The membrane-proximal part of FcεRIα contributes to human IgE and antibody binding – implications for a general structural motif in Fc receptors

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Abstract The high affinity receptor for human IgE (FcεRI) on tissue mast cells and blood basophils is responsible for immediate hypersensitivity reactions. Binding of human IgE (hIgE) to FcεRI has been shown to be mediated via three independent regions in the extracellular part of the α-subunit of FcεRI (ecFcεRIα). By site-directed mutagenesis we investigated the contribution of amino acids within the ecFcεRIα FG loop (residues Lys^{154}–Leu^{165}) to binding to hIgE and two monoclonal anti-FcεRIα antibodies (15/1, 5H5/F8). The mutated receptors were expressed and secreted from eukaryotic cells as amino-terminal fusion to HSA. We show that the proposed loop region contributes partly to hIgE binding and that the epitope of mAb 15/1, which inhibits hIgE/FcεRIα interaction, maps to this region whereby a single W156A mutation results in complete loss of mAb 15/1 binding. In contrast, hIgE binding is not affected by the W156A mutation indicating that different amino acid residues within the loop are recognized by the mAbs 15/1 and hIgE. MAb 5H5/F8 does not recognize a receptor mutant truncated to Ile^{170}. By screening a random dodecapeptide library displayed on bacterial flagella the epitope for mAb 5H5/F8 was mapped to P^{173}REKY^{177} whereas one of the 15/1 binding clones displayed a peptide with an amino acid sequence homologous to Leu^{158}–Ile^{167}. Based on the epitopes identified for the inhibitory mAb 15/1 and the non-inhibitory mAb 5H5F8 and on binding data obtained with polyclonal antisera raised against two ecFcεRIα peptides, we propose a structural element in the membrane proximal part of ecFcεRIα which forms a 3D structure which might facilitate specific and efficient attachment of hIgE.

Key words: Human high affinity IgE receptor; IgE binding site; Epitope mapping; Alanine substitution mutagenesis; Homology alignment

1. Introduction

The binding of IgE to the high affinity Fcε receptor (FcεRI) on mast cells and basophils is mediated by the α-subunit of the tetrameric receptor complex (for review see [1]). The β-chain and the disulfide-linked γ-chains appear to function primarily in signal transduction [2]. For high affinity binding of hIgE ($K_D = 10^{-10}$) the extracellular portion of the FcεRIα subunit (ecFcεRIα) is sufficient [3] which consists of two Ig-like domains: α(1), membrane-distal, and α(2), membrane-proximal. Although in humans IgE binding to FcεRIα is mediated via the α(2) domain, high affinity binding is only achieved in the presence of α(1) [4]. In contrast to hIgE where single amino acid residues within the Cε3 region have been identified to be involved in receptor binding [5,6], the binding site on the receptor is less well characterized (for review see [7]). According to an existing homology-based 3D model [8], each of the regions postulated by Hulett et al. [9] to be involved in hIgE binding contains a surface-exposed loop connecting anti-parallel β-strands. Several lines of evidence suggest that the C-C′-F-G face of the α(2) domain is involved in the hIgE/FcεRIα interaction (reviewed in [1]). A synthetic cyclized C-C′ peptide mimic (Ile^{119}–Tyr^{129}) was shown to inhibit IgE binding to FcεRIα [10]. Recently, Cook et al. [11] demonstrated that a K117D mutation in the C strand reduced the affinity for IgE by a factor of 30. Mapping of Ab specific epitopes on a receptor protein or its ligand has been used as an experimental approach to characterize amino acid regions involved in the ligand-receptor interaction [12,13]. MAbs against FcεRIα have also been used as tools to identify stretches of the α(2) domain which are assumed to take part in hIgE binding; e.g. inhibitory mAb 15A5 [14] binds to a peptide corresponding to Gly^{100}–Val^{115} in α(2). Another inhibitory mAb, 15/1, has been described as a neutralizing antibody in various investigations [15,16].

In this report, the contribution of amino acid residues within the FG loop to hIgE binding and their allocation to epitopes recognized by monoclonal anti-FcεRIα Abs 15/1 and 5H5/F8 was investigated.

2. Materials and methods

2.1. Materials

ecFcεRIα was produced in insect cells and purified at Novartis Basle. FcεRIα-specific mAb 15/1 [15] was obtained from J.-P. Kinet, Harvard Medical School, Boston, MA. The anti-ecFcεRIα mAb 5H5F8 has been described [17]; rabbit polyclonal anti-FcεRIα antiserum was produced at Novartis Vienna and human IgE was obtained from B. Stadler, University Bern.

