

Efficient Folding of the FcεRI α-chain Membrane-proximal Domain D2 Depends on the Presence of the N-terminal Domain D1

Luca Vangelista¹, Michela Cesco-Gaspere¹, Dorian Lamba^{1,2} and Oscar Burrone^{1*}

¹International Centre for Genetic Engineering and Biotechnology, Padriciano 99 I-34012 Trieste, Italy

²Istituto di Cristallografia C.N.R., Sezione di Trieste Basovizza Strada Statale 14 Km. 163.5, I-34012 Trieste Italy

Human high affinity receptor for IgE is a membrane glycoprotein multi-chain complex presenting two extracellular Ig modules in its α-chain (D1D2). The receptor IgE binding region is located within the membrane-proximal module D2, while the N-terminal module D1 appears to promote an optimal receptor conformation for IgE binding. To understand the structural relationship between the two modules, we dissected FcεRI α-chain into its discrete Ig units and expressed them in mammalian cells. Unexpectedly, D2 was secreted as a disulphide-linked dimer, while D1 was monomeric. Active secretion and full glycosylation of dimeric D2 suggest a native-like conformation of the protein, justifying the escape from the endoplasmic reticulum/Golgi quality control systems. We then propose a domain-swapping model for D2, in which two interdigitated polypeptide chains assume the overall conformation of two Ig modules, as observed for rat CD2 N-terminal domain. Fusion of an unrelated Ig fold moiety at the N terminus of D2 did not interfere with its dimerisation. While D1D2 assumes a correct fold, co-expression of both isolated domains in the same cell did not restore monomeric folding of D2. Thus, D1 appears to assist the appropriate folding of FcεRI α-chain, acting as an uncleavable intramolecular chaperone-like block towards D2.

© 2002 Elsevier Science Ltd. All rights reserved

Keywords: FcεRI; domain swapping; intramolecular chaperone; folding *in vivo*; molecular modelling

*Corresponding author

Introduction

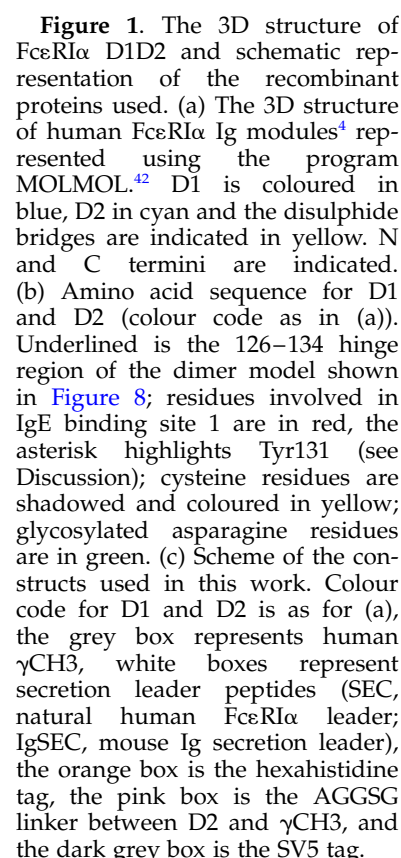
FcεRI, the high affinity receptor for IgE, is a membrane glycoprotein multi-chain complex with a key role in allergic immune disorders.¹ In humans, this receptor has been found in several cell types, either as αβγ₂ or αγ₂ chain variants. While β and γ-chains are responsible for signalling

inside the cell, the extracellular portion of the α-chain presents two highly glycosylated immunoglobulin (Ig) modules (D1 and D2) containing the IgE binding site. FcεRI α-chain (FcεRIα) glycosylation is of primary importance for *in vivo* folding and for the prevention of receptor aggregation, both in membrane and soluble forms.^{2,3} The three-dimensional (3D) structure determination of human FcεRIα⁴ revealed that the two Ig modules are in close proximity, with an extended contact interface forming an acute angle between them. The tight contact and the inter-domain acute angle are unique among proteins containing Ig modules in tandem. These structural features were soon confirmed to be a common theme in the Fc receptor (FcR) family.^{5–7} The crystal structure of the complex between human IgE CH3–CH4 domains and the Ig pair of human FcεRIα confirmed the binding site to be located within the receptor membrane-proximal Ig module.⁸ Moreover, a similar binding mode was found for the corresponding fragments of human IgG–FcγRIII,^{9,10}

Present address: L. Vangelista, Mauro Baschiroto Institute for Rare Diseases, via Bizio 1, 36023 Costozza Vicenza, Italy.

Abbreviations used: Ig, immunoglobulin; D1, domain 1; D2, domain 2; FcεRIα, α-chain of the high affinity IgE Fc receptor; FcγRIII, low affinity IgG Fc receptor; CH, heavy chain constant domain; IgSEC, Ig secretion leader; FCS, fetal calf serum; IRES, internal ribosome entry site; HRP, horse radish peroxidase; H₆, hexahistidine; Endo H, endoglycosidase H; PNGase F, peptide-N-glycosidase F; mAb, monoclonal antibody; PDB, RCSB Protein Data Bank; Sf9, *Spodoptera frugiperda* cells.

