



IgE-binding properties and selectivity of peptide mimics of the FcεRI binding site

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ABSTRACT

FcεRIα found on the surface of mast cells and basophiles mediates allergic diseases, anaphylaxis and asthma through binding of IgE. Disrupting this interaction with anti-IgE mAbs has proven an efficient approach to control these diseases. The crystallographic structure of the complex formed between the IgE-Fc and FcεRIα extracellular domain has shown that recognition is mediated by residues in the second Ig-like domain of the receptor (D2) and in the loop connecting the D1 and D2 domains. In an attempt to obtain specific IgE antagonists, we have designed and prepared a polypeptide named IgE-Trap that partially reproduces the IgE receptor-binding sites and binds with micromolar affinity to soluble IgE. The polypeptide contains loops C'–E [residues 129–134] and F–G [residues 151–161] from the D2 domain joined by a linker, and loop B–C [residues 110–113]. Peptide binding to IgE has been assessed by SPR analyses and the data fit with a biphasic model of interaction, in agreement with the two-site mechanism reported for the native receptor. The polypeptide binds to immobilized IgE in a dose-dependent manner with a K_D estimated to be around 6 μM, while it does not recognize IgG nor IgA. Polypeptide sub-domains involved in IgE binding have also been defined, showing that loop C'–E connected to loop B–C, but also the isolated loop B–C alone suffice to bind immunoglobulins E with high selectivity though with reduced affinity compared to IgE-Trap. ELISA and cytometric assays on RBL2H3 cells demonstrate that the interacting peptides are able to displace the binding of IgE to receptor, confirming affinity and specificity of these ligands and suggesting a potential application as modulators of disorders associated with inappropriate IgE production.

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1. Introduction

The human high affinity IgE receptor, hFcεRI, found on the surface of mast cells and basophiles, mediates allergic diseases, anaphylaxis and asthma through its binding to IgE (Kinet, 1989; Metzger et al., 1989). hFcεRI contains four distinct polypeptide

Abbreviations: BSA, bovine serum albumin; DIEA, di-isopropylethylamine; DMF, dimethylformamide; DCM, dichloromethane; DTT, di-thiothreitol; DMSO, dimethylsulphoxide; Fmoc, fluorenylmethoxycarbonyl; FPLC, fast purification liquid chromatography; FITC, fluorescein isothiocyanate; HBTU, 1-H-benzotriazolium,1-[bis(dimethylamino)methylene]-hexafluorophosphate(1-),3-oxide; HOBt, N-hydroxybenzotriazole; HPLC, high performance liquid chromatography; IPTG, isopropyl β-D-thiogalactoside; LC-MS, liquid chromatography–mass spectrometry; PBS, phosphate buffered saline; PMSF, phenyl-methyl-sulphonyl-fluoride; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; 4-VP, 4-vinyl-pyridine; TIS, tri-isopropylsilane; TFA, trifluoroacetic acid; EDC, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride; NHS, N-hydroxy-succinimide.

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chains: an extracellular α-chain, a β-chain and a dimer of γ-chains. The extracellular portion of the α-chain binds with high affinity to the Fc region of IgE ($K_D = 10^{-9}$ M) (Cook et al., 1997), whereas the β- and γ-chains are responsible for down-stream signal propagation through phosphorylation of their intracytoplasmatic immune-receptor tyrosine-based activation motifs (ITAM) (Kinet, 1999; Kochan et al., 1988; Kraft and Kinet, 2007). The α-chain is N-glycosylated at seven sites, but sugars are not intrinsically necessary for proper folding or for binding to immunoglobulins (Garman et al., 2000). Instead they are deemed to be necessary for mediating the correct interaction between the α-chain and the ER folding machinery in the endoplasmic reticulum (Letourneur et al., 1995) and to stabilize the receptor's secondary and tertiary structures (Garman et al., 1998). Receptor cross-linking through allergen/antibody interactions leads to the activation of an intracellular signal cascade mediated mainly by the SRC family kinase Lyn and Fyn as well as Src kinases (Scharenberg et al., 1995; Turner and Kinet, 1999). In mast cells, this signal cascade leads to degranulation and the release of histamine, cytokines and other mediators of the allergic response. The pharmacological therapy for allergy

and asthma makes use of drugs that block histamine release, of bronco-dilators and corticosteroids that essentially reduce symptoms and the concomitant inflammatory reaction. However, given the increasing incidence of allergic diseases, that nowadays affect more than 20% of the western world population (Cookson, 1999; Meltzer and Grant, 1999), the search for non-symptomatic drugs that block the early events of allergic response is becoming a very important field of investigation. The recent introduction on the market of an anti-IgE antibody (Corren et al., 2009; MacGlashan, 2009) has provided the proof of concept that blocking FcεRI signalling is a successful therapeutic strategy and that preventing IgE binding to the receptor or at least modulating its binding kinetics, is a useful way for counteracting these disorders. Based on this background knowledge, several studies now focus on the development of potent and selective IgE receptor antagonists, with several groups having already developed small peptides targeting the IgE-FcεRI interaction derived from screening phage display libraries or designed on the basis of known receptor agonists (Buku et al., 2001, 2003, 2004, 2005, 2008; McDonnell et al., 1996, 1997; Nakamura et al., 2001, 2002; Rossi et al., 2008; Stamos et al., 2004). However, because of the high affinity and selectivity of the IgE receptor complex, developing effective antagonists still remains a challenging task. The crystallographic structure of the FcεRIα/FcIgE complex has elucidated the molecular mechanism and the stoichiometry of this interaction (Garman et al., 2000, 2001; McDonnell et al., 2001). The reported structure shows that the receptor is comprised of two Ig-like domains, D1 and D2, that form an acute angle like an inverse V and that IgE recognition is mediated by a cluster of aromatic residues localized on D2 and on the linker connecting D1 and D2. The D1 domain, instead, seems to contribute to the high affinity by stabilizing D2 and the linker conformation (Basu et al., 1993). Remarkably, sugar residues present on both the ligand and the receptor, also have only a stabilizing role and do not actively participate in the interaction (Garman et al., 2000, 2001).

In this study, we have attempted to obtain IgE binders derived from the receptor-binding sites thus having a neutralizing activity against receptor activation. Peptides have been prepared by solid phase synthesis and tested by both SPR and ELISA techniques, showing that they bind to human and mouse IgE. Despite their affinity in the micromolar range, binding appears highly selective, as they do not cross-react with IgG and IgA and also block binding of human IgE to the human D2 domain and the binding of mouse IgE to rat receptors on cell membranes.

2. Experimental procedures

2.1. Materials

Oligonucleotides were synthesized by Sigma-Genosys (Sigma-Aldrich, Milano, Italy). pETM vectors were from EMBL (Heidelberg, Germany). Pfu DNA polymerase was from Stratagene (Milano, Italy). Restriction enzymes were from New England Biolabs (Milano, Italy). All molecular biology kits were from Qiagen (Milano, Italy). *Escherichia coli* bacterial strains were from Novagen (Milano, Italy). IPTG was from Inalco (Milano, Italy). Reagents for bacterial medium were from Beckton-Dickinson (Milano, Italy). All reagents for SDS-PAGE, columns and ÄKTA FPLC were from GE Healthcare (Milano, Italy). Sigma-fast *o*-phenylenediamine dihydrochloride Tablet Sets were from Sigma-Aldrich (Steinheim, Germany). The BIAcore 3000 SPR system for Real Time kinetic analysis and related reagents were from GE Healthcare (Milano, Italy). All other reagents and chemicals were commercially available by Sigma-Aldrich or Fluka (Steinheim, Germany). The hFcεRI cDNA was kindly provided by Prof. Pucillo from the University of Udine. Reagents for peptide synthesis (Fmoc-protected amino

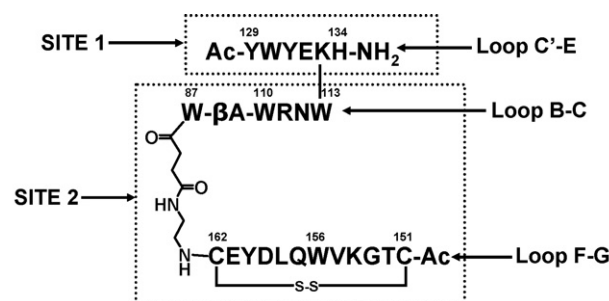


Fig. 1. Schematic representation of the IgE-Trap structure. The loop C'-E contains residues from the IgE-binding site 1. Loop B-C contains residues from the region 110–113 and a tryptophan from the linker region (Trp87). A linker joins this last region with the loop F-G containing residues 151–161. Loop B-C and loop F-G reproduce the IgE-binding site 2. In the cyclic variant (IgE-Trap_{ox}), a disulfide bond bridges Cys151 to a cysteine in position 162 replacing the native serine. In the linear variant (IgE-Trap_{red}) the disulfide bond is in the reduced form.

