shown.

Peptide Minimization and Analogues. Truncations of peptide IGE04 showed smaller inhibitors were possible (Figure 2). IGE06, a 15-residue peptide with an acetylated N-terminus and a free C-terminus, was particularly soluble, and inhibited IgE binding to cells expressing human IgE receptors, with an IC_{50} of 1.8 μM. SPR steady-state binding measurements confirmed that the peptides bind to FcεRIα, rather than to IgE. IGE06 was found to have a binding affinity of 1−2 μM (Figure 3A), in excellent agreement with the IC_{50} value determined from the cell-based inhibition assays (Figure 3B,C).

No significant differences in affinity were observed with modifications of the N-terminal amine or C-terminal acid (IGE05, IGE07, and IGE10; Figure 2). IGE11, a 14-residue peptide, truncated by one residue at the N-terminus and three residues at the C-terminus, exhibited an IC_{50} within 2-fold of that for the 18-residue peptide IGE04. However, IGE09 and IGE12, which have further truncations compared to IGE06, demonstrated significant reductions of 10−20-fold in the level of binding inhibition; IGE13, truncated by four N-terminal residues, showed no detectable inhibition (Figure 2). Thus, the three residues preceding the first Cys and a hydrophobic residue (Met ≈ Ile) following the second Cys are needed for high-affinity binding in this series.

In an initial Ala scan, using phage-displayed peptides, Ala substitution of Val13 led to a 13-fold reduction in the level of binding, while substitutions at Leu2, Pro3, Arg4, and Thr6 each resulted in an only 2−6-fold loss in the level of binding (data not shown).

Synthetic peptides tested the role of individual side chains in receptor binding (Figure 2) and in stabilizing peptide structure (see below). Ala substitutions at Gly8 (IGE16), Pro9 (IGE17), and Trp12 (IGE28) caused 160-, >500-, and 120-fold reductions in the level of binding, respectively. Ala substitutions at nearby positions Trp10 (IGE24) and Gly11 (IGE25) also caused significant (40−80-fold) reductions in affinity. On the other hand, replacement of residues Thr6 (IGE14), Glu7 (IGE15), and Val13 (IGE30) with Ala resulted in only 4−8-fold losses in affinity for each. Tyr, a larger hydrophobic residue introduced for potential radiolabeling, was not tolerated as well as Ala in place of Val13 (IGE31). The specificity of the peptide's side chain interactions with FcεRIα is demonstrated particularly by a series of "proline analogues" which show that the Pro9 side chain is difficult to replace without loss of activity (Figure 2).

Binding of the peptides to human FcεRIα appears to be highly specific. For species specificity testing, we radiolabeled murine IgE and rat IgE, and tested binding on murine (MC/9; ATCC) or rat (RBL-2H3; ATCC) cell lines expressing the high-affinity IgE receptor as described above. No inhibition of murine IgE or rat IgE binding to murine or rat cells, respectively, could be detected with peptide concentrations as high as 50 μM (data not shown).

Structure Determination. Analysis of IGE06 by NMR spectroscopy revealed strong evidence that this peptide adopts a well-defined three-dimensional structure in solution. Backbone and side chain coupling constants and ^{1}H resonance frequencies are significantly different from those expected in an unstructured peptide (e.g., $^3J_{HN-H\alpha}$ values are ≥9.0 Hz for Cys5, Thr6, Trp10, Val13, and Cys14, and $^3J_{H\alpha-H\beta}$ values indicate that the side chains of residues Cys5, Thr6, Trp10, and Cys14 have fixed χ_1 angles). Moreover, peaks in NOESY and ROESY spectra indicate that there are many proton−proton contacts (<5 Å) between residues that are not adjacent in the primary sequence. The structure of IGE06 was calculated from a total of 109 distance and 16 dihedral angle restraints as described previously (*32*). The final ensemble of 20 models satisfies the input data very well, having no distance or dihedral angle restraint violations greater than 0.1 Å or 2°, respectively. The structure also has good covalent geometry as judged by the program PROCHECK with 72 ± 11% of the residues in the most favored region of $\phi-\psi$ space, with none in the disallowed or generously allowed regions (*39*).