E-mail address of the corresponding author: burrone@icgeb.trieste.it



To investigate a possible folding relationship between the two Ig modules of human FcεRIα, we engineered the N-terminal D1 and the membrane-proximal D2 modules as single units and compared their behaviour with the D1D2 pair. The 3D structure of human FcεRIα D1D2⁴ is shown in Figure 1(a), and the amino acid sequence of D1 and D2 is shown in Figure 1(b). We used two C-terminally tagged variants of the D1D2 pair (Figure 1(c)). One is fused to a hexahistidine tag¹³ and the other is fused to the human IgG1 CH3 domain and named sdα (soluble dimeric α-chain).¹⁴ D1 starts with Val1, the natural N terminus of the receptor α-chain, while its C-terminal

boundary, ending with Ser85, was selected to be in the short inter-module loop, according to D1D2 3D structure.⁴ D2 starts with Asp86 and ends with Ala172, a C terminus used successfully in many previous reports on soluble D1D2.^{13–16} Ending the module with Ala172 allows the exclusion of the stalk region connecting the Ig pair to the transmembrane region of Fc ϵ RI α . Both modules have been N-terminally fused to a leader peptide for endoplasmic reticulum (ER) translocation, in order to promote proper biosynthesis and secretion by mammalian cells.

The N-terminal Ig module

Fc ϵ RI α D1 was preceded N-terminally by an immunoglobulin secretion leader peptide (IgSEC)¹⁷ and by a 15 residue peptide tag (SV5) for the easy recognition by a specific monoclonal antibody (Figure 1(c)).¹⁸ Transfection of this construct into CHO cells led to the efficient secretion of the protein as a fully glycosylated monomer (Figure 2). Western blot analysis of SV5-D1 using 9E1, a monoclonal antibody specific for human D1,¹⁴ presented a broad band both under reducing (apparent molecular mass of 25–35 kDa) and non-reducing (20–40 kDa) conditions (Figure 2(a)). Treatment of D1 with peptide-N-glycosidase F (PNGase F) and subsequent Western blot analysis with 9E1 or anti-SV5 mAbs, evidenced a well-defined band both under reducing ($\sim$ 20 kDa) and non-reducing ($\sim$ 17 kDa) conditions (Figure 2(b)), indicating that the protein was heavily glycosylated. Monomeric SV5-D1 migrated in SDS-PAGE at a higher molecular mass than that expected for the deglycosylated polypeptide chain ($\sim$ 11 kDa). Cells supernatant containing secreted SV5-D1 was treated with endoglycosidase H (Endo H) with no effect on the migration pattern of the protein, indi-

cating that the protein was terminally glycosylated (data not shown).

The membrane-proximal Ig module

To differentially recognise the two Ig modules, D2 was fused C-terminally to the CH3 domain of human IgG1 (γ CH3), easily detected by anti-human IgG antibodies (Figure 1(c)). As mentioned above, the protein was N-terminally fused to IgSEC. After transfection, CHO cells produced and secreted the protein mainly in the form of a disulphide-bonded dimer (Figure 3). [³⁵S]methionine-labelled D2- γ CH3 was immunoprecipitated with anti-human IgG antibodies from CHO supernatant and analysed by SDS-PAGE. Under non-reducing conditions, two bands of approximately 40 kDa and 80 kDa were detected, indicating that the protein was secreted in both a monomeric and a dimeric form (Figure 3(a)). This was further confirmed under reducing conditions where only the 40 kDa band was detected. Only $\sim$ 10% of the secreted protein was in the expected monomeric form while the band of the dimer represented $\sim$ 90% of the secreted material. At the same time, the D1D2- γ CH3 version, named sd α ,¹⁴ was used as control and appeared as a monomer of $\sim$ 65 kDa under both reducing and non-reducing conditions (Figure 3(a)).

Analysis of the carbohydrate content revealed that the dimer was terminally glycosylated. In fact, secreted D2- γ CH3 was Endo H resistant, while the material was deglycosylated after PNGase F treatment. Moreover, deglycosylated D2- γ CH3 appeared, under reducing conditions, as a monomer split into two bands of 2–3 kDa difference in the 25 kDa range, whereas sd α was present as a single band of $\sim$ 33 kDa (Figure 3(a)).

The intracellular [³⁵S]methionine-labelled D2- γ CH3 showed significant difference in the molecular mass of the monomer ($\sim$ 30 kDa) and dimer ($\sim$ 60 kDa) (Figure 3(b)) compared to the secreted proteins, indicating that the glycosylation process was as yet incomplete, as confirmed by its sensitivity to Endo H treatment (data not shown). Moreover, the presence of both monomer and dimer in the intracellular environment and detection of the two bands in the $\sim$ 25 kDa range after deglycosylation with PNGase F, confirmed that D2- γ CH3 dimerisation and its possible degradation were intracellular events preceding secretion (Figure 3(b)). A Western blot of secreted D2- γ CH3 essentially confirmed the results obtained by metabolic labelling. Two bands of approximately 18 kDa and 40 kDa, corresponding, respectively, to the deglycosylated monomer and dimer, were obtained under non-reducing conditions. Under reducing conditions, the broad 40 kDa glycosylated monomer was also resolved, following PNGase F treatment, as two polypeptides of about 25 kDa (Figure 3(c)). These two bands might be the result of a protease sensitivity of D2 in the absence of