acids and resins, activation and deprotection reagents) were from Novabiochem (Laufelfingen, Switzerland) and InBios (Pozzuoli, Italy). Solvents for peptide synthesis and HPLC analyses were from Romil (Dublin, Ireland); reversed phase columns for peptide analysis and the LC-MS system were from ThermoFisher (Milan, Italy). Human IgE were from Chemicon International, while mouse IgE were from Sigma-Aldrich. Non-aggregated polyclonal human IgG and IgA were a kind gift of Dr. A. Verdoliva of Tecnogen SpA (Caserta, Italy). Solid phase peptide syntheses were performed on a fully automated multichannel peptide synthesizer Syro I (Multisynthech, Germany). Preparative RP-HPLC were carried out on a Shimadzu LC-8A, equipped with a SPD-M10 AV detector and with a Phenomenex C18 Jupiter column (50 mm × 22 mm ID; 10 μm). LC-MS analyses were carried out on an LCQ DECA XP Ion Trap mass spectrometer equipped with an OPTON ESI source, operating at 4.2 kV needle voltage and 320 °C with a complete Surveyor HPLC system, comprised of MS pump, an autosampler and a photo diode array (PDA). Narrow bore 50 mm × 2 mm C18 BioBasic LC-MS columns were used for these analyses. All ELISA assays to screen the synthetic peptides, were carried out using a fully automated system (Hamilton Robotics, Milano, Italy) comprising a liquid handler, a robotic arm, a washer and an automated Synergy 4 multi-wavelength reader (BIOTEK Instruments, Inc., Highland Park, VT, USA). Dulbecco's modified eagle's medium (DMEM) and fetal bovine serum (FBS) were from BioWhittaker (Versevier, Belgium); L-glutamine was from Gibco (Milano, Italy). FITC-avidin was from Santa Cruz Biotechnology (CA, USA).

2.2. Peptide synthesis

Peptides were prepared by solid phase synthesis as C-terminally amidated and N-terminally acetylated derivatives following standard Fmoc chemistry protocols (Fields and Noble, 1990). A Rink-amide MBHA resin (substitution 0.53 mmol/g) and amino acid derivatives with standard protections were used in all syntheses. A lysine with a 4-methyl-trityl (Mtt) protection was used to link the side chain of Lys133 to the C-terminus of Trp113 (see Fig. 1). A β-alanine was instead used to connect Trp110 and Trp87. A linker comprising a succinic acid and an ethylenediamine moiety was introduced between Trp87 and the C-terminus of loop B-C (Garman et al., 2000, 2001). The synthesis was performed at room temperature (RT) starting from His134 and completing, in a first step, the loop C'-E (129–134). After acetylation using acetic anhydride in DMF (0.5 M, 5% DIEA, 20 min at room temperature), selective deprotection of Lys (Mtt) was achieved by repeated treatments with a dichloromethane (DCM)/trifluoroacetic acid (TFA)/tri-isopropylsilane (TIS) (94:1:5, v/v/v) mixture for

Table 1

Names, sequences and corresponding and MW of peptides used in this study.

Peptide	Sequence	MW
IgE-Trap	See Fig. 1 Ac-Y-W-Y-E-K-H-NH₂ Ac-W-βA-W-R-N-W	3474.20
Loop (C'-E + B-C)		1909.12
Loop C'-E	Ac-Y-W-Y-E-K-H-NH ₂	1009.12
Loop B-C	Ac-G-W-βA-W-R-N-W-NH ₂	1016.14
Loop F-G	Ac-C-T-G-K-V-W-Q-L-D-Y-E-C-NH ₂	1484.70

2 min at RT. Trp87, β-Ala, Trp110, Arg111, Asn112 and Trp113 were incorporated under canonical conditions of peptide synthesis (HBTU/HOBt/DIEA pre-activation, 5-fold excess of Fmoc-protected amino acids). Succinic acid was incorporated by treatment with a 10-fold excess of succinic anhydride (0.5 M in DMF containing 1 M DIEA). Ethylenediamine was then introduced after on resin pre-activation of the carboxylate group with conventional reagents (HBTU/HOBt/DIEA) and reaction with a 2-fold excess of reagent in DMF at RT for 20 min. Loop F-G was subsequently assembled using classical conditions of peptide synthesis. Cleavage of peptides from the solid support was performed by treatment with a TFA/TIS/water (90:5:5, v/v/v) mixture for 90 min at room temperature, affording the crude peptide after precipitation in cold di-ethylether. The cyclic peptide (named IgE-Trap_{ox}) was obtained by spontaneous intramolecular oxidation of cysteines in 100 mM carbonate buffer pH 8.0 for 16 h at RT at the concentration of 10^{-5} M. Cyclization progression was monitored by RP-HPLC. The solution was finally acidified and lyophilized. Products were purified to homogeneity by RP-HPLC using a C18 Jupiter column (50 mm × 2.2 mm) applying a linear gradient of 0.1% TFA in acetonitrile from 5% to 70% over 20 min (flow rates of 20 mL/min). Peptide purity and integrity were confirmed by LC-MS mass measurements using a Surveyor LC system coupled to an LCQ Deca XP mass spectrometer. Characterizations were conducted under standard conditions of peptide analysis. The shorter polypeptides were similarly prepared. Final peptides are reported in Table 1, with names, sequences and molecular weights. The corresponding reduced precursor of IgE-Trap, IgE-Trap_{red}, was also purified and characterized.

2.3. Cloning, expression and purification of the FcεRI extracellular domain (ECD)

The hFcεRI ECD D2 domain residues 84–170 were appropriately amplified by PCR from the full length hαFcεRI cDNA (sequence code NM_002001 from PubMed) and cloning into pETMA11 vector. 100 ng of FcεRI-D2-pETMA11 was transformed into BL21(DE3)trxB *E. coli* competent cells and the recombinant protein expressed as an N-terminal polyhistidine tagged protein at 22 °C in the presence of 0.2 mM IPTG. After incubation for 16 h, the bacterial culture was centrifuged and the pellet was re-suspended in 10 mL of 30 mM Tris-HCl, 1 mM PMSF, 0.1% Triton X-100, 1 μg/mL lysozyme, 3 M urea, 1 mM DTT pH 8.0 (lysis buffer). After incubation for 30 min at room temperature, cells were sonicated on ice for 10 min (10' s on, 10 s off) and centrifuged for 30 min at 12,000 rpm at 4 °C. Total cell protein fraction and soluble fraction (supernatant of cell lysate) were analyzed by SDS-PAGE. Proteins were detected in gels by Coomassie brilliant blue R-250 staining or transferred onto a nitrocellulose membrane for Western blot analysis using HRP-anti-polyhistidine. The protein was purified on a Nickel affinity (Ni-NTA) column, eluting with increasing concentrations of imidazole. Purified protein fractions were dialyzed over night at 4 °C against 30 mM Tris-HCl, 150 mM NaCl, 1 mM DTT 2 M urea pH 8.0, then folding was induced by rapid dilution in the same buffer and slowly decreasing the urea concentration by performing subse-

quent dialysis against 30 mM Tris-HCl, 150 mM NaCl, 1 mM DTT pH 8.0. The last step of dialysis was carried out in 30 mM Tris-HCl, 150 mM NaCl, 5 mM reduced glutathione, 0.5 mM oxidized glutathione pH 8.0 buffer over night at 4 °C. Any precipitate formed during the refolding procedure was removed by centrifugation at 12,000 rpm for 30 min at 4 °C. The recovered material was analyzed by 15% SDS-PAGE under reducing and non-reducing conditions and by LC-MS.

2.4. Circular dichroism spectroscopy

CD spectra were obtained at room temperature on a Jasco J-715 dichrograph, calibrated at 290 nm with an aqueous solution of D(+)-10-camphor sulphonic acid, using 0.1 mm quartz cuvettes. Spectra of the FcεRI-D2 domain were acquired in 20 mM phosphate buffer pH 8.0 with $C = 8.0 \times 10^{-6}$ M. Spectra were recorded using a 0.1-mm path length quartz cuvette. Data were collected at 0.2-nm intervals with a 20 nm/min scan speed, a 2 nm bandwidth, and a 16 s response, within the spectral range of 260–190 nm. The recorded spectra were then signal-averaged over at least three scans, and the baseline was corrected by subtracting the spectrum of the buffer. Spectra were then transformed in molar ellipticity.