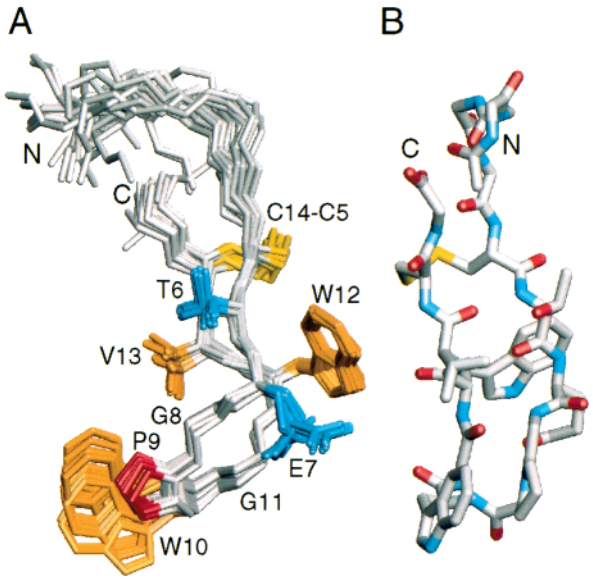

FIGURE 4: Solution structure of IGE06. (A) The ensemble of 20 structures, showing side chains for residues 5−14 only. Side chains are colored on the basis of their importance for peptide structure or function, as judged by the effects of substitutions (from most important to least, red > orange > blue). (B) All heavy atoms of the representative structure for IGE06 are shown, except for the side chains of residues 1−4 and 15, which are not shown for clarity. The view shown in panel B represents a 90° rotation from that shown in panel A. The backbone rmsd from the mean coordinates of the ensemble for residues 5−14 is 0.31 ± 0.06 Å (0.58 ± 0.09 Å for all heavy atoms).

IGE06 is composed of two β-strands comprised of residues 5−8 and 11−14, connected by a type I β-turn centered at residues Pro9 and Trp10 with Gly11 adopting a positive ϕ angle; Arg4 and Met15 extend the β-strands in some of the models (Figure 4). The three N-terminal residues (Asn1, Leu2, and Pro3) are not well-defined by the NMR data and appear to be more flexible in solution than residues Cys5−Cys14; the major species in solution has Pro3 in the trans conformation, although the cis form can also be detected.

Analysis of peptide variants of IGE06 showed that many, but not all, retained this structure (Figure 2). In IGE06, the side chain of Trp12 packs against the disulfide bond and contributes to the stability of the peptide as evidenced by comparison of NMR data obtained from a peptide with Trp12 substituted with Ala (IGE28). The disulfide bond is essential for stabilizing the hairpin conformation; there is no evidence of stable structure when excess reductant (dithiothreitol) is added to the NMR sample. Gly11 (in position 4 of the type I β-turn) adopts a positive ϕ angle; this backbone conformation can only be readily attained by glycine, which explains why the structure of a peptide with Gly11 replaced with Ala is significantly less stable (IGE25). However, D-amino acids readily adopt this conformation as seen in peptides with Gly11 replaced with D-Ala (IGE26) or D-Glu (IGE27). Importantly, NMR analysis of peptides having alanine substitutions for Gly8, Pro9, and Trp10 (IGE16, IGE17, and IGE24, respectively) indicates that these substitutions do not disrupt the hairpin structure, yet have a significant impact on FcεRIα binding affinity (Figure 2).

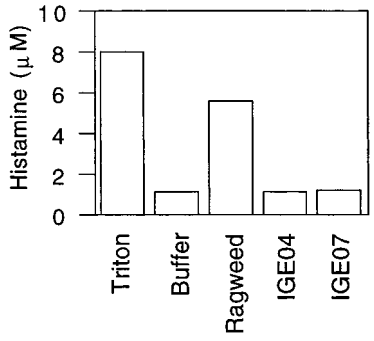

FIGURE 5: Peptides do not cause signaling through the high-affinity IgE receptor. Basophils expressing human FcεRIα were incubated with Triton (to release total cellular histamine), buffer control, ragweed with IgE, 500 μM peptide IGE04, or 500 μM peptide IGE06. The level of histamine release was measured as described for the experiment whose results are shown in Figure 3C.