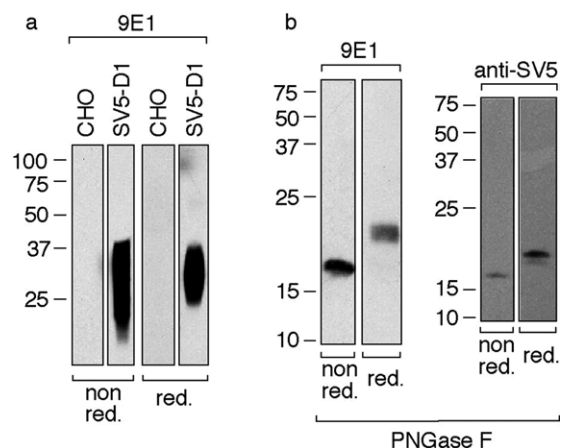


Figure 2. Analysis of isolated Fc ϵ RI α D1. Samples were resolved by SDS-PAGE under reducing and non-reducing conditions and analysed by Western blot using either the D1-specific mAb 9E1 or an anti-SV5 tag mAb, as indicated. (a) Supernatants from non-transfected or SV5-D1-transfected CHO cells. (b) Supernatant containing SV5-D1 treated with PNGase F.

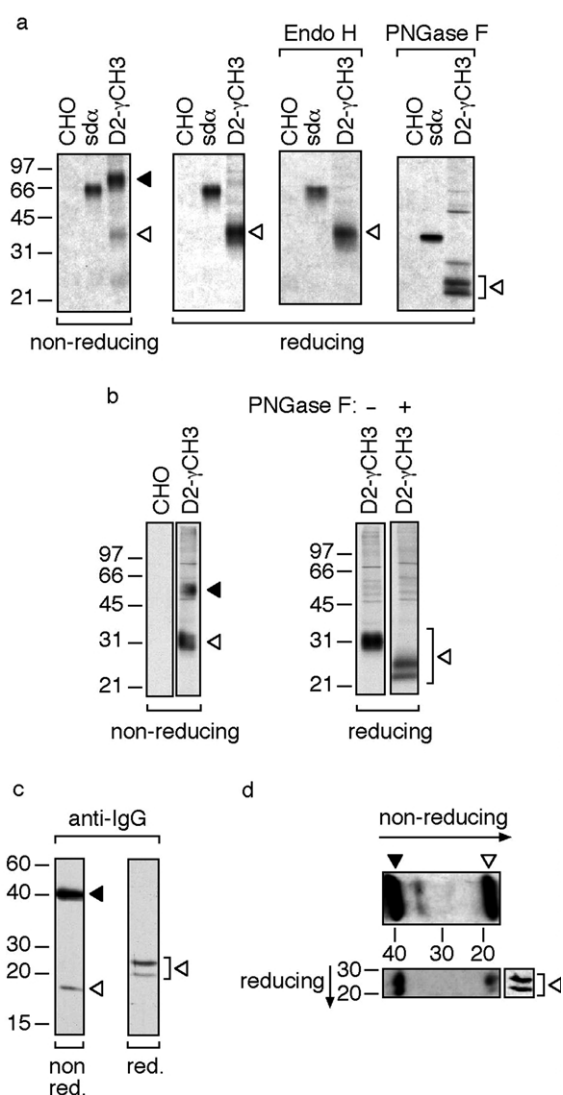


Figure 3. Analysis of D2-γCH3 expression. (a) [³⁵S]methionine-labelled D2-γCH3 and control sdα (D1D2-γCH3), immunoprecipitated from CHO-expressing supernatants, were analysed by SDS-PAGE under reducing and non-reducing conditions, either untreated or treated with endoglycosidase H and PNGase F, as indicated. (b) Analysis of the intracellular material from samples shown in (a). (c) Western blot analysis of secreted D2-γCH3 after PNGase F treatment, using HRP-conjugated anti-human IgG antibodies. (d) Bi-dimensional non-reducing/reducing electrophoresis of the sample shown in (c), analysed by Western blot. The upper and right boxes are reported as reference for the first (non-reducing) and second (reducing) dimension of PNGase F-treated supernatant. Empty and filled triangles indicate monomeric and dimeric D2-γCH3, respectively.

D1. Further analysis of these two products was carried out by bi-dimensional non-reducing/reducing electrophoresis of deglycosylated secreted D2-γCH3. In this way we could identify and estimate the relative amount of the two components in the dimeric and monomeric forms of the protein. From Figure 3(d) it is evident that the majority of the low molecular mass component

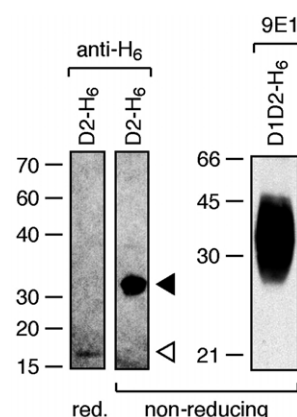


Figure 4. Dimerisation of histidine-tagged D2. Western blot analysis of Sf9 cells supernatant containing D2-H₆ or D1D2-H₆ using HRP-conjugated anti-C-terminal hexahistidine mAb or mAb 9E1, under reducing and non-reducing conditions, as indicated. Empty and filled triangles indicate monomer and dimer, respectively.

participates in the formation of the dimer (present as ~50%), whereas in the monomer the high molecular mass form is predominant (~90%). Similar results were obtained when D2-γCH3 was expressed in the mouse plasmacytoma cell line Sp2/0, indicating that dimerisation is a favoured step in the folding of this protein.