2.5. Binding and competition assays

Real time binding assays were performed on a Biacore 3000 Surface Plasmon Resonance (SPR) instrument (GE healthcare, Milan, Italy). Human IgE, human IgG and human IgA immobilization were achieved on CM5 Biacore sensor chips using EDC/NHS chemistry at pH 5.0 in 10 mM acetate buffer (flow rate 5 μL/min) according to the manufacturer's instructions (Johnsson et al., 1991). Residual reactive groups were deactivated by treatment with 1 M ethanolamine hydrochloride, pH 8.5. Reference channels were prepared for each biosensor by activating with EDC/NHS and deactivating with ethanolamine. All binding assays were carried out in HBS buffer (10 mM Hepes, 150 mM NaCl, 3 mM EDTA, pH 7.4) at a flow rate of 30 μL/min. Analyte injections of 140 μL (IgE-Trap), 90 μL (loop C'-E + B-C) or 130 μL (loop B-C) were performed at the indicated concentrations. Data were manipulated to obtain kinetic and thermodynamic parameters using the BIA evaluation analysis package (version 4.1, GE Healthcare, Milano, Italy). Data fitting was carried out using monophasic or biphasic models and evaluating the goodness of fitting by the analysis of residuals (Cook et al., 1997; Henry et al., 1997). Non-specific binding was subtracted from the specific binding prior to analysis. Binding to human IgG and human IgA was performed at 30 μM for IgE-Trap and loop (C'-E + B-C) and at 60 μM for loop B-C.

To perform ELISA assays, 96-well microtiter plates (Nunc, Milan, Italy) were coated with human IgE at fixed concentrations (2 and 5 μg/mL) in PBS buffer (10 mM Na₂HPO₄, 2 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl pH 7.4) for 16 h at 4 °C. Some wells were filled with buffer alone and were used as blank. After coating, plates were washed three times with PBS-T buffer (PBS containing 0.04% Tween-20) and blocked with 250 μL PBS containing 1% BSA, for 2 h at 37 °C. After washing, solutions at increasing concentrations of D2 between 0.50 and 25 μM in PBS or Bis-Tris 25 mM pH 6.5 were added to coated wells and incubated 1 h at 37 °C. After washing again with PBS-T, 100 μL of anti-His-HRP conjugated antibody (Santa Cruz Biotechnology, CA, USA) diluted 1:1000 with PBS was added. After 1 h incubation at 37 °C, plates were washed again with PBS-T and 100 μL of chromogenic substrate, *o*-phenylendiamine 0.4 mg/mL in 50 mM sodium-phosphate-citrate buffer pH 5.0 containing 0.4 mg/mL hydrogen peroxide, were added and the reaction was stopped by adding 50 μL of 2.5 M H₂SO₄ in each well. The absorbance was measured at 490 nm.

For the ELISA competition assays, human IgE, diluted in PBS1×, were coated overnight on microtiter plates at 2.0 µg/mL (10 nM). Recombinant receptor concentration was maintained at 10 µM and mixed with competitor peptides at increasing concentrations as indicated in the legend of Fig. 8. The subsequent steps were carried out as previously described. Data points were in triplicate. Assays were performed at least three times obtaining reproducible results.

2.6. IgE biotinylation

Mouse IgE (0.5 mg) were dissolved in 1 mL of PBS, pH 7.4, and treated with 10 mM of succinimidyl-6-(biotinamido)hexanoate (Pierce, Italy) in DMSO. After 1 h of incubation on ice, 1 mL of Tris 50 mM pH 8 was added to deactivate residual active groups. The biotinylated-IgE were extensively dialyzed 4 °C against PBS pH 7.4.

2.7. Cell culture and flow cytometry

The rat basophilic leukemia (RBL2H3) cell line was maintained in DMEM supplemented with 10% heat-inactivated FBS, containing 2 mM L-glutamine at 37 °C in a humidified atmosphere at 5% CO₂. For competitive binding experiments, RBL2H3 cells were readily detached by gentle scraping with PBS/EDTA 0.05%, centrifuged at 100 × g for 5 min, washed with PBS, 0.2% BSA and re-suspended in the same buffer to a final density of 5 × 10⁶ cells/mL (Buku et al., 2001). Increasing concentration of peptide between 0 and 600 µM was pre-incubated with 13 nM biotinylated-IgE at 4 °C for 1 h. The mix was added to cell suspensions for 45 min followed by the addition of 10 µL FITC-avidin. After 30 min the cells were washed twice with PBS/BSA and analyzed with a flow cytometer equipped with a 488-nm argon laser (FACSCalibur, Becton Dickinson). For each sample, 20,000 events were acquired and analyzed using the Cell Quest software.

3. Results

3.1. Peptide design

The peptide IgE-Trap was designed on the basis of the crystallographic structure of the IgE-FcεRI complex reported by Garman

et al. (2000) (PDB code 1F6A). A sketch of the peptide structure is reported in Fig. 1, where the two receptor-binding sites and loops involved in the interaction with IgE are evidenced by boxes and arrows. The polypeptide recapitulates both receptor sites which are shared by three distinct loop regions, named loop C'–E, loop B–C, and loop F–G, in agreement with the nomenclature reported in Garman et al. Loop C'–E (boxed) contains residues 129–134 (YWYEKH), which comprise most of site 1. Here, Asn133 originally present in the receptor sequence and not involved in contacts with the IgE-Fc, was mutated to a lysine in order to exploit the side chain amino group to join residues 110–113 (WRNW) from loop B–C. The ε-NH₂ of lysine was thus connected to the C-terminus of Trp113. An additional tryptophan, putatively mimicking Trp87, on the N-terminus of Trp110 was linked using a β-alanine as spacer. Residues of loop F–G (Cys151–Ser162, loop 3) were connected to Trp87 by a linker comprising a succinic acid and an ethylenediamine moiety. Loops B–C and F–G were used to mimic the receptor-binding site 2. In order to reduce the conformational flexibility of loop F–G, Ser162 was replaced by an isosteric cysteine and a disulfide bridge was inserted. To increase the polypeptide's stability, the N-terminal and C-terminal ends were acetylated and amidated, respectively.

3.2. Peptide synthesis

Cyclic IgE-Trap, named IgE-Trap_{ox}, was obtained with an overall yield of about 5% after cyclization and purification. The linear precursor (named IgE-Trap_{red}) was also prepared and purified. The yield was about 10%. The other peptides (see Table 1) were obtained in higher yields (ranging from about 20 to 50%). Molecular weights were consistent with the expected values. Disulfide cyclization of IgE-Trap was assessed by high resolution mass spectrometry and confirmed by the lack of reactivity following iodoacetamide treatment (not shown).

3.3. Peptide binding to IgE

The capacity of peptides to bind to immobilized human IgE was determined by Real Time kinetic analysis using the SPR technique.

