

A minimal receptor-Ig chimera of human FcεRI α-chain efficiently binds secretory and membrane IgE

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We constructed a soluble minimal receptor-Ig chimera in which the two extracellular domains of human FcεRI α-chain (D1 and D2) were fused to the dimerizing C-terminal domain of human IgG1 heavy chain (γ1-CH3). The protein was expressed and actively secreted by Chinese hamster ovary (CHO) cells as a fully glycosylated soluble dimeric protein. It showed efficient binding both to human membrane-bound IgE isoforms and to the two secretory IgE isoforms. Moreover, the dimeric receptor binds IgE with the expected 1:2 stoichiometry. The receptor-Ig chimera, in 2-fold molar excess, inhibited engagement of secretory IgE to rat basophilic leukemia cells expressing the human αβγ receptor. Full self-nature and inability to bind Fcγ receptors make this protein an attractive candidate for clinical applications and a novel biotechnological tool for atopic allergy research.

Keywords: binding/chimera/dimerization/FcεRI/IgE

Introduction

Human high affinity receptor for IgE (FcεRI) is at the forefront of atopic allergy research for its importance in the binding of human IgE antibodies in both effector and antigen presenting cells, where it is assembled on the cell surface as a αβγ₂ or αγ₂ chain complex, respectively (Kinet, 1999). FcεRI mediates immediate and chronic atopic allergy manifestations driven by allergen–IgE recognition (Kinet, 1999). FcεRI belongs to a family of homologous receptors including FcγRI/II/III, all part of the multichain immune-receptors family comprising T- and B-cell receptors (TCR and BCR, respectively). The IgE binding site (the Ig-like domains D1 and D2) lies on the extracellular portion of FcεRI α-chain (Garman *et al.*, 2000). Recombinant soluble forms of this receptor moiety have been investigated in different expression systems (Blank *et al.*, 1991; Haak-Frendscho *et al.*, 1993; Robertson, 1993; Scarselli *et al.*, 1993; Vangelista *et al.*, 1999), with the aim of competing IgE engagement to endogenous FcεRI.

An immunoadhesin containing FcεRI α-chain D1D2 fused to the Fc portion of IgG1 was produced (Haak-Frendscho *et al.*, 1993) and tested for its efficacy in immunotherapy (Saban *et al.*, 1994). However, the interaction of immunoadhesins with IgG Fc receptors (FcγRs) may lead to deleterious unwanted effects (Junghans, 1997). FcγRs interaction should not occur if the second constant IgG1 domain (γ1-CH2) is absent in the chimeric protein, as the binding site resides in this domain

together with part of the antibody hinge region (Sondermann *et al.*, 2000).

We propose here the fusion of human FcεRI α-chain D1D2 to human IgG1 third constant heavy chain domain γ1-CH3 to obtain a soluble dimeric receptor (named sdα) lacking Fcγ receptor binding activity.

Fusion to γ-CH3 has been independently reported in the field of antibody engineering by two groups to obtain bivalent scFv fragments, named minibodies (Hu *et al.*, 1996) and SIPs (Small Immune Proteins) (Li *et al.*, 1997). In a recent development, the ε-CH4 domain (the γ-CH3 homologous domain in IgE) from secretory IgE isoforms, IgE_{S1} and IgE_{S2} (Batista *et al.*, 1996a), was used to generate ε-SIP variants (J.Sepulveda and O.Burrone, unpublished work). Fusion of ε-CH4_{S2} to a tumor-specific scFv presented enhanced tumor localization and reduced serum clearance with respect to the monomeric scFv (L.Borsi *et al.*, in preparation).

Two minimal structure–function blocks, the binding unit and the dimerizing unit, form the novel chimeric receptor-Ig variant described in this work. sdα was actively secreted and fully glycosylated by mammalian cells and displayed excellent IgE binding activity.

Materials and methods

Cell lines and media

Chinese hamster ovary (CHO) cells were grown in α-MEM medium (Life Technologies, Gaithersburg, MD) containing 40 μM deoxyribonucleosides and 40 μM ribonucleosides, supplemented with 10% (v/v) FCS (Life Technologies). Mouse myeloma J558L cells transfected with secretory human Cε_{S1} and Cε_{S2} have been described previously (Batista *et al.*, 1995, 1996a). WEHI 231 cells transfected with human membrane IgE isoform long or short have been described previously (Batista *et al.*, 1996b). Rat basophilic leukemia cells transfected with human FcεRI α-, β- and γ-chains (RBL SX38, kindly provided by M.H.Jouvin) have been described previously (Wiegand *et al.*, 1996) and were supplemented with 800 μg/ml Geneticin (G-418 sulfate, Life Technologies).

Construction of sdα

Human FcεRI α-chain was generated by RT-PCR using upstream primer RX1 (5'-AAGCTTCAAGATGGCTGCCAT-GGAATCC-3') and downstream primer mutalpha (5'-TAAACTGCAGCTTTTATTACAGTAAT-3'). A HindIII/PstI fragment of 595 bp, encoding the first 197 residues of the FcεRI α-chain, was cloned into the corresponding sites of the previously described vector, pUC18-VC-SIP/1 (Li *et al.*, 1997), encoding human IgG1 CH3 domain fused to an upstream peptide linker sequence (AGGSG). To generate pcDNA3-sdα, a HindIII/XbaI 953 bp fragment, encoding the final chimeric product, was ligated into the corresponding sites of pcDNA3 (Invitrogen, Groningen, The Netherlands) and used for transfection.