To rule out that the covalent dimerisation of D2-γCH3 could be driven or enhanced by the C-terminal IgG1 domain, we analysed a previously reported histidine-tagged version of D2 (D2-H₆), secreted by Sf9 insect cells.¹³ Immunoblotting with an anti-C-terminal hexahistidine mAb showed that D2-H₆ was a covalent dimer of ~34 kDa, which, under reducing conditions, appeared as a monomer of 16–17 kDa. On the other hand, a hexahistidine-tagged version of D1D2 (D1D2-H₆), secreted by Sf9 cells,¹³ consisted only of the monomeric form (Figure 4). Transfection of D1D2-H₆ into CHO cells led to the secretion of a monomer with higher migration compared to the Sf9-secreted protein, due to the difference in the carbohydrate moieties between insect and mammalian cells (not shown).

We also investigated the IgE binding properties of D2-γCH3, since the receptor binding site resides entirely within the D2 module.⁸ Implementing a previously described ELISA protocol,¹⁴ we found that comparable amounts of sdα (D1D2-γCH3), D2-γCH3 and an irrelevant γCH3 fusion protein¹⁹ showed significant IgE binding activity only for the D1D2-γCH3 version of the receptor (Figure 5). Thus, we can conclude that D2-γCH3 is unable to bind IgE.

Co-expression of FcεRIα modules

All the results described above suggested that proper monomeric folding of D2 was dependent upon the presence of the N-terminal partner D1.

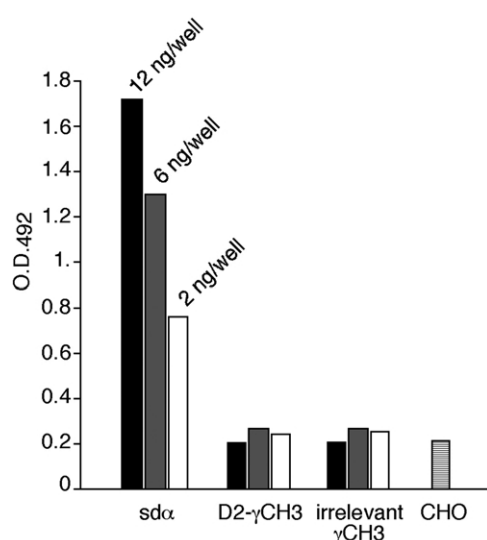


Figure 5. IgE binding assay. sdα, D2-γCH3 and an irrelevant γCH3 fusion protein²⁰ (used as negative control), were captured on an ELISA plate and incubated with IgE. Bound IgE was detected using anti-human IgE antibodies. CHO cells supernatant was used as a negative control (striped bar). Black, grey and white bars correspond to different protein concentrations, as indicated.

Assuming a chaperone-like activity by FcεRIα D1 towards D2, we investigated a possible folding assistance *in trans*, co-expressing the two Ig modules in the same cell as separate units.

CHO cells were co-transfected with the two plasmids encoding SV5-D1 and D2-γCH3. All clones expressing both proteins showed that D2 was indifferent to the presence of the N-terminal moiety *in trans* and was yet secreted as a disulphide-linked dimer (Figure 6).

We then aimed at obtaining a higher expression level of D1 in respect to D2, to favour any possible folding assistance within the ER. To achieve this, we constructed a bi-cistronic system in which, at the 5' end, D1 was under cap-dependent protein

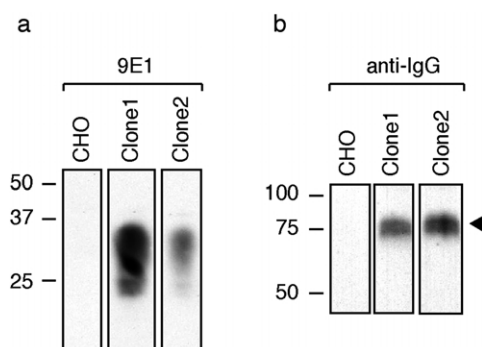


Figure 6. Co-expression of D1 and D2 as isolated units. Supernatants from CHO-transfected cells co-expressing SV5-D1 and D2-γCH3 were resolved under non-reducing conditions and analysed by Western blot. (a) SV5-D1 was revealed using mAb 9E1; (b) D2-γCH3 was detected by HRP-conjugated anti-human IgG antibodies. The filled triangle indicates the D2-γCH3 dimer.

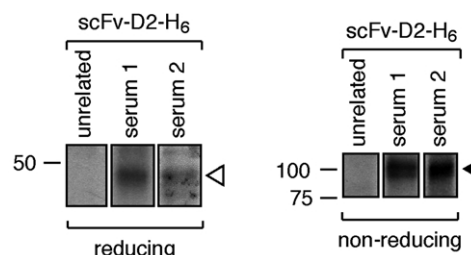


Figure 7. Analysis of scFv-D2-H₆ secretion. [³⁵S]methionine-labelled scFv-D2-H₆ immunoprecipitated from the CHO-expressing supernatant, was analysed by SDS-PAGE under reducing and non-reducing conditions, as indicated. The supernatant was immunoprecipitated using serum 1 (recognising folded/unfolded scFv), serum 2 (recognising only folded scFv) or an unrelated serum (control). Empty and filled triangles indicate monomeric and dimeric scFv-D2-H₆, respectively.

synthesis, followed by D2 under the control of an internal ribosome entry site (IRES).²⁰ Despite this, we observed again secretion of D2 as a dimer, with D1 secreted as a monomer (data not shown).