2.2. Cyanogen bromide cleavage of ecFcεRIα

100 μg ecFcεRα was subjected to cyanogen bromide cleavage [18] and products were separated by HPLC. Elution was performed on a 0.1% trifluoroacetic acid/acetonitrile gradient. The fragment Gly^{100}–Leu^{179}, containing α(2), was isolated and used in ELISA to test for binding of hIgE and mAb.

2.3. Endoproteinase Arg-C cleavage of ecFcεRIα

100 μg ecFcεRIα was diluted 1:1 with 2×incubation buffer (200 mM Tris-HCl, pH 7.6, 20 mM $CaCl_2$) and incubated with endoproteinase Arg-C (Boehringer Mannheim) according to the manufacturer's protocol. The reaction was brought to pH 3.0 with 25% trifluoroacetic acid and separated by reversed phase chromatography (Beckman model 126). Elution was performed with an increasing tri-

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Abbreviations: FcεRI, human high affinity Fc receptor for IgE; FcεRIα, α-subunit of the human high affinity Fc receptor for IgE; hIgE, human IgE

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fluoroacetic acid gradient (5–70%). Eluted peak fractions were lyophilized and tested in ELISA for binding to hIgE and to mAbs. Amino acid analysis was carried out with 0.5–1 nmol peptide hydrolyzed in vapor phase (at 110°C for 24 h) with 6 M HCl containing 0.1% phenol. The amino acid composition of this mixture was determined with an LKB 2131 Alpha Plus instrument.

2.4. Mutagenesis

Receptor mutants were expressed in COS-7 cells as amino-terminal fusion to HSA (HSA-ecFcεRIα). 5×10^6 cells were electroporated with 30 μg DNA with a Bio-Rad Gene Pulser (250 μF, 400 V), then transferred to a 80 cm^2 culture flask (Nunc) with 25 ml medium (DMEM H21, 10% fetal calf serum, 1% penicillin/streptomycin, 1% L-glutamine) and incubated for 72 h at 37°C with 5% CO_2.

Two unique *Hin*dIII restriction sites were introduced in FcεRIα by PCR by mutating nucleotides a643g, g644c, g646t and c679g, a680c (sequence according to [19]) which resulted in the amino acid changes V155L and N166K, I167L (numbering according to [20]). Mutants were generated using splice overlap extension PCR with *Hin*dIII site flanked primers (bold). Mutant 1 (*Hin*dIII cassette, HSA-ecFcεRIα Val^1–Ile^{170}, V155L, N166K, I167L) forward: 5′ TCA TAC TAG TGA ATT CAC CAT GAA GTG GGT AAC C 3′, reverse: 5′ CCT CGA GGT GAA TTC TTA TCA TAT TAC AGT **AAG CTT** GAG GGG CTC AGA CTC ATA GTC CAG CTG CCA **AAG CTT** GCC CGT ACA GTA GTA GG 3′. Mutant 3 (FcγRII) forward: 5′ CAC TGC ACA GGA **AAG CTT** GGC TAC ACG CTG TTC TCA TCC AAG CC 3′, reverse: 5′ CAC TTG GAC AGT **AAG CTT** CAC AGG CTT GGA TGA GAA CAG CG 3′. Mutant 4 (S162A) forward: 5′ CAC TGC ACA GGA **AAG CTT** TGG CAG CTG GAC TAT GAG GCC GAG CCC 3′, reverse: 5′ CAG TTG GAC AGT **AAG CTT** GAG GGG CTC GGC CTC ATA GTC CAG CTG CCA 3′. Mutant 5 (Q157A, S162A) forward: 5′ CAC TGC ACA GGA **AAG CTT** TGG GCT CTG GAC TAT GAG GCT GAG CCC 3′, reverse: 5′ CAG TTG GAC AGT **AAG CTT** GAG GGG CTC AGC CTC ATA GTC CAG AGC CC 3′. Mutant 6 (W156A, D159A, E161A, E163A) forward: 5′ CAC TGC ACA GGA **AAG CTT** GCC CAG CTG GCT TAT GCT TCT GCT CCC 3′, reverse: 5′ CAG TTG GAC AGT **AAG CTT** GAG GGG AGC AGA AGC ATA AGC CAG CTG GGC 3′. Mutant 7 (W156A, Y160A) forward: 5′ CAC TGC ACA **AAG CTT** GCC CAG CTG GAC GCT GAG TCT GAG CCC 3′, reverse: 5′ CAG TTG GAC AGT **AAG CTT** GAG GGG CTC AGA CTC AGC GTC CAG CTG GGC 3′. Mutant 8 (W156A) forward: 5′ CAC TGC ACA GGA **AAG CTT** GCC CAG CTG GAC TAT GAG TCT GAG CCC 3′, reverse: 5′ CAG TTG GAC AGT **AAG CTT** GAG GGG CTC AGC CTC ATA GTC CAG CTG GGC 3′. PCR fragments were *Hin*dIII digested and ligated in the *Hin*dIII cut expression cassette of mutant 1. Mutant 2 (loop deletion) was created by cutting plasmid DNA encoding mutant 1 with *Hin*dIII and religation. Each mutant receptor DNA sequence was confirmed by double-stranded dideoxy sequencing [21].