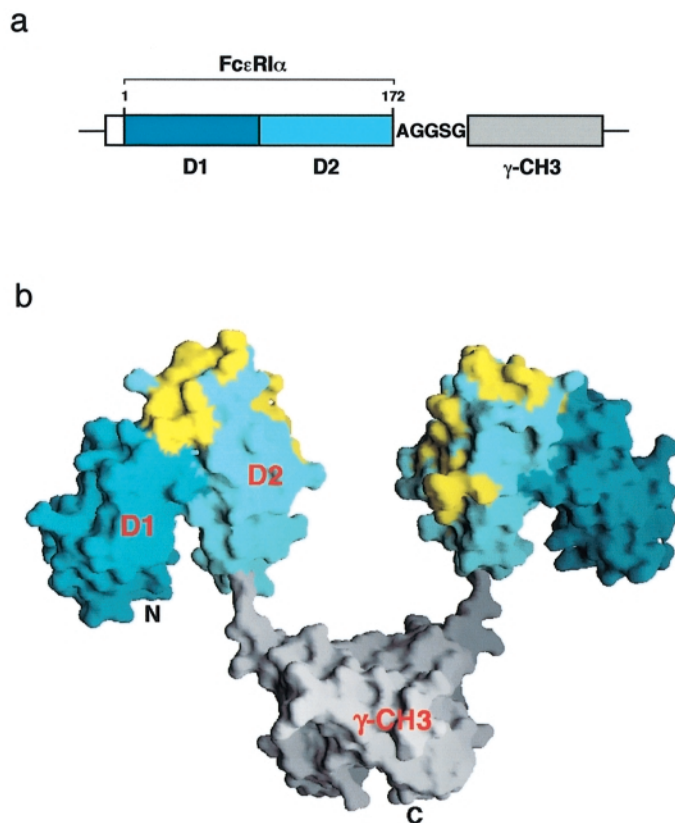


Fig. 1. Schematic and 3D model representation of sdα. (a) Scheme of the construct. The white box represents FcεRI α-chain leader sequence for ER translocation. AGGSG is the five-amino acid linker joining FcεRI α-chain domains D1D2 to the CH3 domain of human IgG1. (b) 3D model of sdα was prepared using Swiss-PdbViewer (Guex and Peitsch, 1997) and displayed as a GRASP (Nicholls *et al.*, 1993) surface representation. The PDB coordinate entry for γ-CH3 dimer is 1fc1 (Deisenhofer, 1981) and the D1D2 entry is 1f2q (Garman *et al.*, 1998). The yellow surface indicates amino acids involved in IgE binding according to Garman *et al.* (Garman *et al.*, 2000). N and C indicate sdα termini. As the linker between receptor and Ig modules gives flexibility to the molecule, the orientation of the two D1D2 portions was chosen arbitrarily.

CHO transfection and selection by ELISA

1×10^6 CHO cells were resuspended in cold PBS (phosphate-buffered saline: 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , 137 mM NaCl, 3 mM KCl, pH 7.2) and electroporated with 10 μg of pcDNA3-sdα, previously linearized by *Pvu*I, with a single pulse at 25 μF , 1000 V in a Gene Pulser (Bio-Rad, Hercules, CA). After electroporation, cells were kept for 5 min on ice and plated in 10 ml of complete α-MEM medium, supplemented with 2% (v/v) Origen Hybridoma Cloning Factor (IGEN International, Rockville, MD). Selection of positive clones was carried out in the presence of 400 $\mu\text{g}/\text{ml}$ Geneticin. Screening of sdα-secreting cells was executed by coating a 96-well enzyme-linked immunosorbent assay (ELISA) plate (Nunc Maxisorp, Roskilde, Denmark) with goat polyclonal anti-human IgG (Fc) antibodies (Pierce, Rockford, IL), 3 $\mu\text{g}/\text{well}$ in 50 mM Na_2CO_3 – NaHCO_3 pH 9.5, overnight at 4°C. Cells supernatants were added and incubated for 1 h at room temperature, followed by horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Fc) antibodies (Pierce) for 1 h at room temperature. Detection was performed using 2 mg/ml *o*-phenylenediamine (Sigma, St. Louis, MO) and 0.03% (v/v) H_2O_2 as peroxidase substrate, in 100 mM K_2HPO_4 – KH_2PO_4 ,

pH 6.0. The O.D._{492 nm} was read with a Bio-Rad 550 microplate reader.

Metabolic labeling, immunoprecipitation, deglycosylation and electrophoresis

sdα-secreting CHO cells were cultured in 6 cm dishes until ~80% confluence, washed and incubated with 2 ml/dish DMEM medium without methionine for 20 min at 37°C. [^{35}S]L-Methionine (10 mCi/ml, 1000 Ci/mmol, Amersham Pharmacia Biotech, Uppsala, Sweden) was then added to a final concentration of 100 $\mu\text{Ci}/\text{ml}$. After 4 h of labeling, the supernatants were collected and cells were washed with PBS and lysed in 500 μl TNN buffer [50 mM Tris–HCl, pH 8.0, 250 mM NaCl, 0.5% (v/v) NP-40] containing 20 mM *N*-ethylmaleimide (Fluka, Buchs, Switzerland), 1 mM PMSF (Calbiochem, La Jolla, CA) and a protease inhibitor cocktail for mammalian cell extracts (Sigma) for 10 min on ice. Cell lysates were then centrifuged for 10 min at 14 000 r.p.m. at 4°C and the supernatants were collected. Extracellular and intracellular fractions were immunoprecipitated with rabbit anti-human IgG (Dako, Glostrup, Denmark) and agarose-immobilized Protein A (Repligen, Cambridge, MA). Agarose beads were then washed with TNN and RIPA buffer [0.1 M Tris–HCl, pH 8.0, 0.1 M NaCl, 5 mM MgCl_2 , 1% (v/v) NP-40, 1% (w/v) deoxycholate, 0.1% (w/v) SDS]. Proteins were eluted with 2% (w/v) SDS for 5 min at 95°C and analyzed by 10% SDS–PAGE under reducing (2.5% β-mercaptoethanol) and non-reducing conditions. The eluted proteins were treated with endoglycosidase H (Endo H_p) and peptide-*N*-glycosidase F (PNGase F) (New England Biolabs, Beverly, MA) under reducing conditions and analyzed by 10% SDS–PAGE.