N-terminal fusion of D2 with an unrelated Ig fold moiety

A single chain antibody variable domains (VL-VH) fragment (scFv) was fused at the D2 N terminus. CHO cells were transfected with the scFv-D2-H₆-encoding plasmid. Cell supernatant, containing ³⁵S-labelled proteins, was immunoprecipitated using two different mouse anti-idiotypic sera recognising the scFv, regardless of its folding (serum 1) or only as properly folded (serum 2).²¹ The scFv moiety contributes with ~30 kDa to the mass of the fusion protein while the glycosylated D2 module has a 20–25 kDa mass (according to Figure 3). As shown in Figure 7, the scFv-D2-H₆ was secreted as a disulphide-bonded dimer. In addition, the idiotype in the scFv-D2 protein was detected as properly folded, since the protein was immunoprecipitated by an anti-idiotypic serum recognising the folded VL-VH assembly.²¹

These results indicate that an unrelated N-terminal Ig fold moiety does not assist folding of D2, strengthening a specific function by D1 in chaperoning D2 folding.

Prediction of D2 folding *via* intertwining

A domain-swapping event,^{22,23} in which extensive inter-digitation of two polypeptide chains generates a native-like Ig fold,²⁴ could justify the observed active processing, secretion and S–S bonding of FcεRIα D2 dimer. This mechanism would account for the lack of intervention by ER/Golgi-resident quality control mechanisms, since the D2 dimer would resemble the native protein. We thus propose the 3D model of an intertwined FcεRIα D2 (Figure 8), on the basis of the

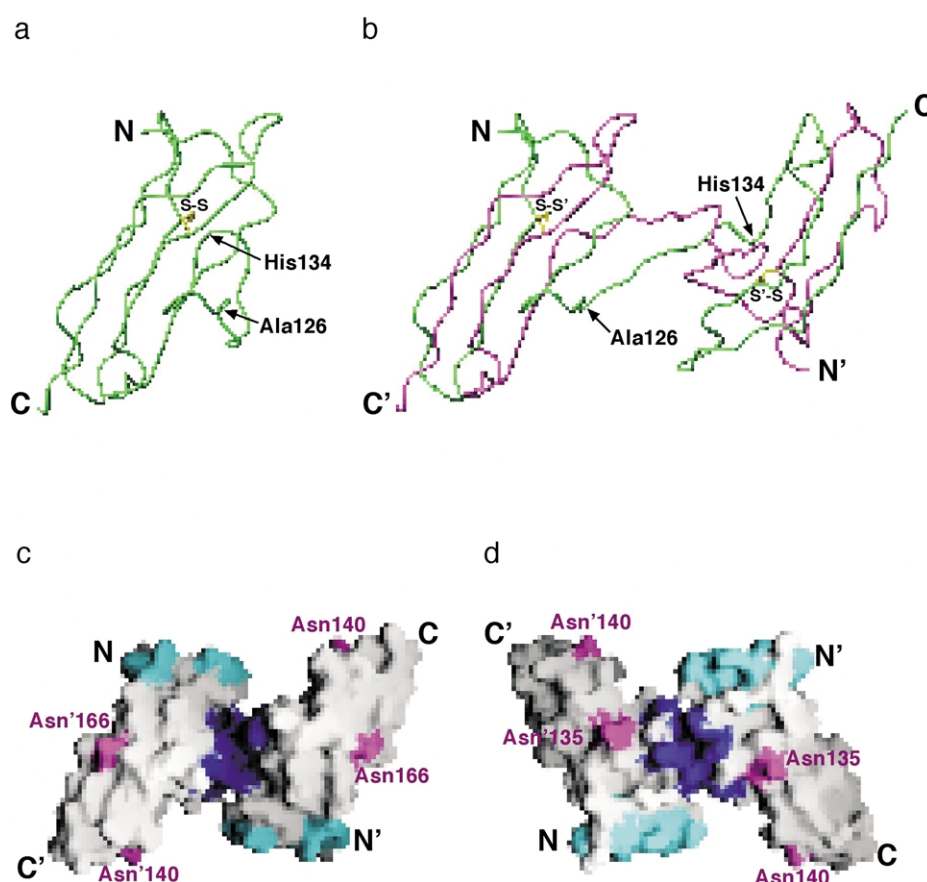


Figure 8. The 3D model of dimeric D2. (a) Backbone representation of monomeric D2, prepared using the program MOLMOL; the intramolecular disulphide bond (S–S) is indicated in yellow. Arrows indicate the region (Ala126–His134) modelled to allow intertwining of D2. (b) Dimeric D2. Intertwined polypeptide chains are coloured in green and pink. Intermolecular disulphide bonds are indicated as S–S' and S'–S. The Ala126–His134 region is highlighted by arrows. (c) Surface representation of dimeric D2 obtained using the program GRASP.⁴³ Glycosylation sites (Asn135, Asn140 and Asn166) are in pink and IgE binding sites 1 and 2 (as described by Garman *et al.*⁸) are in blue and cyan, respectively. (d) A 180° rotation on the *x* axis of (c). N and C termini are indicated. Termini and residues (with an apostrophe) allow distinction of the different polypeptide chains. The template PDB coordinates used are from entry 1F2Q.⁴

well-characterised case of rat CD2 N-terminal Ig domain (CD2 D1), which was shown to dimerise *via* extensive domain swapping.^{25–27} The different IgSF sets²⁸ were also considered in the modelling, namely, rat CD2 D1 belongs to the V set while FcεRIα D2 falls in the C2 set (see Materials and Methods).