2.5. Concentration determination of receptors in cell supernatants (ELISA)

100 μl of a solution containing 5 μg/ml rabbit polyclonal anti-HSA (Cappel, Durham, NC) in a buffer containing 0.1 M $NaHCO_3$, 0.01% NaN_3, pH 9.6, were immobilized in Strip Plate-8 wells (Costar, Cambridge, MA) in a humidified chamber at 4°C overnight. After washing twice with 300 μl PBS (150 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM K_2PO_4, pH 7.5) containing 0.05% Tween 20 (PBS_T) pH 7.2, 100 μl of COS supernatants was added and incubated for 2 h at room temperature. The wells were washed twice with 300 μl PBS_T and incubated with 100 μl of a goat anti-human HSA-horseradish peroxidase (HRP) conjugate (Cappel, 1:1000 diluted in PBS_T, 2% FCS) for 90 min at room temperature. Plates were washed and subsequently incubated with 100 μl of ABTS (Bio-Rad) substrate. The reaction was stopped after 5 min with 100 μl of 3% oxalic acid and absorbance at 405 nm was read. The readouts were corrected for unspecific binding assayed with supernatants of mock-transfected cells. The concentration was determined using an HSA-standard calibration curve; all samples were assayed in triplicates. Binding of receptor mutants to immobilized mAb and hIgE was tested as described before with 200 ng mAb (15/1, 5H5/F8) or 240 ng hIgE were immobilized instead of the polyclonal anti-HSA conjugate.

2.6. Epitope mapping of mAb 5H5/F8 and 15/1

The pFliTrx random peptide library (Invitrogen, San Diego, CA) panning procedure and colony blot analysis were exactly performed following the protocol of Lu et al. [22]. MAbs bound to colonies in dot blot experiments were incubated with goat anti-mouse IgG conjugated to HRP (Bio-Rad) and developed with the ECL kit (Amersham).

2.7. Production and purification of rabbit antisera

Polyclonal sera were raised in rabbits against the keyhole limpet hemocyanin (KLH) coupled peptides $N^{166}ITVI^{170}$ and $E^{161}SEPLNITVIKAPRE^{175}$. Sera were tested in ELISA for binding to ecFcεRIα and for their ability to inhibit hIgE/FcεRIα interaction, and purified with the AvidChrom Protein A Antibody Purification Kit (Sigma). The obtained IgG preparation was affinity purified using the peptide coupled to activated CH Sepharose 4B (Pharmacia) procedure.

2.8. Binding of anti-peptide antisera to immobilized ecFcεRIα (ELISA)

Strip Plate-8 wells (Costar) were incubated at 4°C overnight with 100 ng ecFcεRIα in 100 μl coating buffer (0.1 M $NaHCO_3$, 0.01% NaN_3, pH 9.6) in a humidified chamber. After blocking, wells were incubated in duplicate with 2, 4 and 8 μg/well of each affinity purified anti-peptide antiserum for 2 h at room temperature and subsequently with a goat anti-rabbit IgG HRP conjugate (Bio-Rad) for 1 h at room temperature. The wells were washed three times with PBS_T and further incubated with 100 μl of the ABTS substrate (Bio-Rad). The reaction was stopped with 100 μl of 2% oxalic acid and color intensities were read at 405 nm.

2.9. Competition ELISA

1 ng biotinylated ecFcεRIα was preincubated with 0.15, 1, 5, 10 μg/well antisera in 100 μl reaction volume – all samples were assayed in triplicate. The mixtures were incubated at 4°C overnight and subsequently 1 h at 25°C. Then the samples were transferred to microtiter plate wells each coated with 150 ng hIgE. After a 2 h incubation the wells were washed as described and incubated with 100 μl of a 1:1000 dilution of streptavidin conjugated to alkaline phosphatase (Gibco) for 1 h at 25°C. Wells were developed with the Alkaline Phosphatase Substrate Kit (Bio-Rad).