Chemical cross-linking and Western blot analysis

sdα-secreting CHO cells were cultured in protein-free hybridoma medium (Life Technologies) for 24 h at 37°C. The cell supernatant was dialyzed against PBS and reacted with 600 μM dithiobis(succinimidylpropionate) (DSP, Pierce) for 2 h at 4°C. The reacted material was again dialyzed against PBS and samples were separated by reducing and non-reducing 10% SDS–PAGE and transferred to a nitrocellulose membrane (Sartorius, Göttingen, Germany). Detection of sdα was performed with 9E1, a mouse monoclonal antibody specific for domain D1 of human FcεRI α-chain (unpublished work) and HRP-conjugated goat anti-mouse IgG (Fc) (Pierce). Bound antibodies were detected by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech) autoradiography. IgE_{S1}-secreting J558L cells were cultured in protein-free hybridoma medium and incubated for 4 h at 4°C with sdα at a 4:1 volume ratio to guarantee excess of IgE. DSP cross-linking and detection was as described above. Samples were applied on to a 20 cm non-reducing slab of 0.5% agarose/2.5% SDS–PAGE as described previously (Brewer *et al.*, 1994) and run for 18 h at 50 mA/1.5 mm gel. Transfer of proteins to nitrocellulose was for 20 h at 50 mA. Molecular weight markers were prestained Precision protein standards (broad range, Bio-Rad), human IgM (Sigma), human IgA (Sigma) and IgE_{S1} supernatant from J558L cells. sdα was revealed as described above, while human IgM, IgA and IgE were revealed using HRP-conjugated rabbit anti-human IgA/IgG/IgM (Dako), goat anti-human IgA (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and goat anti-human IgE (Kirkegaard & Perry Laboratories), respectively. Immunoprecipitation of sdα–IgE_{S1} complexes was carried out using rabbit anti-human IgG

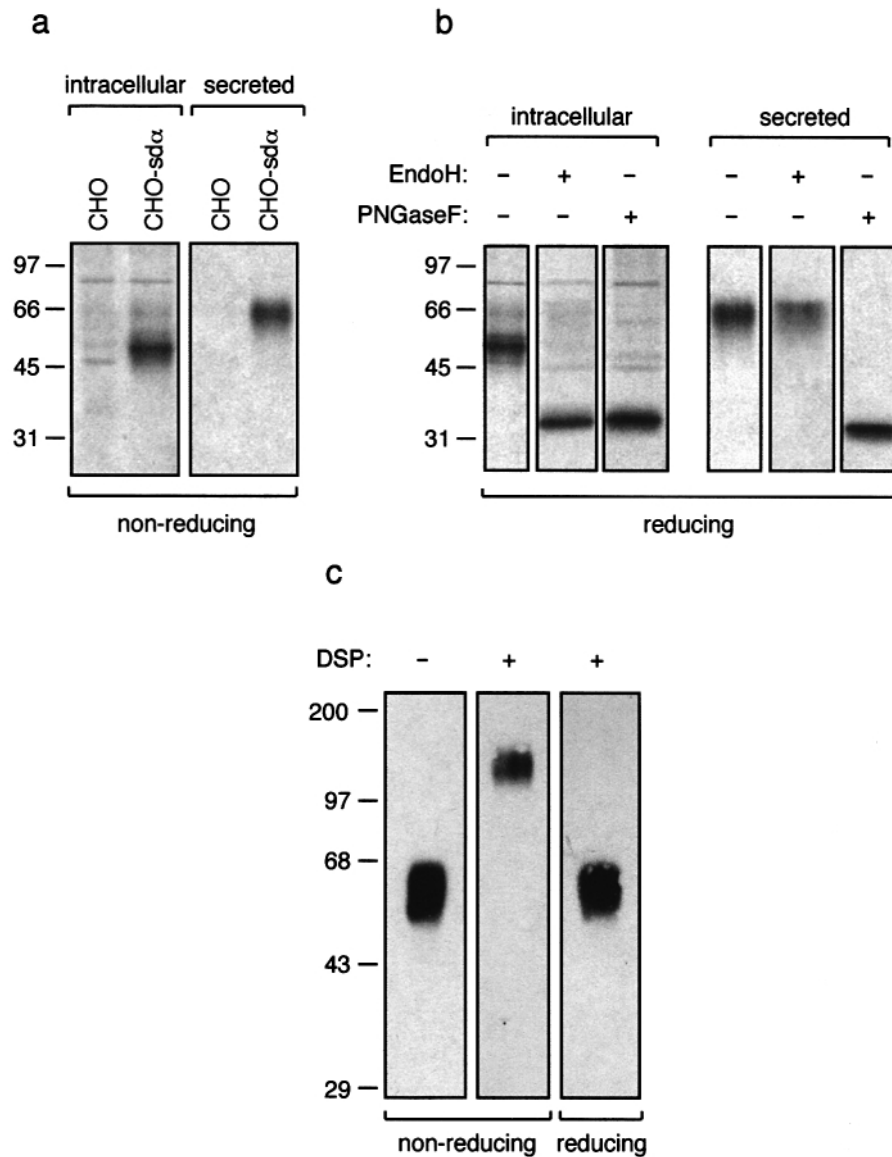


Fig. 2. Characterization of sdα. (a) Non-reducing SDS-PAGE analysis of immunoprecipitated, [³⁵S]methionine-labeled sdα derived from intracellular and culture supernatants of CHO cells or sdα-expressing CHO cells, as indicated. (b) The same materials as shown in (a) were either not treated or were deglycosylated with Endo H or PNGase F, as indicated, and separated by SDS-PAGE under reducing conditions. (c) Western blot analysis of DSP-cross-linked sdα analyzed under reducing and non-reducing conditions, as indicated.