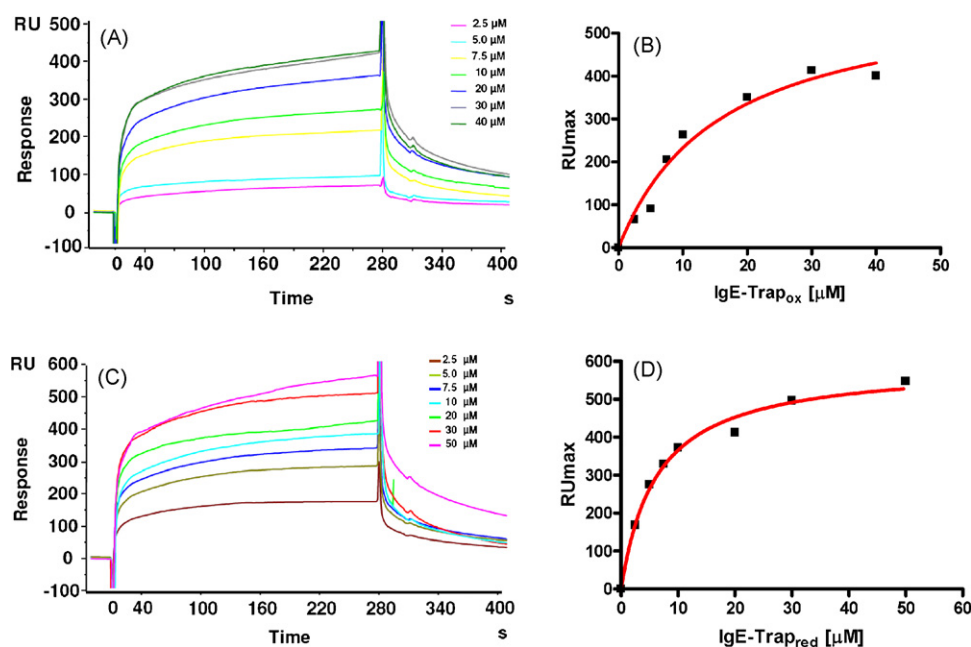


Fig. 2. (A) Sensorgrams relative to the binding of IgE-Trap_{ox} to human IgE immobilized on the surface of a Biacore sensor chip. Concentrations were between 2.5 µM and 40 µM. Experiments were carried out at a 25 °C, at a constant flow rate of 30 µL/min using HBS as running buffer (140 µL injected for each experiment). (B) Plot of RU_{max} from each single experiment versus concentration (µM). Data were fitted by non-linear regression analysis. In (C) and (D), data obtained for IgE-Trap_{red} are reported. Experiments were performed at concentrations between 2.5 µM and 50 µM.

Table 2

Association and dissociation rates and dissociation constants for the active peptides reported in this study. Data for the two IgE-Trap variants and for loop (C'-E+B-C) are referred to a fitting with a biphasic model of binding to IgE. The K_D for loop (B-C) has been obtained by fitting the data with a monophasic model of association with IgE.

A							
Peptide	Mean 1st association rate, K_{a1} ($M^{-1} s^{-1}$)	Mean 1st dissociation rate, K_{d1} (s^{-1})	Mean 2nd association rate, K_{a2} ($M^{-1} s^{-1}$)	Mean 2nd dissociation rate, K_{d2} (s^{-1})	Mean K_{D1} (M)	Mean K_{D2} (M)	Global K_D (M)
IgE-Trap _{ox}	1.63×10^2	1.06×10^{-2}	6.14×10^3	3.69×10^{-1}	$65 (\pm 5) \times 10^{-6}$	$60 (\pm 6) \times 10^{-6}$	$16 (\pm 4) \times 10^{-6}$
IgE-Trap _{red}	3.30×10^4	6.43×10^{-1}	2.87×10^2	1.05×10^{-2}	$19 (\pm 6) \times 10^{-6}$	$37 (\pm 7) \times 10^{-6}$	$6.2 (\pm 0.7) \times 10^{-6}$
Loop (C'-E+B-C)	1.35×10^4	8.70×10^{-1}	1.33×10^2	2.54×10^{-2}	$65 (\pm 6) \times 10^{-6}$	$19 (\pm 5) \times 10^{-5}$	$19 (\pm 4) \times 10^{-6}$
B							
Peptide	Mean association rate, K_{a1} ($M^{-1} s^{-1}$)			Mean dissociation rate, K_{d1} (s^{-1})		K_D (M)	
Loop (B-C)	1.81×10^2			1.50×10^{-1}		$8.3 (\pm 0.5) \times 10^{-4}$	

Human IgE, IgG and IgA were efficiently immobilized to the surface of a CM5 sensor at a similar extent (about 15,000 RU). At first, IgE-Trap_{ox} and IgE-Trap_{red} were tested. Sensorgrams relative to IgE-Trap_{ox} are reported in Fig. 2A, while thermodynamic parameters are shown in Table 2. A preliminary analysis of residuals (see Supplementary Fig. S1A–D) suggested that experimental data were fitted better by a biphasic model of interaction, therefore we hypothesized that two distinct association and dissociation kinetic constants characterized the binding. This observation was in agreement with the fact that the peptide contains two distinct receptor-binding sites for which we obtained, incidentally, two very similar K_D s of about 60 μ M (see Table 2A). Data were further analyzed to obtain a value for the apparent macroscopic dissociation constant. For this purpose, RU_{max} values from each determination were plotted against polypeptide concentrations and the K_D evaluated by data fitting using a non-linear regression analysis (Fig. 2B). In agreement with the “two-site binding” hypothesis, the K_D extrapolated by this approach was lower than the K_D s for the two distinct sites, namely $16 \pm 4.0 \mu$ M compared to about 60 μ M (Table 2), also suggesting a cooperative mechanism. In order to evaluate the contribution to binding provided by the presence of the disulfide bridge, we also analyzed the reduced polypeptide IgE-Trap_{red} (Fig. 2C). Again, a preliminary analysis of residuals (not shown) indicated that experimental data were best fitted by biphasic curves, thus confirming the occurrence of a two-site binding also for this molecule. As reported in Table 2, slightly higher affinities compared to the cyclic variant were observed for both sites; this was also reflected by the somewhat lower macroscopic K_D ($6.2 \pm 0.7 \mu$ M) determined as reported for the cyclic variant (Fig. 2D). In order to dissect the contribution to IgE recognition by the different IgE-Trap regions and to further confirm the occurrence of multiple and cooperative interaction sites, we then analyzed the binding properties of synthetic peptides corresponding to different receptor loops. Peptides, together with sequences and the corresponding elements of secondary structure in the native receptor, are reported in Table 1 and Fig. 1. Sensorgrams relative to IgE binding by the peptide named loop (C'-E+B-C), at concentrations ranging between 2.5 and 50 μ M, are reported in Fig. 3A. The peptide bound in a dose-dependent fashion to the functionalized chip and, in agreement with the fact that residues from two distinct binding sites are present within this sequence, experimental data were again fitted better by biphasic curves (see Supplementary Fig. S2A–D). Importantly, the first K_D was very close to that determined for the whole IgE-Trap_{ox} (see Table 2), suggesting that a site with a higher affinity is present on both peptides. The affinity for the second site was fairly lower (190 μ M), while the global apparent K_D determined for this peptide was $19 \pm 4.0 \mu$ M (Fig. 3B and Table 2), very similar to that determined for the IgE-Trap_{ox} variant. This result, based on the preliminary assumption that two sites were present, suggested that the contribution to binding of

loop F–G, either in the cyclic or reduced form, was negligible. In order to confirm this hypothesis, we then analyzed the binding properties of the isolated cyclic and reduced synthetic loop F–G, which indeed showed no recognition (not shown). To further dissect the contribution to binding from loops C'-E and B-C and confirm the occurrence of two sites in this peptide too, we investigated their separate effects using the corresponding synthetic peptides. Unexpectedly, loop C'-E was unable to bind alone to IgE (not shown), while loop B-C, assayed at concentrations up to 75 μ M (at higher concentrations it was insoluble), bound very weakly ($K_D = 8.3 \pm 0.5 \times 10^{-4}$ M, Fig. 4A and Table 2). Furthermore, although a dose-response trend was observed, the dependence of RU_{max} versus concentration was essentially linear and not saturable in the concentration range investigated (Fig. 4B), indicating a prevalence of less specific and very weak interactions. Importantly, in this case, experimental data were fitted better by a monophasic

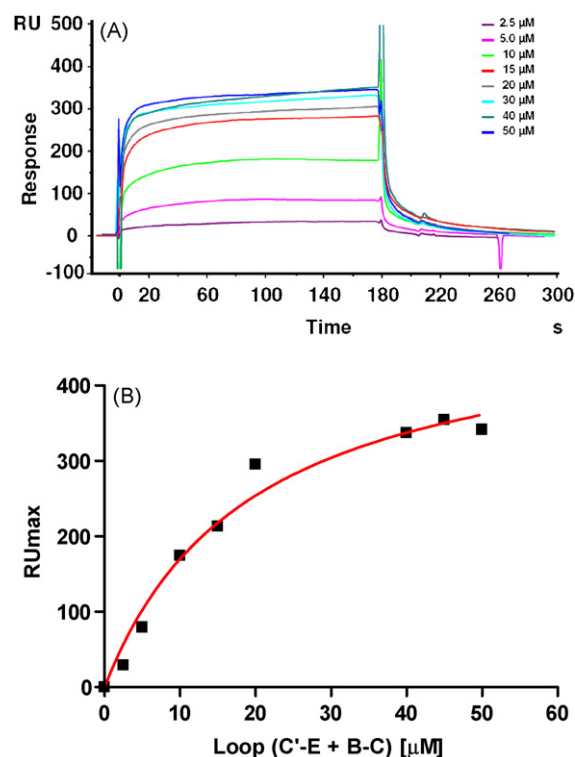


Fig. 3. (A) Sensorgrams relative to the binding of loop (C'-E+B-C) to immobilized IgE. Concentrations were between 2.5 μ M and 50 μ M. Experiments were carried out at 25 °C, at a constant flow rate of 30 μ L/min using HBS as running buffer (90 μ L injected for each experiment). (B) Plot of RU_{max} from each single experiment versus concentration (μ M). Data were fitted by non-linear regression analysis.