3. Results and discussion

The experiments shown in this report focused on assessing the contribution of the FG loop of the extracellular region of FcεRIα (ecFcεRIα) to hIgE binding. Insight into the struc-

Table 1
Binding of Igs to ecFcεRIα fragments generated by chemical or protease cleavage (ELISA)

Cleavage	Fragment isolated	Recognition of fragment by			
		pc.-a serum	human IgE	mAb 15/1	mAb 5H5/F8
BrCN	Glu^{99}–Leu^{179}	+++	–	–	++
Arg-C/1	Tyr^{129}–Lys^{154}	+++	–	–	–
Arg-C/2	Val^{155}–Lys^{171}	+++	–	–	–
Arg-C/3	Val^{118}–Lys^{128}	–	–	–	–
Arg-C/4	Asn^{112}–Lys^{176}	+++	–	–	++

++ indicates binding 10-fold over background and 2-fold lower than the reference polyclonal antiserum (pc.-a +++).

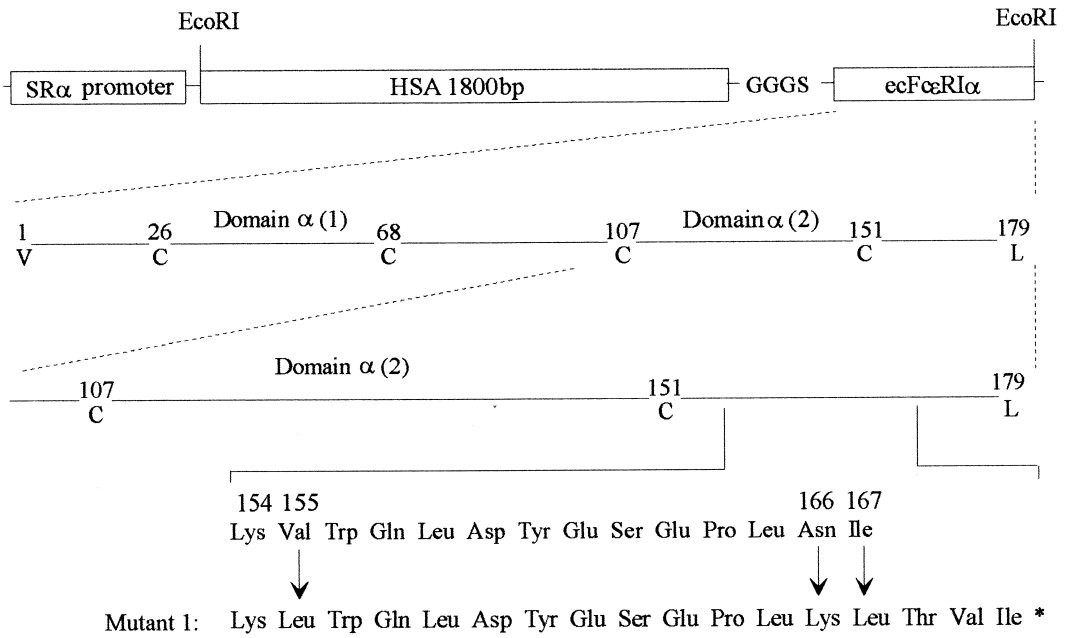

Fig. 1. HSA-ecFcεRIα expression construct for mutagenesis (upper lane). In mutant 1 (bottom lane), two unique *Hin*dIII restriction sites flanking the DNA sequence encoding the FG loop were introduced and the carboxy-terminal end was truncated to Ile[170].

tural characteristics was gained by identification of epitopes within this region recognized by mAb raised against ecFcεRIα and by polyclonal anti-peptide antisera. In a first study recombinant ecFcεRIα was cleaved with Arg-C protease or cyanogen bromide and the fragments were probed for binding to hIgE and various antibodies (Table 1). The experiments revealed the antigenicity of the carboxy terminal segment Val[155]–Lys[171], comprising the FG loop, since this region was recognized by a polyclonal anti-ecFcεRIα antiserum. In contrast, it was found to be able to bind to neither hIgE nor the mAbs tested. The inhibitory mAb 15/1, which is expected to recognize a conformational epitope sensitive to denaturation [16], did not bind to ecFcεRIα fragments generated by protease digestion or chemical cleavage. Under the denaturing chemical conditions applied by cyanogen bromide cleavage also hIgE was not able to bind to fragment Glu[99]–Leu[179] representing α(2). MAb 5H5/F8 has been shown to detect ecFcεRIα in Western blots from SDS-PAGE run under denaturing conditions [17] and is therefore assumed to recognize a stable epitope. The Ab binding pattern obtained for 5H5/F8 points towards recognition of amino acids between Lys[176] and Leu[179].

Hulett et al. [9] demonstrated that each of three independent ecFcεRIα regions transfers IgE binding capability to a receptor chimera when transplanted into the homologous site of the low affinity IgG receptor (FcγRII). Each of the three regions contains a loop according to an existing homology-based 3D model [8].