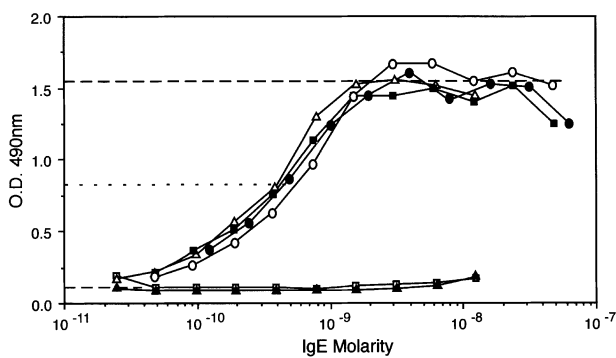


Fig. 3. sdα binding to soluble IgE. ELISA assay on sdα captured on goat anti-IgG-coated wells. Open circles, IgE_{S1}; filled squares, IgE_{S2}; filled circles, IgE from U266 cells; open triangles, commercial IgE; open squares, PBS; filled triangles, irrelevant γ-SIP. Bound antibodies were revealed with HRP-conjugated goat anti-human IgE. Dotted line indicates 50% binding.

(Dako) and agarose-immobilized Protein A (Repligen), as described above.

sdα-IgE_{S1} complexes were purified by affinity chromatography with CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech) coupled to goat anti-human IgE (Kirkegaard & Perry Laboratories). sdα was detected by Western blotting using HRP-conjugated goat anti-human IgG (Fc) (Pierce), as described above.

Secretory IgE binding assay

Secretory IgE binding to immobilized sdα was performed in a solid-phase binding assay (adapted from Basu *et al.*) (Basu *et al.*, 1993) by coating plate wells with goat anti-human IgG (Fc) (Pierce) and incubating with sdα-containing CHO supernatant. After washing, plate wells were incubated overnight at 4°C with secretory IgE_{S1} or IgE_{S2} from J558L cell supernatant, IgE from U266 cells (Nilsson *et al.*, 1970) or serum-purified IgE (Chemicon, Temecula, CA) in PBS. Wells

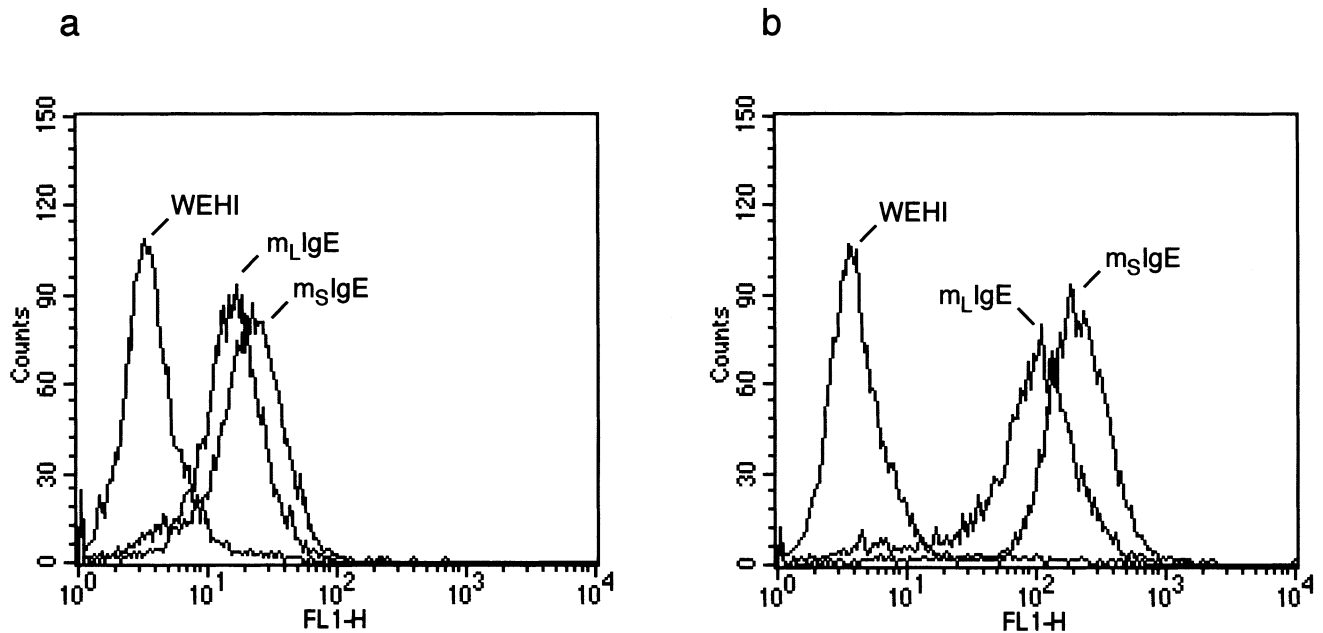


Fig. 4. $sd\alpha$ binding to membrane-bound IgE. Flow cytometry of WEHI 231 cells expressing the long and short membrane IgE isoforms and wild-type cells reacted with (a) $sd\alpha$, followed by FITC-conjugated rabbit anti-human IgG, and (b) FITC-conjugated goat anti-human IgE.