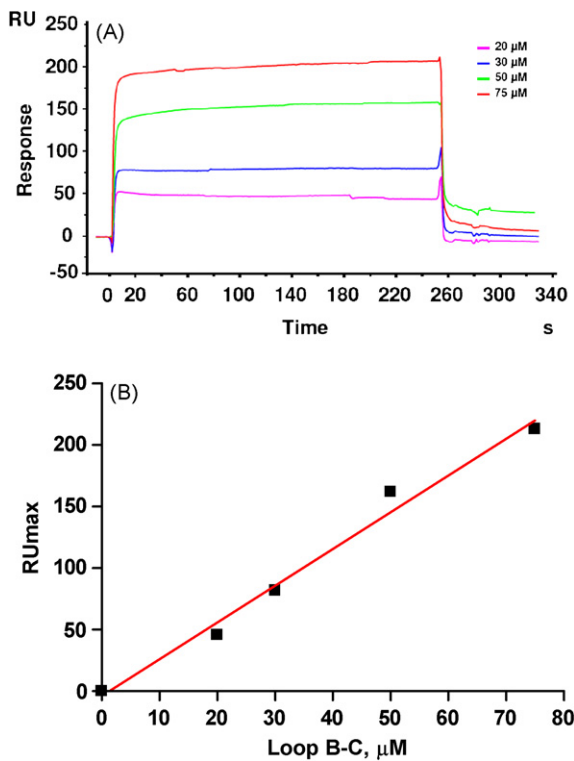


Fig. 4. (A) Sensorgrams relative to the binding of loop B-C to immobilized IgE. Concentrations were between 20 μ M and 75 μ M. Experiments were carried out at 25 °C, at a constant flow rate of 30 μ L/min using HBS as running buffer (130 μ L injected for each experiment). (B) Plot of RUmax from each single experiment versus concentration (μ M). Data could not be fitted by non-linear regression analysis in the explored concentration range.

association model (Fig. S3A–D), in agreement with the assumption that this short peptide can mimic only one site. Collectively, the data support the hypothesis that, most contribution to the binding properties of IgE-Trap is provided by residues belonging to loops C'-E and B-C. Furthermore, despite the low affinity compared to the native receptor, a strong cooperativity occurs between these loops when they are covalently interconnected, while they appear poorly or essentially inactive when are assayed separately. To further characterize the binding properties of these peptides, we performed comparative assays with IgG and IgA, which are the most abundant immunoglobulins and the most similar in primary sequence to IgE. As shown in Fig. 5A and C, the peptides IgE-Trap_{ox}, loop (C'-E + B-C) and loop B-C, did not bind immunoglobulins from other classes, suggesting a very high specificity of these molecules for IgE.

3.4. Preparation of the recombinant D2 domain of the FcεRI receptor

It has been reported that binding of the receptor to IgE is mediated by the D2 domain and by residues located on the linker between D1 and D2. D1, instead, favours an optimal receptor conformation, but does not actively participate in receptor recognition (Vangelista et al., 2002). Glycosylation is also deemed to only slightly affect affinity, being involved in the stabilization of the protein structure and not in direct contacts with IgE (Garman et al., 2000). To carry out binding and competition experiments with the bioactive peptides and to further confirm the specificity of recognition, we designed and prepared a D2 variant starting from Phe84 and including Ile170 (the carboxy-terminal tail involved in membrane anchoring was excluded, Fig. 6A) The protein, expressed in *E. coli*, also bear at its N-terminus a polyhistidine tag that facilitated its purification using a Ni-chel-immobilized affinity resin

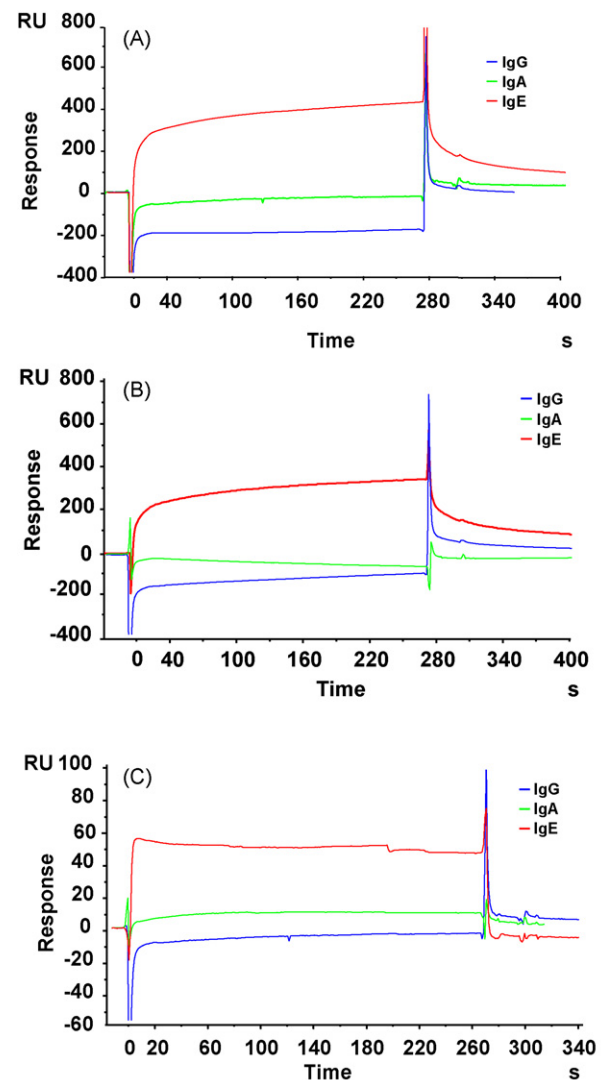


Fig. 5. (A) Comparative sensorgrams for the binding of IgE-Trap to immobilized IgE, IgG and IgA. The peptide only binds to IgE. The peptide was analyzed at a concentration of 30 μ M with a constant flow rate of 30 μ L/min. (B) The same comparative analysis performed with loop (C'-E + B-C) at 30 μ M and with loop B-C at 60 μ M. (C) Experiments were carried out at 25 °C, at a constant flow rate of 30 μ L/min using HBS as running buffer.

(Fig. 6B). A yield of 0.5 mg of folded protein from 100 mL of bacterial cell culture was obtained in typical preparations. The final material was more than 90% pure as estimated by SDS-PAGE (Fig. 6B) and LC-MS analysis (not shown). The experimental molecular weight was in agreement with the theoretical value ($MW_{\text{Exper./Calc.}}$: 13,253/13253.2 amu). Protein refolding was assessed by CD analysis (Fig. 6C); the protein spectrum showed canonical β -sheet bands (minimum at 215 nm and a maximum at about 195 nm), as expected on the basis of the domain structure (Garman et al., 1999, 2000, 2001). The presence of the disulfide bridge was confirmed by LC-MS analysis of a 4-VP-treated sample, which was indeed not affected by the alkylation reaction (not shown).