We focused on the inspection of the membrane proximal FG loop, e.g. by site specific mutagenesis, because: (i) preliminary data suggested that mAb 5H5/F8 recognizes an epitope in the carboxy-terminal part of ecFcεRIα; (ii) we wanted to evaluate the contribution of the FG loop which is part of the 'Hulett fragment' Ser[146]–Val[169] to IgE binding; and (iii) indications exist that binding of the non-inhibitory mAb 15/1 is mediated via this region (unpublished data).

For mutagenesis, a recombinant construct was prepared which is shown in Fig. 1. To facilitate linker mutagenesis by cassette cloning two *Hin*dIII restrictions sites were introduced resulting in amino acid changes V155L and N166K, I167L (mutant 1). Receptor mutants were transiently expressed in COS-7 cells as amino terminal fusion to HSA. The HSA part has no influence on the IgE binding capability of FcεRIα and was used for quantitation of secreted receptor in COS cell supernatants and, more importantly, as a tag for measuring relative binding intensities to the various Abs since this tag remains unaffected by the mutations made in the ecFcεRIα part. Binding of hIgE and mAb 15/1 to mutant 1 was reduced as compared to HSA-ecFcεRIα (Table 2). Because Hulett et al. [8] showed that truncation of FcεRIα to amino acid Val[169] does not interfere with hIgE binding, we argue that the reduced binding of mutant 1 is due to the exchange of the indicated amino acids (V155L, N166K, and I167L), preferentially N166K resulting in the replacement of a polar residue by a charged one. In agreement with the results of the enzymatic cleavage experiments mAb 5H5/F8 failed to bind to mutant 1 indicating that this non-inhibitory mAb recognizes an epitope beyond Ile[170]. Deletion of the loop region Trp[156]–Leu[167] (mutant 2) abolished binding to both hIgE and mAb 15/1, probably due to severe structural disturbances. Replacement of this region with the homologous FcγRIIα sequence (mutant 3) partly restored hIgE binding capacity while mAb 15/1 did not recognize this mutant indicating that an essential part of the 15/1 epitope resides within Trp[156]–Leu[167]. In contrast, hIgE binding seems to be only in part mediated via this region. This is in accordance with published data [9] suggesting three independent FcεRIα regions capable of mediating hIgE binding when transplanted into the homologous sites of FcγRIIα.

Alanine substitution mutagenesis was used to further characterize FcεRIα derived amino acids residing within the FG loop region Trp[156]–Leu[165] important for hIgE and mAb 15/1

A:

15-1	P	S	A	L	W	M	H	G	V	R	V	E
15-2	A	A	A	W	P	T	L	P	H	G	E	E
15-3	V	R	G	P	E	W	P	G	I	V	M	G
15-4	N	I	A	H	S	I	D	L	W	N	E	A
15-5	K	Q	D	T	V	S	E	M	L	G	N	F
15-6	A	V	G	P	L	W	A	E	L	R	G	V
15-7	S	H	F	W	P	G	G	T	A	S	C	N
15-8	L	D	G	E	S	K	E	V	L	G	I	G

FcεRIα: C_{151} T G K V W_{156} Q L D Y E S E P L N I T V I_{170}

B:

5/1	P	H	Q	N	Q	L	P	R	E	S	W	S
5/2	P	R	E	N	W	A	S	S	I	E	A	Q
5/3	P	R	E	G	F	R	D	G	E	I	M	A
5/4	P	R	E	G	Y	R	A	R	V	P	N	Q
5/5	P	R	E	T	Y	D	K	L	S	D	L	R
5/6	P	R	E	H	G	T	L	V	S	T	G	Y
5/7	P	R	E	R	M	G	E	L	G	C	W	R
5/8	P	R	E	R	F	G	V	G	R	D	E	R

FcεRIα: A_{172} P R E K Y W L_{179}

Fig. 2. Peptide sequences identified by screening the FliTrx random peptide library with (A) mAb 15/1 and (B) mAb 5H5/F8. Shaded amino acids belong to a consensus sequence that can be aligned by similar amino acid properties to the ecFcεRIα sequence whereas amino acids typed bold represent identities.