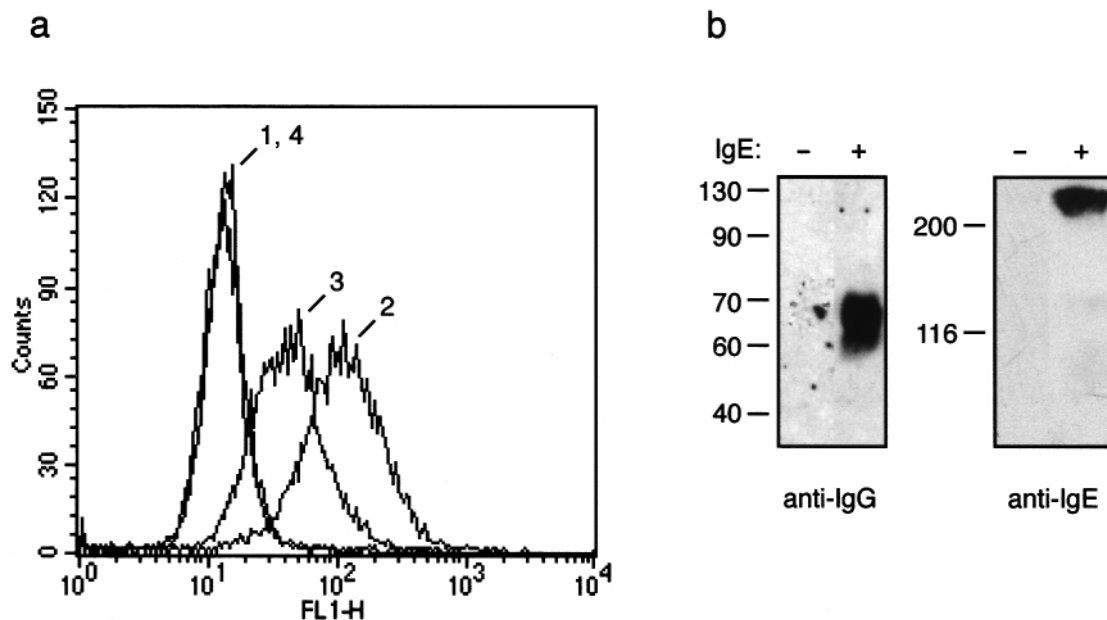


Fig. 5. (a) $sd\alpha$ inhibition of IgE binding to membrane-bound human $Fc\epsilon RI$. RBL SX38 cells incubated with: 1, CHO cells supernatant; 2, IgE_{S1} ; 3, $sd\alpha-IgE_{S1}$ at a 1:1 molar ratio; and 4, $sd\alpha-IgE_{S1}$ at a 2:1 molar ratio. IgE binding was detected using FITC-conjugated goat anti-human IgE. (b) Purification of $IgE_{S1}-sd\alpha$ complex. $sd\alpha$ incubated with (+) or without (-) IgE was loaded on to an anti-IgE affinity column and the eluted material was monitored by Western blotting. $sd\alpha$ and IgE were detected using HRP-conjugated goat anti-human IgG and anti-human IgE, respectively.

were then washed and incubated with HRP-conjugated goat anti-human IgE (Kirkegaard & Perry Laboratories). Plate colorimetry and reading were performed as above.

FACS analysis

Flow cytometry was performed on a FACScalibur, (Becton Dickinson Immunocytometry, Mountain View, CA). IgE antibodies bound to membrane $Fc\epsilon RI$ were detected using FITC-conjugated goat anti-human IgE (Kirkegaard & Perry Laboratories) as described (Lorenzi *et al.*, 1999). Expression of surface IgE isoform long and short on transfected WEHI 231 and A20 cells was monitored using FITC-conjugated goat anti-human IgE (Kirkegaard & Perry Laboratories). Binding of $sd\alpha$ to

human membrane IgE isoforms was performed using FITC-conjugated rabbit anti-human IgG (Fc) (Pierce).

Results

Design of $sd\alpha$

We constructed a chimeric protein gene ($sd\alpha$) coding for the first 197 residues of human $Fc\epsilon RI$ α -chain (comprising the 25 amino acid-long secretion leader peptide and the two extracellular domains D1 and D2) fused, through a flexible five amino acid linker (AGGSG), to human IgG1 CH3 module (107 C-terminal amino acids of $\gamma 1$ -H chain gene). Mature $sd\alpha$, obtained from CHO-transfected cells, consists of a soluble