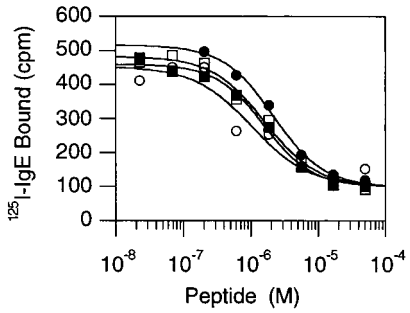

FIGURE 6: Stability of hairpin peptides. IGE06 was incubated for 24 h at 37 °C in murine serum (○), lung lavage (■), or minced lung tissue (□), and then the results were compared with an IGE06 standard (●) in an IgE binding inhibition assay as in Figure 3B.

Biological Assays of Peptide Activity. A cell-based potency assay demonstrated that peptide IGE06 acts as a functional antagonist by inhibiting allergen-induced activation of RBL-48 cells, an FcεRI-expressing basophil cell line (*35*), expressing human FcεRIα. IGE06 demonstrated an IC_{50} of <10 μM (Figure 3C), whereas control peptides had no activity in inhibiting histamine release.

The peptides themselves were tested for activating the receptor by incubating peptides with RBL-48 cells at a high concentration (0.5 mM) and assaying for released histamine. Importantly, no release of histamine was induced following incubation with peptides IGE04, IGE07, and IGE12, or the control peptide (Figure 5). In contrast, cells exposed to IgE and ragweed allergen did cause histamine release. Thus, peptide binding does not lead to receptor activation.

To test for peptide susceptibility to proteolytic digestion, peptides were incubated at 37 °C for 24 h in mouse serum, lung lavage fluid, or homogenized lung tissue, and then assayed for activity in inhibiting [^{125}I]IgE binding to cell-surface-expressed FcεRI. None of these treatments resulted in loss of peptide activity (Figure 6).

DISCUSSION

Direct binding selections from naïve peptide-phage libraries using the high-affinity IgE receptor led to identification of a novel class of disulfide-constrained peptides that form stable β-hairpin structures in solution. Although an immunoglobulin fusion of the entire extracellular domain of FcεRIα provided many possible epitopes for peptide binding, even the initial peptide-phage clones were blocked from binding to FcεRIα by IgE. Synthetic peptides derived from these sequences not only inhibited IgE binding to FcεRIα but also inhibited histamine release from cells in the low micromolar concentration range. Binding was specific for human IgE receptor, with no detectable binding to murine or rat receptors (each ∼50% identical in sequence to the human form). Significantly, the peptides alone did not induce histamine release from cells, even at concentrations exceeding that needed for receptor saturation.

A comparison of the sequences of the selected phage peptide clones with FcεRIα, IgE, and other peptides reported to inhibit IgE binding to receptor failed to reveal similarities. Moreover, a database search (Dayhoff database, March 23, 2001) revealed no peptide or protein sequences that were convincingly similar to that of IGE06. The most closely related peptides were from peptide phage with CXXGP-WXXXC motifs identified for binding to an insulin-like growth factor binding protein (*20*) or for binding to the erythropoietin receptor (*36*). Thus, the peptides described here appear to use novel binding determinants for binding FcεRIα at a site overlapping that for IgE binding.

Design of improved antagonists requires further information about the relationship between peptide structure and function. Remarkably, IGE06 and its analogues form stable β-hairpins with type I turns. Interestingly, phage-peptide selection based solely on optimizing binding affinity appears to have also selected for residues that stabilize the hairpin conformation. For example, Trp was selected at position 12, in the non-hydrogen-bonded position of the β-hairpin adjacent to the disulfide bond. Studies with model systems have shown that Trp is by far the most stabilizing natural amino acid in this position (*40*). Furthermore, residue substitutions that decrease the stability of the hairpin structure also reduce the binding affinity, suggesting that the peptide adopts a similar structure when bound to FcεRIα.