binding. Substitution by alanine deletes all interactions made by atoms beyond the β-carbon and should reveal the contribution by the removed side chain to the binding reaction [12]. The choice of amino acids to be substituted within the FG loop was based on inspection of the ecFcεRIα model that can be obtained from the Brookhaven Protein Data Bank (Code 1ALT) and on a publication evaluating amino acid preferences at protein binding sites [23]. As can be seen from the binding pattern shown in Table 2, Ser^{162} (mutant 4) is critical for hIgE and mAb 15/1 binding. The additional Q157A exchange in mutant 5 completely abolished binding to both molecules. Serine residue 162 seems to be of structural importance because it is highly conserved among Fc receptors suggesting a pivotal role in building up the overall α(2) structure. While for hIgE no other important residue was identified within the FcεRIα region Trp^{156}–Leu^{167}, residue Trp^{156} was found to be essential for mAb 15/1 binding whereas hIgE binding remained unaffected by the mutating Trp^{156} as demonstrated with mutants 6, 7 and 8. Since the yields of mutant proteins generated by transient COS cell expression was too low to allow physico-chemical characterization, e.g. CD spectroscopy, binding to hIgE was taken as indicative of correct folding. Therefore, we argue that the loss of mAb 15/1 binding is not due to a structural rearrangement caused by the W156A mutation but is indeed dependent on the removal of the tryptophan side chain. This is in agreement with Sechi et al. [24] who speculated that aromatic amino acids might be part of the mAb 15/1 epitope. Moreover, a modeling study of α(2) [25] showed that in both association modes that were examined, several tryptophans are still accessible to solvent. Furthermore, tryptophans occur more frequently within protein binding sites than other amino acid residues [23].

To confirm the alanine substitution mutagenesis data obtained with mAb 15/1 and to identify the epitope of mAb 5H5/F8, the FliTrx random peptide library was screened. This library displays random peptides on the surface of *Escherichia coli* on the major bacterial flagellar protein [22]. Screening of the FliTrx library by panning on mAb 15/1 (Fig. 2A) resulted in the identification of binding clones that support the mutagenesis results: one peptide sequence identified (15-8) shows partial sequence homology with the FG loop. Most of the peptide sequences displayed by the other clones contain a tryptophan residue. Only one of these clones (15-7) reacted in Western blot with mAb 15/1 (data not shown). Parts of its sequence (polar/positively charged/hydrophobic/tryptophan) can be aligned to FcεRIα region $S^{153}LVW^{156}$ based on amino acid properties. The importance of this region for mAb 15/1 binding is further stressed by the reduced binding observed with mutant 1.

Screening the FliTrx library with mAb 5H5/F8 revealed a core consensus sequence of the peptides displayed by the binding clones which maps to position Pro^{173}–Tyr^{177} of FcεRIα (Fig. 2B) explaining its inability to bind to mutant 1 truncated to Ile^{170}. This is also in accordance with the results obtained with proteolytically cleaved ecFcεRIα where binding was observed to fragments Gln^{100}–Leu^{179} and to Asn^{112}–Lys^{176} but not to Val^{155}–Lys^{171}. Moreover, these data might explain why mAb 5H5/F8 is not able to bind to FcεRIα expressed on monocytic cells (G. Mudde, personal communication): the vicinity of the cell membrane might render its epitope inaccessible to cell-bound α-chain.

Table 2
Receptor composition and correlation with Ig binding (ELISA)

COS-7 cell expressed	ecFcεRIα amino acid sequence	Binding to		
		IgE	15/1	5H5/F8
HSA ecFcεRIα	K^{154}VWQLDYESEPLNITVIKAPREKYWL^{179}	+++	+++	+++
HSA mutant 1	K^{154}LWQLDYESEPLKLTVI^{170}	++	+	−
HSA mutant 2	K^{154}L————TVI^{170}	−	−	−
HSA mutant 3	K^{154}LGYTLFSSKPVKLTVI^{170}	+	−	−
HSA mutant 4	K^{154}LWQLDYEAEPLKLTVI^{170}	−	+	n.d.
HSA mutant 5	K^{154}LWALDYEAEPLKLTVI^{170}	−	−	n.d.
HSA mutant 6	K^{154}LAQLAYASAPLKLTVI^{170}	++	−	n.d.
HSA mutant 7	K^{154}LAQLDAESEPLKLTVI^{170}	++	−	n.d.
HSA mutant 8	K^{154}LAQLDYESEPLKLTVI^{170}	++	−	n.d

Mutated residues in ecFcεRIα are underlined. For comparison of HSA ecFcεRIα with HSA mutant 1, 100% is defined as binding of HSA ecFcεRIα to hIgE; +++ = 75%, ++ = 55%, + = 35%, − = 10%. Binding of subsequent mutants is set in relation to HSA mutant 1 which is then defined as 100% in an analogous way.