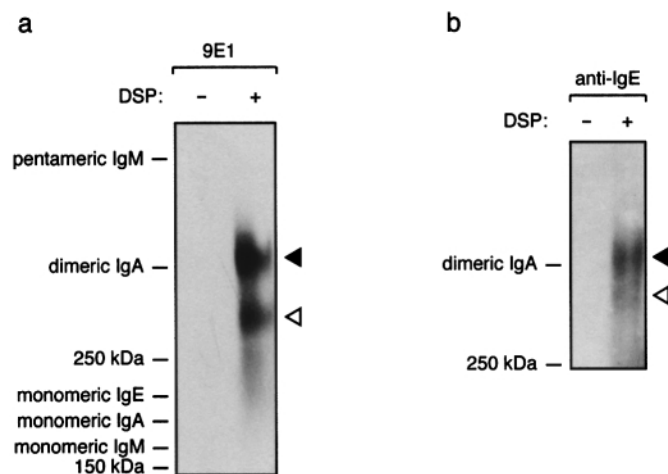


Fig. 6. Western blot analysis of DSP-cross-linked $\text{sd}\alpha$ -IgE_{S1} complexes. Proteins were analyzed under non-reducing conditions. Lanes containing cross-linked (DSP +) and non-cross-linked (DSP -) material are indicated. Molecular weight standards and references set the bands of the $\text{sd}\alpha$ -IgE cross-linked complexes at ~300 and ~500 kDa (open and filled triangles, respectively). (a) Membrane probed with 9E1, a mouse anti-human FcεRI α-chain mAb; (b) membrane of anti-human IgG immunoprecipitated material probed with HRP-conjugated anti-human IgE antibodies.

dimeric receptor containing 284 residues per monomer (Figure 1a). A 3D model of $\text{sd}\alpha$ is proposed (Figure 1b), based on the crystallographic coordinates of human FcεRI α-chain D1D2 (Garman *et al.*, 1998) and human IgG1 CH3 (Deisenhofer, 1981). The linker inserted between domain D2 and γ-CH3 should be unstructured and allow sufficient distance and flexibility to the two components of the chimeric protein. In this way, the receptor moieties should freely dispose to accommodate two IgE antibodies.

Characterization of secreted $\text{sd}\alpha$

CHO cells were transfected with the pcDNA3- $\text{sd}\alpha$ plasmid and clones supernatants screened by ELISA. $\text{sd}\alpha$ expression was investigated by [³⁵S]methionine labeling of the protein followed by immunoprecipitation and SDS-PAGE (Figure 2a, b). As expected, since γ-CH3 dimerization occurs via non-covalent interactions, secreted and intracellular $\text{sd}\alpha$ appeared, under non-reducing conditions, as monomers of ~65 and ~55 kDa, respectively (Figure 2a). The different mobility can be attributed to differences in the degree of terminal glycosylation, which should be complete in the secreted protein. This was confirmed by treating the immunoprecipitated material with endoglycosidases Endo H and PNGase F which deglycosylate high-mannose and complex carbohydrate moieties, respectively. Sensitivity to Endo H was observed only for intracellular $\text{sd}\alpha$ (Figure 2b), where deglycosylation appeared to be complete. Conversely, digestion with PNGase F produced a single band of ~33 kDa for both the intracellular and the secreted protein, as expected for the non-glycosylated polypeptide chain. Absence of Endo H-sensitive $\text{sd}\alpha$ in cells supernatant clearly indicates that the totality of secreted $\text{sd}\alpha$ is terminally glycosylated. From the above observations, it is likely that the transit time between completion of the glycosylation process and $\text{sd}\alpha$ secretion must be very short. $\text{sd}\alpha$ secretion by CHO cells was in the range 1–2 mg/l. The non-covalent dimeric nature of $\text{sd}\alpha$ was investigated by chemical cross-linking of the secreted material using DSP, a bifunctional cross-linker that contains an internal disulfide bridge. Western blot analysis confirmed that DSP-cross-linked $\text{sd}\alpha$ migrates, under non-

reducing conditions, as a dimer of ~130 kDa, whereas, under reducing conditions, it shows the expected mobility of the monomer (Figure 2c).

$\text{sd}\alpha$ binding to secretory IgE

To characterize the biological activity of $\text{sd}\alpha$, we investigated its ability to bind both secretory isoforms of human IgE, IgE_{S1} and IgE_{S2}. These two isoforms derive from alternative splicing of the ε H-chain. Compared with IgE_{S1}, IgE_{S2} contains a distinct ε H-chain C-terminal tailpiece of eight amino acids, with a cysteine in the last position involved in an interchain disulfide bridge (Batista *et al.*, 1996a). We performed a solid-phase binding assay in which $\text{sd}\alpha$ was captured with anti-human IgG and incubated with different concentrations of human IgE from various sources. IgE binding was developed with peroxidase-conjugated anti-human IgE (Figure 3). The assay was highly specific and showed almost identical reactivity for IgE, regardless of the source or isoform. A concentration of $(3\text{--}6)\times 10^{-10}$ M produced 50% binding for all IgE tested. This value is in the range of that reported for the IgE binding to the native membrane receptor [reviewed by Turner and Kinet (Turner and Kinet, 1999)]. As previously reported for cellular FcεRI (Lorenzi *et al.*, 1999), $\text{sd}\alpha$ showed comparable binding for the two secretory IgE isoforms.

$\text{sd}\alpha$ binding to membrane IgE

Human membrane IgE (mIgE) occurs in two variants resulting from alternative splicing. They differ by the length of the extracellular membrane proximal domain (EMPD) (Peng *et al.*, 1992; Zhang *et al.*, 1992; Batista *et al.*, 1996b). The short EMPD is composed of 14 residues while the long EMPD has 66 amino acids. Both IgE isoforms (m_{SH}IgE and m_LIgE) assemble into functional B-cell receptors (Batista *et al.*, 1996b). To investigate whether $\text{sd}\alpha$ was able to bind IgE when expressed on the surface of B-cells, we performed flow cytometry assays on WEHI 231 (Figure 4) and A20 cells (data not shown), stably expressing the two membrane IgE variants. Remarkably, $\text{sd}\alpha$ was able to bind specifically both m_{SH}IgE and m_LIgE in the two cell types, as shown for WEHI 231 cells (Figure 4a). Binding of $\text{sd}\alpha$ was proportional to the mIgE expression level, as determined with anti-IgE antibodies (Figure 4b). This result clearly indicates that, in human membrane-bound IgE, the FcεRI binding site, which resides in domain ε-CH3, is available for recognition by our soluble receptor.