The resulting 3D model structure of the FcεRIα D2 dimer agrees with the features observed experimentally. First, the intermolecular disulphide bonding is perfectly allowed in the dimer model as the atomic distance between Cys107 and Cys'151 (as well as Cys'107 and Cys151) would be identical with that of the monomer (2.05 Å) (see S–S in Figure 8(a) and (b)). Second, the three D2 N-glycosylation sites (Asn135, Asn140 and Asn166) are fully exposed to the solvent and far from the two pseudo-domains interface (Figure 8(c) and (d)), thus excluding any possible steric hindrance between the polypeptide and the carbohydrate chains. At the same time, full and terminal glycosylation of the dimer should contribute to its solubility and active secretion, as reported for

soluble D1D2² and for the processing of complete FcεRIα.³ Finally, the observed absence of IgE binding activity by D2-γCH3 is explained by the fact that the amino acids involved in the hinge region of intertwined D2 are those determining the reported IgE binding site 1.⁸ Actually, the IgE binding site 1 is buried in the pseudo-domains interface (Figure 8(c) and (d)).

Discussion

The high affinity receptor for IgE, FcεRI, is a multi-chain membrane glycoprotein which mediates the allergic response driven by allergen-specific IgE antibodies. IgE antibodies are readily bound to FcεRI on the surface of granulocytes and antigen-presenting cells.¹ Upon allergen challenge from the external environment, IgE binding triggers FcεRI signal transduction *via* membrane clustering of the receptor. IgE–FcεRI binding affinity is among the highest in natural protein–protein interactions ($K_d \sim 10^{-10}$ M). IgE binding is provided by

the FcεRI α-chain (FcεRIα), composed of two extracellular Ig domains (D1 and D2), a short stalk region followed by a transmembrane domain and a cytoplasmic tail. In human FcεRIα, D1 (the N-terminal Ig module) has been reported to contribute to the appropriate orientation of D2 (the membrane-proximal Ig module, containing the IgE binding site), indirectly promoting high IgE affinity.¹¹ In an attempt to elucidate the relationship between D1 and D2, we dissected the receptor α-chain into two discrete modular units and analysed them independently or in co-expression.

Biosynthesis of D1 in mammalian cells led to the active secretion of a monomeric, terminally glycosylated protein, indicating that D1 contains all the information necessary for its autonomous (isolated unit) folding in mammalian cells.

Conversely, D2 was actively secreted by mammalian cells as a terminally glycosylated protein but, unexpectedly, it assembled as a covalently linked dimer *via* intermolecular disulphide bonding (Figure 3). Since intracellular dimeric D2 was sensitive to deglycosylation by endoglycosidase H, dimerisation should occur within the endoplasmic reticulum, representing the preferred final conformation for this Ig module when deprived of N and C-terminal constraints (isolated module).

Dimerisation of D2 is unlikely to be the result of a mere misfolding of the polypeptide chain. The protein appeared not to be retained and degraded intracellularly, rather the D2 dimer was efficiently glycosylated and secreted. As bio-processing of the module appeared regular, we envisaged the possible presence of a native-like conformation in the dimer. A conformation of dimeric D2 resembling its native Ig fold would explain the escape from the ER/Golgi quality control mechanisms, leading to the active glycosylation and secretion in the extracellular environment. Therefore, we proposed an extended domain-swapping event^{22,23} in which a complete interdigitation of two polypeptide chains would generate an intertwined dimer, assuming the final folded conformation, at all similar to two identical Ig modules facing each other (Figure 8). Our prediction is on the basis of the extensively documented case of rat CD2 N-terminal Ig module (CD2 D1), whose crystallographic 3D structure revealed an alternative fold assuming the above-mentioned dimerisation *via* intertwining.²⁵ Subsequent mutagenesis on rat CD2 D1 led to further characterisation of the process generating this peculiar fold.^{26,27} We thus designed a 3D structural model of human FcεRIα D2, on the basis of the deposited crystallographic coordinates,⁴ using CD2 D1 coordinates as a template for the dimerisation mode.

A major difference, apart from the IgSF classification of the two Ig modules, is the absence of the canonical Ig fold cysteine residues in CD2 D1, present in FcεRIα D2. In addition, while intertwining of rat CD2 D1 occurs in the cytoplasm of *Escherichia coli*, FcεRIα D2 dimer is the result of an *in vivo* bio-processing within the ER of mammalian cells. Our

results strongly suggest that the escape of the D2 dimer is unlikely to be the result of a saturation of ER-resident retention–degradation systems, rather the completion of a stepwise biosynthesis ending with the active secretion of the protein.