3.5. Binding assays between IgE and the recombinant D2 domain

The recombinant receptor domain was at first tested for binding with IgE. An ELISA assay was thus set up in order to use it in subsequent competition experiments with the synthetic peptides. The assay was performed by coating human IgE at 25 nM (5 μ g/mL) and the recombinant receptor in solution at concentra-

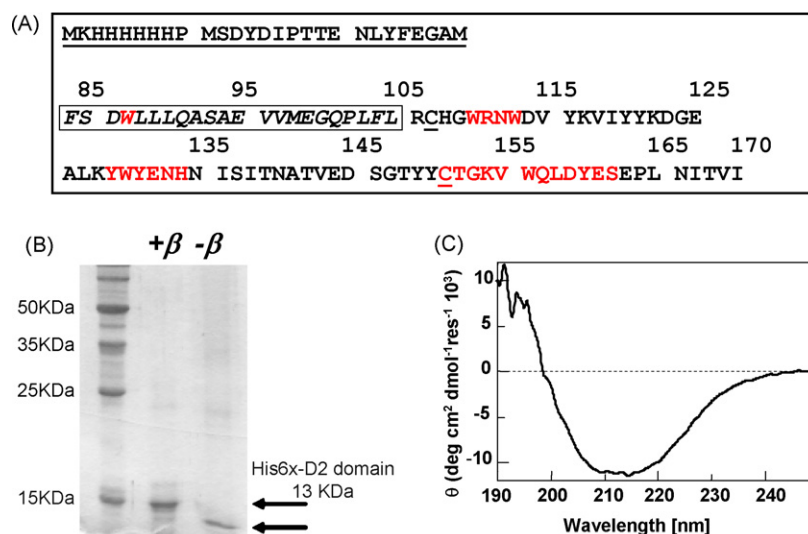


Fig. 6. (A) Sequence of the recombinant D2 domain of FcεRI. Residues used to design the peptides are reported in red. Residues corresponding to the linker region are in italic and boxed. Cysteines involved in the disulfide bridge are underlined. Residues coming from the pETMA11 vector containing the polyhistidine linker are reported in the first line of the sequence. (B) SDS-PAGE characterization of the recombinant protein in the presence and absence of β-mercaptoethanol. The reduced form of the protein migrates slightly slower. (C) CD spectrum of the protein in 20 mM phosphate buffer, pH 8.0. The polypeptide, analyzed at a concentration of 8.0 μM shows the typical spectrum of β-sheet rich structures. Molecular weight markers are reported on the gel left margin. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

tions ranging between 50 nM and 20 μM. As shown in Fig. 7A, the receptor bound to immunoglobulins in a dose-dependent manner, but showed a clear two-step curve with a first saturation point at about 500 nM and a second saturation point at concentrations higher than about 10 μM (Fig. 7B and C). Fitting of data by non-linear regression over the two concentration ranges, provided the respective K_D s of 0.11 ± 0.04 μM and 4.1 ± 0.1 μM. This behaviour, reproducibly observed over at least three independent experiments, is indicative of two independent sites, but with much lower affinities compared to the full length receptor and to previous data (Cook et al., 1997; Henry et al., 1997). However, given the lack of the adjacent D1 domain and the presence of the polyhistidine tag at the N-terminus where the linker between D1 and D2 is located, it is likely that residues Ser85, Asp86 and Trp87, which contribute to the receptor-binding site 2 (Garman et al., 2000), are not properly disposed or are partially inaccessible, thereby causing a reduced affinity. This hypothesis is consistent with the large difference of K_D values determined for the two distinct sites. ELISA competition assays using the recombinant D2 domain and IgE were then carried out in order to confirm that peptides bind the immunoglobulins on the same sites as the receptor. In this experiment, 10 nM human IgE (2.0 μg/mL) were coated on the plates and 5.0 μM D2 was used in solution, since, at this receptor concentration, both binding sites are saturated. For the dose-dependent competition assay, we explored peptide:receptor molar ratios between 1 and 100. Competitor concentrations therefore ranged between 5.0 μM and 500 μM. Results reported in Fig. 8A and C, show that all peptides efficiently block the interaction, confirming that peptides actually mimic the receptor-binding sites. Noticeably, IC_{50} values are consistent with the relative binding affinities determined by SPR. Indeed, IgE-Trap_{ox} displayed an IC_{50} of 36.0 ± 0.1 μM, loop (C'-E+B-C) an IC_{50} of 103.0 ± 0.2 μM, whereas loop B-C alone showed an IC_{50} of only 379.0 ± 0.2 μM.

3.6. Inhibition of biotinylated-IgE binding to RBL2H3 cells by loop (C'-E+B-C)

The competitive binding experiments was performed with increasing concentration of loop (C'-E+B-C) at a fixed concentra-

tion of 13 nM biotinylated-IgE. As shown in Fig. 9, the peptide at 60 μM reduced the binding of biotinylated-IgE to 60% of the control value. The positive control was defined as the fluorescence intensity of 13 nM biotinylated-IgE bound to the cells. The results are expressed as percentage fluorescence intensity of the sample minus the fluorescence of the cells incubated with FITC-avidin alone. In the same assay 100-fold excess of non-labelled-IgE was used to verify the specificity of IgE binding to FcεRI receptor on RBL2H3. The addition of a competition mix containing 1 mM of a scrambled peptide provided a proof of the specificity of loop (C'-E+B-C) for binding to IgE (not shown).

4. Discussion

Therapeutic protocols for allergy are still largely based on symptomatic drugs which are directed at reducing the degree of inflammation, vasodilation, and congestion (Jardieu, 1995). More recently, new strategies targeting the IgE-FcεRI molecular complex that triggers the allergic cascade, have been undertaken. These efforts have culminated so far in the development of the anti-IgE monoclonal antibody named Omalizumab which has proven to be of broad clinical efficacy in reducing the severity of several allergic diseases (Ames et al., 2004; Corren et al., 2009; MacGlashan, 2009). With the aim of identifying smaller antagonists capable of binding to either the immunoglobulin or the receptor, several techniques have been employed, including combinatorial approaches (Nakamura et al., 2001, 2002; Rossi et al., 2008), the use of truncated IgE-Fc or receptor loops (Rigby et al., 2000b), or by modifying known receptor agonists (Buku et al., 2003). Most of these molecules disrupt IgE binding and despite their low affinity (McDonnell et al., 1996; Rossi et al., 2008), have proved effective in preventing activation of cells or even in *in vivo* assay (Rossi et al., 2008). Elucidation at the atomic level of the interaction interface between IgE and the FcεRI has open the way to the design of new, effective drugs blocking receptor signalling and thus to new opportunities for therapeutic intervention at the molecular level (Garman et al., 2000). However, the presence of two distinct interaction sites and the convex nature of the receptor make the design of receptor-binding peptides quite difficult. In addi-

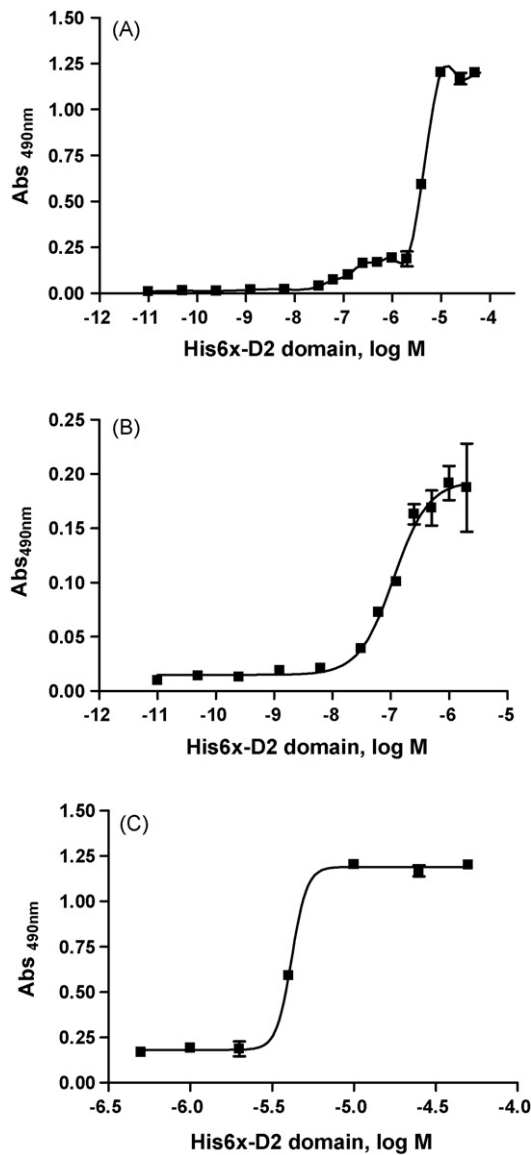


Fig. 7. (A) ELISA binding assay between coated human IgE and the soluble recombinant D2 domain of human FcεRI fused with an N-terminal polyhistidine tag (His6x-D2). The binding curve shows a clear two-step dependence from protein concentration with a first saturation point for values higher than about 500 nM and a second saturation point for concentrations higher than 10 μM. In (B) and (C), the two steps are highlighted by spitting the plot between 0.01 nM and 2.0 μM (B) and between 2.0 μM and 50 μM (C). Experiments were in quadruplicate and were repeated at least three times. Data $\pm$ SD are reported.