$\text{sd}\alpha$ inhibits IgE binding to membrane FcεRI

To investigate the potential of $\text{sd}\alpha$ for biomedical applications, we performed a simple IgE binding inhibition assay using RBL SX38 cells. These cells, which express the complete human high affinity receptor for IgE (FcεRIαβγ₂), were incubated either with IgE_{S1} or with a mixture of IgE_{S1} and $\text{sd}\alpha$ and then analyzed by flow cytometry using FITC-conjugated anti-IgE antibodies (Figure 5a). As shown, an $\text{sd}\alpha$:IgE molar ratio of 1:1 resulted in 50% inhibition, and a 2:1 ratio completely abolished IgE binding to cell surface FcεRI. As expected, IgE previously bound to the membrane receptor was unable to bind $\text{sd}\alpha$ (data not shown), confirming the 1:1 FcεRI-IgE stoichiometry. To confirm further the $\text{sd}\alpha$ -IgE interaction, the two components were reacted in solution and the complex was applied to an affinity column coupled with anti-IgE. $\text{sd}\alpha$ was found to be specifically retained by the column only when pre-incubated with IgE (Figure 5b). These results are consistent with an efficient IgE scavenging activity of $\text{sd}\alpha$.

sd α binds two IgE molecules

A crucial requirement to assess sd α efficacy is the determination of its bivalency towards IgE. We addressed this issue by incubating sd α with an excess of IgE_{S1}, in order to guarantee full occupancy of sd α binding sites. Binding of sd α to IgE_{S1} was followed by DSP cross-linking and the stoichiometry of the interaction was monitored by Western blotting (Figure 6). An IgE molecule has a molecular weight of ~200 kDa, whereas sd α is a dimer of ~130 kDa (as shown in Figure 2c). We therefore expected a band of ~330 or ~530 kDa, according to an sd α –IgE binding stoichiometry of 1:1 or 1:2, respectively. To achieve an efficient separation of the above molecular weights we performed a non-reducing 0.5% agarose/2.5% SDS–PAGE (Brewer *et al.*, 1994), using human IgM (monomer ~180 kDa, pentamer ~900 kDa), human IgA (monomer ~200 kDa, dimer ~400 kDa) and human IgE (monomer ~200 kDa) as molecular weight references. Monomeric IgA migrates in this system at higher apparent molecular weight (~200 kDa) with respect to monomeric IgM, thus J chain-containing dimeric IgA should migrate at an apparent molecular weight close to or slightly higher than 400 kDa. Following cross-linking of sd α –IgE complexes, two bands of ~300 and ~500 kDa were detected using 9E1 (Figure 6a), a monoclonal antibody specific for domain D1 of human Fc ϵ RI α -chain (unpublished work). The presence of 20–30% of the cross-linked material as a 1:1 complex could be due to incomplete efficiency of the chemical cross-linker. This result indicates that the two binding sites of sd α are available for simultaneous recognition by IgE. Immunoprecipitation of sd α –IgE cross-linked complexes with anti-human IgG antibodies (recognizing the sd α γ -CH3 domain) and probing with anti-human IgE antibodies showed the two ~300 and ~500 kDa bands (Figure 6b), further confirming the sd α –IgE stoichiometry of 1:2.

Discussion

Increasing knowledge of structural biology helps in the design of refined chimeric proteins, coupling structural requirements to functional needs. We introduce here sd α , the first member of a novel family of minimal receptor-Ig chimeras, modeled on the human IgE high affinity receptor (Fc ϵ RI) α -chain domains (D1D2) by fusion to the dimerizing C-terminal domain of human IgG1 (γ -CH3).

sd α was terminally glycosylated and actively secreted by CHO cells as a non-covalent homodimer. The protein showed excellent IgE binding activity, comparable to the natural membrane receptor. In a competition assay, IgE binding to cells bearing human Fc ϵ RI was inhibited by sd α , strongly suggesting a possible use of sd α as an *in vivo* IgE scavenger. In agreement with a previous study (Lorenzi *et al.*, 1999), sd α was shown to bind both IgE isoforms, IgE_{S1} and IgE_{S2}. As expected, the binding stoichiometry of sd α –IgE interaction is 1:2, in agreement with the dimeric nature of the recombinant receptor. In addition, both membrane-bound human IgE isoforms long and short were recognized efficiently by sd α , thus proving the ability of this soluble receptor to interact with IgE on the surface of B-cells. In a previous study, a monomeric version of soluble Fc ϵ RI α -chain was shown to promote inhibition of IgE production on IgE-bearing B-cells (Yanagihara *et al.*, 1994). Since this may imply functional interaction with membrane-bound IgE and considering the sd α cross-linking potency, we are currently investigating cellular responses following sd α binding to IgE-bearing B-cells.