The proposed model represents an intertwined dimer, which explains our experimental evidence. Firstly, secretion by mammalian cells should require a certain degree of order in the protein, leading to occlusion of hydrophobic surfaces for the release from ER/Golgi-resident quality control. Secondly, as reported for both soluble D1D2 and the complete membrane-anchored FcεRIα,^{2,3} terminal glycosylation is necessary for efficient folding and secretion or transport to the cell membrane, respectively. Glucose trimming is a main prerequisite for the subsequent checkpoint of ER-resident lectin-like chaperones, such as calnexin and calreticulin.^{29,30} In the case of human FcεRIα, interaction with calnexin has been reported,³ further confirming the importance of glycosylation-mediated chaperone assistance. The structural model of dimeric D2 presents solvent exposure of the glycosylation sites within the two pseudo-Ig domains (Figure 8). Therefore, the discussed mechanism of glycosylation-mediated chaperone assistance would be allowed in the modelled dimer. Thirdly, the intermolecular disulphide bonding of the model presents the appropriate atomic distance.

Moreover, the dimer conformation proposed here explains the IgE binding properties of isolated human FcεRIα D2. In fact, the present work and all previous studies reporting the analysis of isolated D2, evidenced absence³¹ or significant decrease^{13,32,33} in IgE binding activity by the module, although IgE recognition in human FcεRI is attributable exclusively to D2.⁸ The model presented highlights the fact that the hinge connecting the two pseudo-domains involves IgE binding site 1 and is buried by the two pseudo-domains. Specific residues in IgE binding site 1 are the determinants for Ig Fc higher affinity of FcεRI compared to all other receptors in the FcR family.^{8,9,34} The hinge region selected for the design of the intertwined D2 model comprises strand C'. This was done to reach the closest analogy between the experimental hinge region in intertwined rat CD2 D1 and the corresponding region in human FcεRIα D2. Noteworthy, a recent survey on 11 crystallographic forms of human FcεRIα D1D2³⁴ evidenced that the D2 C' strand assumes different conformations in different crystal forms. In one form this region of D2 reaches the extreme of assuming a D strand conformation, thus forming hydrogen bonds with the opposite sheet of the Ig fold, relative to the C' strand.³⁴ Accordingly, the conformational variability of strand C' would further justify our decision to disrupt it to form the hinge connecting the two pseudo-domains of an intertwined dimer.

Next, we looked at human FcεRIα D1 in search for a possible role played by this domain in the

folding assistance to its C-terminal partner, D2. All soluble versions of the Ig pair D1D2 have been reported to be monomeric and possess full IgE binding activity. Thus, a role for D1 in assisting the folding of D2 can already be assumed. Recently, a new category of chaperone-like moieties has been proposed,¹² where the activity is given by an uncleaved intramolecular block helping the proper folding of the remainder of the protein. To date, only the dihydrofolate reductase has been shown to possess such an internal chaperone block, although there are other potential candidates.¹² In light of our results, we propose Fc ϵ RI α D1 as an uncleaved N-terminal chaperone-like block acting towards D2 to promote its native (monomeric) state. Furthermore, we found that D1 assisted D2 folding only in the context of the D1D2 pair (*cis*), whereas this was not the case when both modules were co-expressed in the same cell as separate units (*trans*).

In order to understand whether the putative D1 chaperone-like activity towards D2 is a specific feature of the Fc ϵ RI α N-terminal domain, we fused D2 at its N terminus with an unrelated Ig fold moiety (namely a scFv). This fusion was decided on the basis of the fact that a scFv is able to assume its fold autonomously, a prerequisite for a putative chaperone to assist the folding of a protein. Dimerisation of D2, even in the context of the N-terminal scFv fusion (Figure 7), reinforces an exclusive role played by D1 in assisting proper folding of D2.

In perspective, the high structural homology of D1D2 within the Fc receptors family including Fc ϵ RI, Fc γ Rs and members of the recently reported FcR homologs,³⁵ suggests that D1 assistance to D2 folding could be a common mechanism for successful domain assembly. Indeed, interchanges of human Fc ϵ RI α D1 with the corresponding D1 from either Fc γ Rs (Rigby *et al.*¹¹) or rat Fc ϵ RI α D1³² were not shown to be dimeric and resulted nearly as effective in allowing D2 IgE binding activity. Following analysis of the D1D2 inter-domain interface, also in terms of residue conservation within the FcR family, it appears that many conserved and interacting residues are present, both in D1 (i.e. W13, F17, E(D)20) and D2 (i.e. L(V)89, Q91, R106, H108, W110). However, only a direct experimental investigation will prove a general D1 assistance towards D2 in the FcR α -chains. Alternatively, D2 dimerisation could occur exclusively for Fc ϵ RI. In fact, the region putatively involved in the intertwined dimer hinge comprises three aromatic residues (Tyr129, Trp130 and Tyr131), specific for Fc ϵ RI within its Fc receptor family.^{8,9} In monomeric D2, this triad forms a hydrophobic patch which, in the dimer model, becomes buried between the two pseudo-domains, within a polar/charged residue environment.

Moreover, residues 129–131 participate in IgE binding site 1 and have been proposed to be responsible for the recently observed C' strand flexibility in D2.³⁴ Finally, Tyr131 is likely the most important

determinant in providing Fc ϵ RI exclusive high affinity,^{34,36} thus its inaccessibility in the model would be in agreement with our observations.