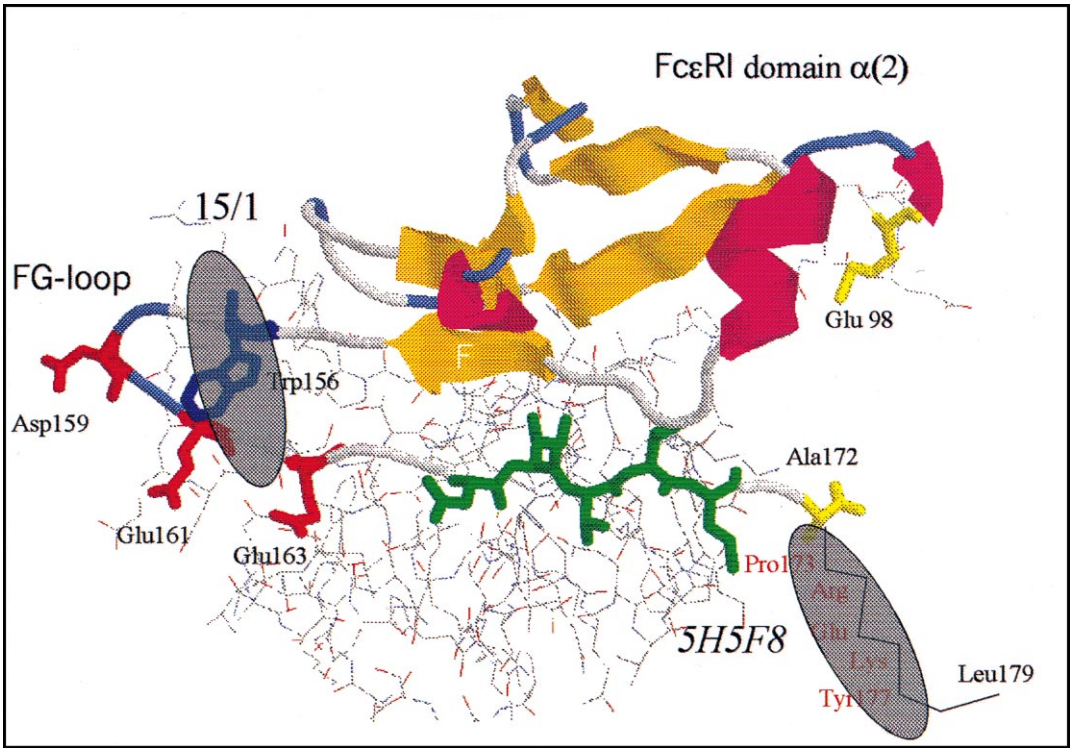

Fig. 3. Brookhaven Protein Data Bank (Code 1ALT) homology-based model of ecFcεRIα (Val^{1}–Ala^{172}). The α(1) domain is shown in the background whereas α(2) is drawn in a way that incorporates the results of this report. The NITVI amino acid stretch is colored green and the receptor amino acid sequence is schematically elongated up to Leu^{179}.

The most striking result of the cleavage and mutagenesis experiments was the observation that the epitopes recognized by mAbs 15/1 and 5H5/F8 were found to lie in close vicinity to each other, not only by the primary amino acid sequence of α(2) but also as deduced from the three-dimensional model (Fig. 3). Despite this obvious closeness, the two mAb display quite contrary biological activities: 15/1 efficiently inhibits hIgE binding whereas 5H5/F8 is non-inhibitory. Therefore, these epitopes must by structurally separated in FcεRIα in a way that hIgE binding is blocked after mAb 15/1 binding, but is still possible after mAb 5H5/F8 binding. Additionally, 15/1 does not compete with 5H5/F8 for receptor binding (data not shown) indicating that their epitopes are not overlapping. Taking into account that there is no steric hindrance for hIgE binding by mAb 5H5/F8 bound to the receptor, it is reasonable to propose a model where the two Ab epitopes are separated due to the tertiary structure of FcεRIα, e.g. by a 'bend'.

Support for this hypothesis was gained by the generation of polyclonal rabbit antisera against the α-chain peptides E^{161}SEPLNITVIKAPRE^{175} and N^{166}ITVI^{170}. The first sequence was chosen because it fulfills the following criteria: (i) it links the two mAb epitopes, (ii) the amino-terminal part is assumed to have a well defined structure containing the highly conserved serine residue Ser^{162} as shown in Fig. 5, (iii) the sequence has a high probability to be surface exposed (secondary structure prediction data according to a Chou-Fasman algorithm, data not shown) and – according to alanine scanning mutagenesis results – is part of the 15/1 epitope. The short peptide was selected to assess the importance of the NITVI sequence since our data strongly suggested this region to be involved in mAb 15/1 and hIgE binding. For rabbit immunization both peptides were chemically coupled to KLH. Immune sera were pre-purified over a protein A column and the obtained IgG fractions were further purified by affinity chromatography using the peptide coupled to a Sepharose matrix. Both purified polyclonal anti-peptide Ig fractions recognized ecFcεRIα in ELISA (data not shown) and inhibited the hIgE/ecFcεRIα interaction in a concentration dependent manner (Fig. 4). We therefore propose the existence of a turn or a bend in the receptor structure within Glu^{161}–Glu^{175}. Such a bend would not only explain the unpublished findings that 5H5/F8 does not recognize cell-bound