Our minimal receptor-Ig chimera represents an evolution in the engineering of soluble IgE receptors [reviewed by Vangelista and Burrone (Vangelista and Burrone, 2001)]. In fact, fusion with IgG CH3 domain improves many aspects of a soluble receptor. Dimerization should be a means of increasing the apparent affinity. Fusion to a stable serum protein domain (γ -CH3) could confer an increased half-life on the receptor α -chain, as observed for scFvs (L. Borsi *et al.*, in preparation) and provides a tag for the easy purification of the soluble receptor. In addition, sd α being a fully human protein, it should be recognized as a self-constituent *in vivo*.

sd α lacks Fc γ receptor binding, as the recognition site for these receptors resides in γ -CH2 (Sondermann *et al.*, 2000), absent in our chimera. Binding by Fc γ Rs may lead to unwanted counter side effects in clinical trials (Junghans, 1997). We therefore propose a protein design which abolishes the unwanted effects of Fc γ Rs engagement, while retaining the advantages of previous Ig Fc chimeras. However, binding by the neonatal receptor (FcRn), responsible for the extended IgG *in vivo* half-life (Junghans and Anderson, 1996), seems unlikely for sd α , as the FcRn binding site resides largely on γ -CH2 (West and Bjorkman, 2000).

Acknowledgements

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References

- Basu, M., Hakimi, J., Dharm, E., Kondas, J.A., Tsien, W.H., Pilson, R.S., Lin, P., Gilfillan, A., Haring, P. and Braswell, E.H. (1993) *J. Biol. Chem.*, **268**, 13118–13127.
- Batista, F.D., Efremov, D.G. and Burrone, O.R. (1995) *J. Immunol.*, **154**, 209–218.
- Batista, F.D., Efremov, D.G. and Burrone, O.R. (1996a) *Proc. Natl Acad. Sci. USA*, **93**, 3399–3404.
- Batista, F.D., Anand, S., Presani, G., Efremov, D.G. and Burrone, O.R. (1996b) *J. Exp. Med.*, **184**, 2197–2205.
- Blank, U., Ra, C.S. and Kinet, J.P. (1991) *J. Biol. Chem.*, **266**, 2639–2646.
- Brewer, J.W., Randall, T.D., Parkhouse, R.M. and Corley, R.B. (1994) *J. Biol. Chem.*, **269**, 17338–17348.
- Deisenhofer, J. (1981) *Biochemistry*, **20**, 2361–2370.
- Garman, S.C., Kinet, J.P. and Jardetzky, T.S. (1998) *Cell*, **95**, 951–961.
- Garman, S.C., Wurzburg, B.A., Tarchevskaya, S.S., Kinet, J.-P. and Jardetzky, T.S. (2000) *Nature*, **406**, 259–266.
- Guex, N. and Peitsch, M.C. (1997) *Electrophoresis*, **18**, 2714–2723.
- Haak-Frendscho, M., Ridgway, J., Shields, R., Robbins, K., Gorman, C. and Jardieu, P. (1993) *J. Immunol.*, **151**, 351–358.
- Hu, S., Shively, L., Raubitschek, A., Sherman, M., Williams, L.E., Wong, J.Y.C., Shively, J.E. and Wu, A.M. (1996) *Cancer Res.*, **56**, 3055–3061.
- Junghans, R.P. (1997) *Trends Biotechnol.*, **15**, 155.
- Junghans, R.P. and Anderson, C.L. (1996) *Proc. Natl Acad. Sci. USA*, **93**, 5512–5516.
- Kinet, J.P. (1999) *Annu. Rev. Immunol.*, **17**, 931–972.
- Li, E., Pedraza, A., Bestagno, M., Mancardi, S., Sanchez, R. and Burrone, O. (1997) *Protein Eng.*, **10**, 731–736.
- Lorenzi, R., Jouvin, M.H. and Burrone, O.R. (1999) *Eur. J. Immunol.*, **29**, 936–945.
- Nicholls, A., Bharadwaj, R. and Honig, B. (1993) *Biophys. J.*, **64**, A166.
- Nilsson, K., Bennich, H., Johansson, S.G. and Ponten, J. (1970) *Clin. Exp. Immunol.*, **7**, 477–489.
- Peng, C., Davis, F.M., Sun, L.K., Liou, R.S., Kim, Y.W. and Chang, T.W. (1992) *J. Immunol.*, **148**, 129–136.
- Robertson, M.W. (1993) *J. Biol. Chem.*, **268**, 12736–12743.
- Saban, R., Haak-Frendscho, M., Zine, M., Ridgway, J., Gorman, C., Presta, L.G., Bjorling, D., Saban, M. and Jardieu, P. (1994) *J. Allergy Clin. Immunol.*, **94**, 836–843.
- Scarselli, E., Esposito, G. and Traboni, C. (1993) *FEBS Lett.*, **329**, 223–226.

- Sondermann,P., Huber,R., Oosthuizen,V. and Jacob,U. (2000) *Nature*, **406**, 267–273.
- Turner,H. and Kinet,J.P. (1999) *Nature*, **402**, B24–30.
- Vangelista,L. and Burrone,O. (2001) In Cooper,M.D., Takai,T. and Ravetch,J.V. (eds), *Activating and Inhibitory Immunoglobulin-like Receptors*. Springer, Tokyo, pp. 55–62.
- Vangelista,L., Laffer,S., Turek,R., Grönlund,H., Sperr,W.R., Valent,P., Pastore,A. and Valenta,R. (1999) *J. Clin. Invest.*, **103**, 1571–1578.
- West,A.P.,Jr and Bjorkman,P.J. (2000) *Biochemistry*, **39**, 9698–9708.
- Wiegand,T.W., Williams,P.B., Dreskin,S.C., Jouvin,M.H., Kinet,J.P. and Tasset,D. (1996) *J. Immunol.*, **157**, 221–230.
- Yanagihara,Y., Kajiwara,K., Ikizawa,K., Koshio,T., Okumura,K. and Ra,C. (1994) *J. Clin. Invest.*, **94**, 2162–2165.
- Zhang,K., Saxon,A. and Max,E.E. (1992) *J. Exp. Med.*, **176**, 233–243.

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