tion, receptor-binding compounds can lead to unwanted agonistic activities. Instead, blocking the circulating IgE by soluble factors is a validated approach with a proven clinical efficacy (Corren et al., 2009; MacGlashan, 2009). On this background, starting from the receptor structure we have designed small polypeptides that mimic the IgE-binding interface and thereby specifically interact with IgE. Despite their relatively low binding capacity (low micromolar range) compared to the full length receptor ($K_D = 10^{-9}$ M), these peptides show a cooperative two-site mechanism of binding, as suggested by the lower value (2–3 times) of the macroscopic K_D compared to the single-site K_D s and by a preliminary analysis of residuals for the fitting of experimental binding curves. The occurrence of a cooperative mechanism, which implies the existence of at least two interacting sites, is also suggested by the lack of activity shown by the isolated loops C'–E and F–G, which, instead, synergically contribute to binding when covalently connected with other

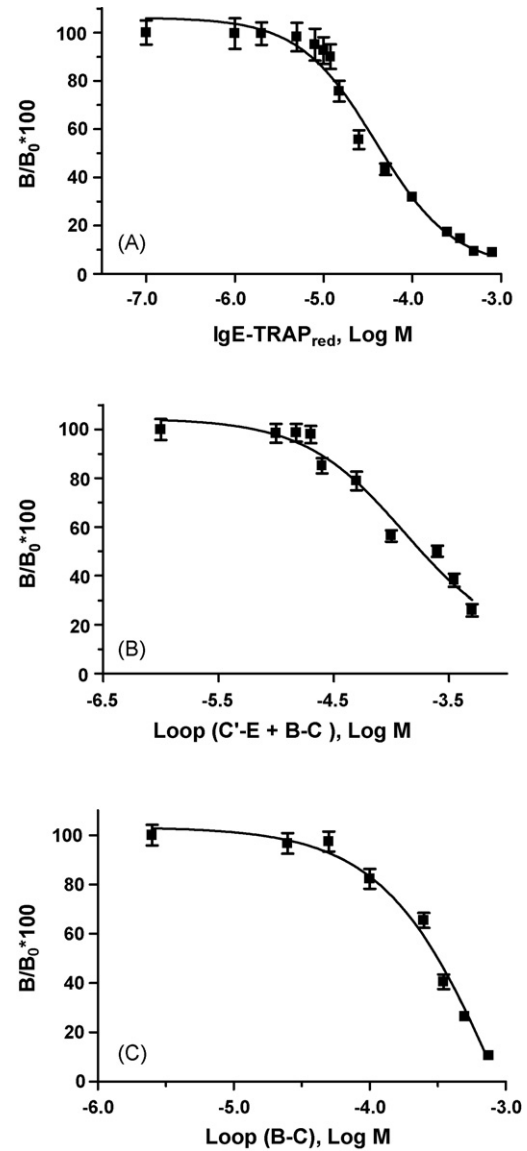


Fig. 8. Competitive binding assays between the recombinant D2 domain and the synthetic bioactive peptides to immobilized human IgE. (A) Competition assay with IgE-Trap_{red}. The experiment was performed at concentrations ranging between 10^{-7} M and 10^{-3} M. The IC_{50} was 36.0 ± 0.1 μM. (B) The same experiment carried out with loop C'–E + B–C at concentrations ranging between 1 μM and 500 μM. The estimated IC_{50} was 103.0 ± 0.2 μM. (C) Competition assay with the peptide corresponding to loop B–C. Concentrations of the competing peptide were between 3 μM and 750 μM. The estimated IC_{50} was 379.0 ± 0.2 μM. For all experiments human IgE were coated at 10 nM while the recombinant receptor domain was maintained at 10 μM. Data on the vertical axis are reported as $B/B_0 \times 100$, where B is the absorbance at a given concentration of competitor and B_0 is the absorbance from wells without competitors.

peptide regions. The presence of loop C'–E, containing only residues from site 1, confers a 100-fold affinity increase to loop B–C, while it is totally inactive alone. This finding suggests that site 1 on the IgE is not readily accessible to the peptide, this hypothesis being corroborated by the occurrence of the slow association kinetics for one of the two bindings ($K_{a2} = 1.33 \times 10^2$ M⁻¹ s⁻¹) (Table 2) and the concurrent slow dissociation ($K_{d2} = 2.54 \times 10^{-2}$ s⁻¹). Notably, this observation is in agreement with previous evidences provided by Wan and co-workers (Wan et al., 2002) suggesting a poor accessibility of the corresponding site 1 on the IgE and a mechanism of bending of the immunoglobulin upon receptor engagement.

The presence of the cyclic loop F–G in IgE-Trap_{ox} does not greatly affect the binding of the peptide to IgE, which indeed displays the

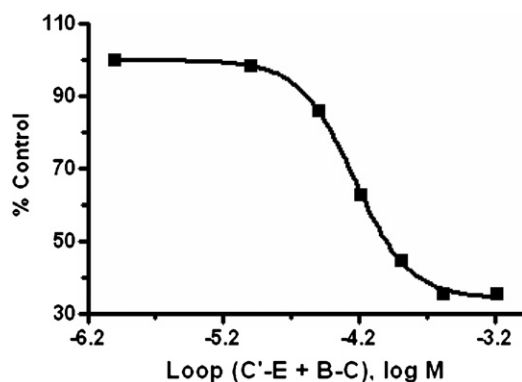


Fig. 9. Flow-cytometric analysis of inhibition of biotinylated-IgE binding to RBL2H3 cells. The number of cells is plotted against the logarithm of fluorescence intensity of the bound ligand. Biotinylated-IgE were coupled with FITC-avidin and incubated with cells. Fluorescence intensity was determined by FACS scan analysis in the absence of competitors and was assumed as the 100% control. Increasing concentrations of peptide loop (C'-E + B-C) between 0 μ M and 600 μ M were incubated with the cells under the same conditions and fluorescence intensity again determined. Data shown are the average from at least three independent determinations.

same global K_D both with and without it. Conversely, leaving this loop in the reduced form, slightly enhances the affinity (about 3-fold), suggesting that, while the more rigid cyclic moiety is not capable of adopting a conformation suitable for binding, the linear peptide does and contributes to slightly increase its global affinity.

The soluble and functional D2 domain of human Fc ϵ RI has also been prepared as recombinant protein and utilized to confirm the ability of these peptides to reproduce the binding sites. The protein also shows a two-site mechanism of interaction, but despite the presence of the linker region (residues 84–105), binding affinities are still low compared to the full length receptor. Indeed, two independent sites are observed with K_D s of about 100 nM and 4 μ M. The IgE-binding peptides block the interaction between human IgE and D2 with IC_{50} values consistent with the relative binding affinities, thus confirming that recognition occurs on the expected regions. Competition has been demonstrated also on rat cells expressing the receptor using the loop (C'-E + B-C). Remarkably, while IgE-Trap or the smaller loop (C'-E + B-C) described here, shows a reduced affinity compared to receptor-binding peptides reported in previous studies (Buku et al., 2008; Nakamura et al., 2001, 2002), they show IC_{50} for IgE displacement from cell-bound receptors, in the same order of magnitude as the more active peptides (about 70 μ M compared to 20 μ M reported for the MCD analogues). The peptides we report here also have affinities higher or similar to IgE-binding compounds with a proven *in vivo* activity (Rigby et al., 2000a; Rossi et al., 2008). In addition, the peptides show a very high selectivity for IgE, being incapable of binding IgG and IgA. This feature is of particular interest for *in vivo* applications, as unspecific binding to IgG, highly abundant in blood and in other tissues, would subtract the molecule from its target. Similarly, IgA are the most abundant immunoglobulins in external secretions (saliva, mucus, gastric fluids, sweat, and tears), therefore the lack of binding will ensure no subtraction in case of oral administration. Finally, given the small molecular size, they are also not expected to be immunogenic and could be used in *in vivo* experiments at high dosages without modifications, therefore they have a potential as modulators of disorders associated with inappropriate IgE production.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molimm.2009.07.025.

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Fig. S1. Comparative analysis of monophasic and biphasic fitting models for the peptide IgE-Trap_{ox}. **A:** Comparison of binding phases and **B:** corresponding distribution of errors for the two curves. **C:** Comparison of dissociation phases and **D:** distribution of errors for the two curves. Residuals are much lower and more spread out for the biphasic models of association and dissociation.