Conversely, individual Ala substitutions at Gly8, Pro9, or Trp10 do not disrupt the hairpin structure, yet each results in a significant decrease in receptor binding affinity, implying that these residues make direct contact with receptor. For Trp10, the contact requirements are not extremely stringent as a large hydrophobic group, 2-naphthylalanine, substitutes as well (Figure 2). Residues 8−11 form a type I β-turn that can only readily be accommodated in a hairpin, provided the residue at the position of Gly11 adopts a positive φ angle. Indeed, replacement of Gly11 with L-Ala destabilizes the structure of the peptide and causes a 40-fold decrease in affinity, whereas replacement with D-Ala or D-Glu has little effect on structure or activity. Taken together, the contributions to the binding affinity made by Gly8, Pro9, and Trp10 appear to require the specific geometry presented in IGE06.

The largest impact on binding was found for substitutions of Pro9. No detectable binding was observed when Pro9 was replaced with alanine (IGE17) despite the fact that the structure and stability of the turn were indistinguishable from those of IGE06 (not shown). As of yet, attempts to improve the affinity with analogues at Pro9 (Figure 2) have not resulted in increased binding affinity, but have confirmed the importance of this residue, and suggest that there is a proline-shaped pocket on the surface of the receptor where this residue binds. Interestingly, in the crystal structure of

the IgE−FcεRIα complex, there is a proline on IgE (Pro426) that packs intimately between two tryptophans (Trp87 and Trp110) on the receptor, forming a so-called "proline sandwich" (*8*). This type of interaction appears to be conserved in all Fc receptor−Ig complexes, as the two tryptophans are conserved in all Fc receptors, and the proline is present in both IgE and IgG (*41*). It is certainly possible that IGE06 inhibits IgE from binding its receptor by occupying this important site of interaction on the IgE receptor. Other binding determinants, perhaps stemming from interactions with Val13 and residues near the N-terminus of the peptide, could confer the observed specificity for human FcεRIα.

Structural analysis of the IGE06−FcεRIα complex will determine the molecular details of the peptide−receptor interaction and may provide insights into the design of small-molecule antagonists of IgE. Such molecules could lead to orally bioavailable drugs, with advantages of relatively low production cost and ease of delivery, for the treatment of allergic diseases. While the smallest active peptides described here are 14 residues in length (MW ≈ 1690), with contributions from the N-terminal as well as central-loop residues, it is clear that a subset of side chains, especially Pro9 and Trp10, contribute a large share of the energy of interaction with FcεRIα. Even though these side chains are hydrophobic in nature, the peptides have selectivity for the human receptor, and stringent requirements (especially Pro9; Figure 2) for binding recognition.

In the absence of further structural information about the peptide−receptor complex, we cannot rule out the possibility that inhibition of IgE results from a conformational change in the receptor induced by binding of the peptide. However, little conformational change in FcεRIα was observed between the free and IgE-bound forms (*7*, *8*). It therefore seems unlikely that peptide binding could induce such a change. The complete inhibition of IgE binding at saturating concentrations of peptide and the agreement between apparent peptide affinities determined in direct-binding and IgE-binding inhibition experiments (Figure 3A,B) are consistent with a competitive inhibition model. Thus, the peptides appear to function as competitive inhibitors of IgE.

The inherent stability of these peptides in serum and lung lavage samples, as well as their failure to elicit receptor activation even at high peptide concentrations in vitro, suggests that related peptides might be developed as therapeutic agents themselves, an idea raised by similar studies on other systems (see refs *18*, *20*, *36*, and *42*). Although the present peptides are of modest affinity, successive mutagenesis and binding selections, including peptide insertions, can often lead to improved peptide affinity (*36*−*38*). In addition, structural information about the peptide−receptor interface may provide the basis for engineering improved binding affinity by altering functional groups in synthetic peptide analogues. Our data do not address drug-development issues such as half-life, delivery technology, possible immunogenicity, or other safety issues, such as peptide or antibody-induced aggregation, which may have severe consequences. However, the inherent stability of the FcεRIα-binding hairpin peptides described here should facilitate the investigation of FcεRI antagonists in vivo, including issues of safety and possible feedback regulation of IgE synthesis.

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SUPPORTING INFORMATION AVAILABLE

Resonance assignments and coupling constants for IGE06 at 288 K and pH 5.7. This material is available free of charge via the Internet at http://pubs.acs.org.

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