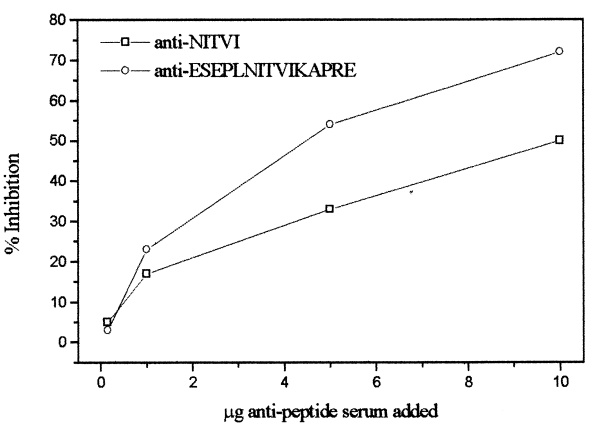

Fig. 4. Inhibition of ecFcεRIα binding to hIgE by purified polyclonal anti-ecFcεRIα antisera (ELISA). Reduced ecFcεRIα binding was calculated as %, relative to 100% binding obtained with pre-immune serum and shown as % inhibition.

Amino acid position :	156											166								170								179
																						mAb 5H5H8						
huFcεRIα	C	T	G	K	V	W	Q	L	D	Y	E	S	E	P	L	N	I	T	V	I	K	A	P	R	E	K	Y	W	L
mFcεRIα	C	K	G	Y	L	R	Q	V	E	Y	E	S	D	K	F	R	I	A	V	V	K	A	Y	K	C	K	Y	Y	W
ratFcεRIα	C	T	G	Y	L	N	K	V	E	C	K	S	D	K	F	S	I	A	V	V	K	D	Y	T	I	E	Y	R	W
huFcγRIα	C	S	G	M	G	K	H	R	-	Y	T	S	A	G	I	S	V	T	V	K	E	L	F	P	A	P	V	L	N
mFcγRIα	C	S	G	T	G	R	H	R	-	Y	T	S	A	G	V	S	I	T	V	K	E	L	F	T	T	P	V	L	R
huFcγRIIa	C	T	G	N	I	G	Y	T	L	F	S	S	K	P	V	T	I	T	V	Q	V	P	S	M	G	S	S	S	P
huFcγRIIb	C	T	G	N	I	G	Y	T	L	F	S	S	K	P	V	T	I	T	V	Q	A	P	-	-	-	S	S	S	P
huFcγRIIc	C	T	G	N	I	G	Y	T	L	Y	S	S	K	P	V	T	I	T	V	Q	A	P	-	-	-	S	S	S	P
mFcγRII	C	K	G	S	L	G	R	T	L	H	Q	S	K	P	V	T	I	T	V	Q	G	P	K	S	S	R	S	L	P
huFcγRIII	C	R	G	L	F	G	S	K	N	V	S	S	E	T	V	N	I	T	I	T	Q	G	L	A	V	S	T	I	S
mFcγRIII	C	K	G	S	L	G	S	T	Q	H	Q	S	K	P	V	T	I	T	V	Q	D	P	A	T	T	S	S	I	S
ratFcγRIII	C	K	A	Y	L	G	R	T	M	H	V	S	K	P	V	T	I	T	V	Q	G	S	A	T	A	S	T	S	S
guinea pig FcR IgG1,2	C	T	G	L	I	G	R	T	S	H	T	S	P	P	V	T	I	T	V	Q	G	P	K	S	S	D	S	D	S

Fig. 5. Alignment of amino acid sequences of Fc receptor α-subunits of various species (based on [25]). Numbering is sequential, ranging from Cys^{151} to Leu^{179}, and based on the sequence of human FcεRIα. The mAb 5H5/F8 epitope is indicated. For mAb 15/1 binding, Trp^{156} was found to be essential by mutagenesis experiments. Panning the FliTrx random peptide library with mAb 15/1 identified clones with amino acid homology to Leu^{158}–Ile^{167} (underlined). Residue Ser^{162} is completely conserved over the receptors. The sequences related to FcεRIα NITVI are shaded.

FcεRIα whereas 15/1 strongly binds to the receptor expressed on various cell types, but might also provide a model for the efficient presentation of the complex high affinity binding region within the membrane-proximal domain of FcεRIα to its ligand IgE. Homology alignment (Fig. 5) revealed that this region is well conserved among various Fc receptors and may therefore represent a general structural element to guarantee the exposition of binding residues which lie near to the cell membrane thus allowing access and exact positioning for specific and efficient attachment of their respective ligands.

Future studies carried out with recombinant receptor mutants expressed on the cell surface will provide more insight into the structural importance of this stretch of a few amino acids and help to find out whether it might represent a target for the design of agents which inhibit binding of hIgE to its high affinity receptor.

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