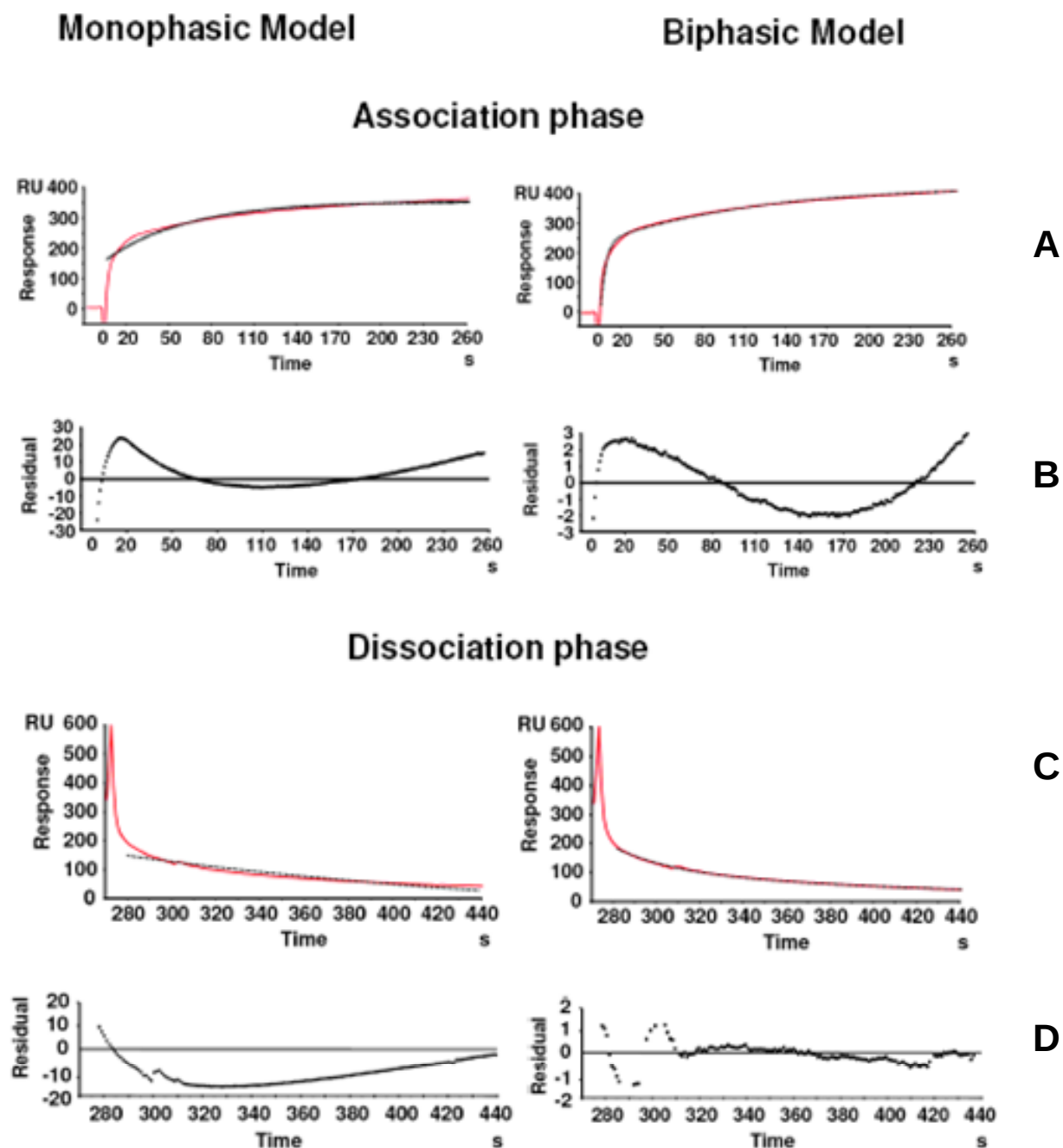


Fig. S2. Comparative analysis for monophasic and biphasic fitting models for the peptide comprising Loops (C'-E + B-C). **A:** Comparison of binding phases and **B:** corresponding distribution of errors for the two curves. **C:** Comparison of dissociation phases and **D:** distribution of errors for the two curves. Residuals are much lower and more spread out for the biphasic models of association and dissociation.

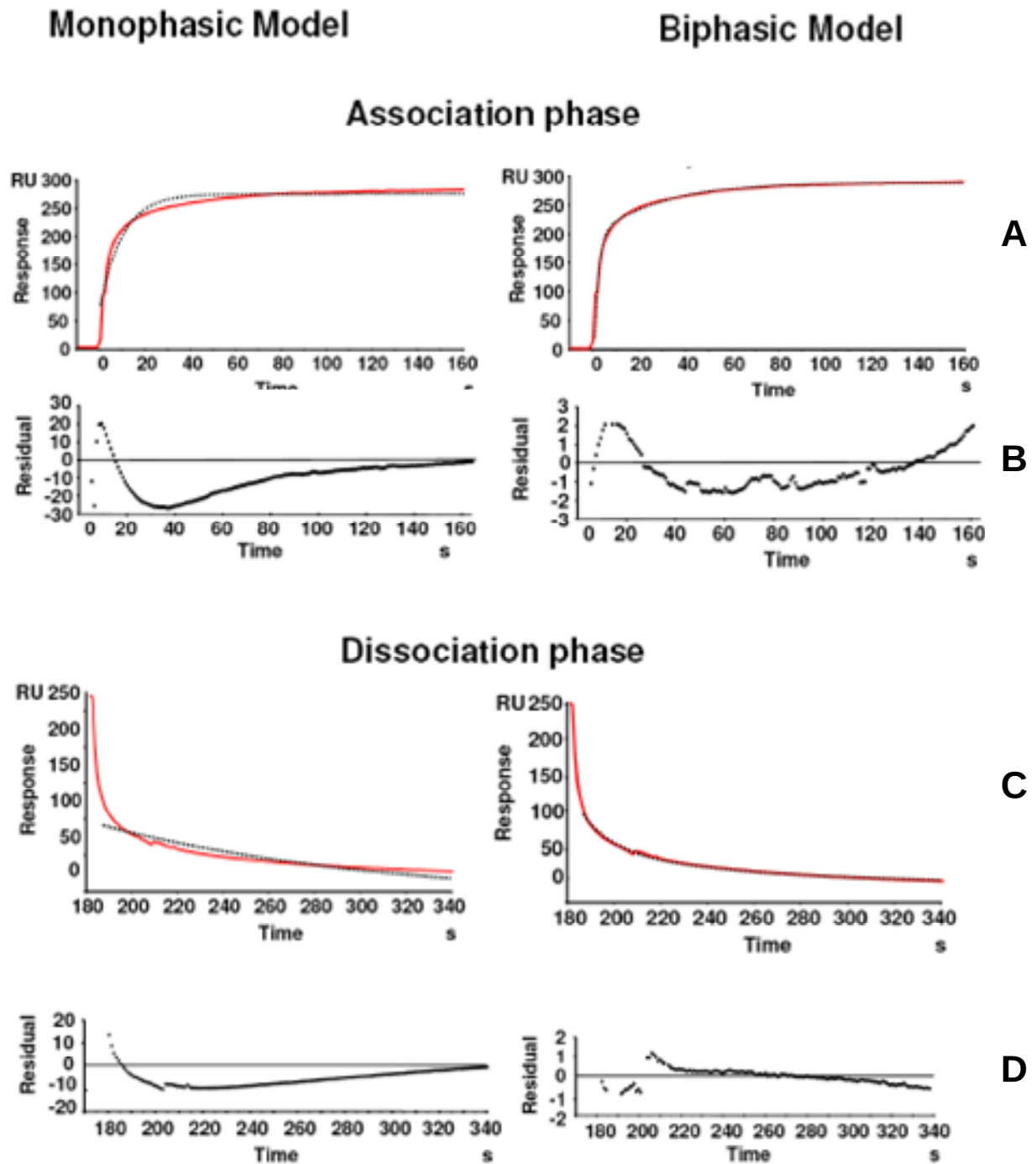


Fig. S3. Comparative analysis for monophasic and biphasic fitting models for the peptide comprising Loop (B-C). **A:** Comparison of binding phases and **B:** corresponding distribution of errors for the two curves. **C:** Comparison of dissociation phases and **D:** distribution of errors for the two curves. Residuals are much lower and more spread out for the monophasic models of association and dissociation.