Materials and Methods

Cell lines and media

Chinese hamster ovary (CHO) cells were grown in α -MEM medium (Life Technologies, Gaithersburg, MD, USA) containing 40 μ M deoxy-ribonucleosides and 40 μ M ribonucleosides, supplemented with 10% (v/v) fetal calf serum (FCS) (Life Technologies). IgE_{s1} produced by mouse myeloma transfected J558L cells have been described.^{37,38} Mouse plasmacytoma Sp2/0 cells were grown in RPMI medium (Life Technologies), supplemented with 10% (v/v) FCS.

Cloning

The C-terminally hexahistidine-tagged Fc ϵ RI α variants D1D2 and D2 for insect cells expression (pVL1392-D1D2-H₆ and pVL1392-D2-H₆), and the Fc ϵ RI α chimera sd α (pcDNA3-sd α) for mammalian cells expression, have been described.^{13,14}

pcDNA3-D1D2-H₆ was obtained by ligating the *Bgl*II/*Eco*RI 631 bp fragment (containing D1D2 with the natural N-terminal Fc ϵ RI α leader sequence for endoplasmic reticulum translocation) deriving from pVL1392-D1D2-H₆ into the *Bam*HI/*Eco*RI sites of pcDNA3, making use of *Bgl*II and *Bam*HI cohesive ends.

Human γ CH3-fused D2 (D2- γ CH3) was rescued from the pcDNA3-sd α vector,¹⁴ using a natural *Stu*I site occurring after the first six N-terminal amino acid residues of the D2 domain. pcDNA3-sd α was *Stu*I/*Xba*I-digested and the resulting 601 bp fragment ligated into the respective sites of a modified pUT-SEC vector.¹⁷ Modification of pUT-SEC was performed by annealing of *Apa*LI/*Xho*I sticky oligonucleotides (5'-TGCACTCGGA-CTGGCTGCTCCTTCAGGCCTC-3' and 5'-TCGAGAGG-CCTGAAGGAGCAGCCAGTCCGAG-3') and ligation in the respective vector sites. The above oligonucleotides contain a suitable *Stu*I site to recover the missing N-terminal boundary of D2.^{4,13} *Hind*III/*Kpn*I digestion of pUT-SEC-D2- γ CH3 released a fragment, which, by ligation into a *Hind*III/*Kpn*I-linearised pcDNA3 plasmid (Invitrogen, Groningen, The Netherlands), yielded the pcDNA3-D2- γ CH3 construct.

Construction of isolated Fc ϵ RI α D1, N-terminally fused to a SV5 tag (EGKPIPNNLLGLDGS),¹⁸ was obtained by annealing *Apa*LI/*Kpn*I sticky oligonucleotides encoding the SV5 tag (5'-TGCACTCGGAAGGCA-AACCAATCCCAAACCCACTGCTGGGCCTGGATT CCGGAGGTAC-3' and 5'-CTCCGGAATCCAGGCCCA-GCAGTGGGTTTGGGATTGGT TTGCCTTCCGAG-3', containing a *Bsp*EI site upstream of *Kpn*I) and ligation in the respective sites of the pUT-SEC vector, to obtain pUT-SEC-SV5. Domain 1 of human Fc ϵ RI α was PCR amplified from pcDNA3-sd α using the 5' *Bsp*EI-containing oligonucleotide (5'-GATTCCGG AGTCCCTCAGAA-ACCTAAG-3') and the 3' *Sac*I-containing oligonucleotide (5'-AATGAG CTCTCATTAAGTGAAGACTTCCAGGTA-3'). The PCR product was *Bsp*EI/*Sac*I-digested and ligated into the corresponding sites of pUT-SEC-SV5. Digestion of the resulting pUT-SEC-SV5-D1 with *Hind*III/*Eco*RI and ligation of the fragment into the corresponding sites of pcDNA3 yielded the pcDNA3-SV5-D1 vector.

Construction of the SV5-D1-IRES-D2-γCH3 co-expression cassette was obtained by purifying the 592 bp *EcoRI/BamHI* fragment from the BSICZSVPA vector,³⁹ containing the encephalomyocarditis virus internal ribosome entry site (EMCV IRES). Ligation of the above fragment into the corresponding *EcoRI/BamHI* sites of pUC19 yielded a pUC19-IRES vector. Digestion of pUT-SEC-D2-γCH3 with *XbaI* and partial digestion with *NcoI* released a 758 bp fragment encoding for the pUT-SEC mouse Ig secretion leader (IgSEC)¹⁷ fused to the complete D2-γCH3 cDNA (IgSEC-D2-γCH3). Ligation of the above IgSEC-D2-γCH3 *XbaI/NcoI* fragment into the corresponding sites of pUC19-IRES and subsequent *EcoRI/XbaI* digestion released a fragment corresponding to the IRES-IgSEC-D2-γCH3 cassette. Ligation of the above fragment into the *EcoRI/XbaI* sites of pcDNA3-SV5-D1 yielded the final pcDNA3-SV5-D1-IRES-D2-γCH3 construct.

To obtain the N-terminal fusion of the scFv (from the B-cell lymphoma BCL1¹⁹) to D2-H₆ the pUT-SEC vector modified with the *ApaI/XhoI* sticky oligonucleotides containing the *StuI* site was *StuI/XbaI*-digested. The linearised vector was ligated with the fragment *StuI/XbaI* excised from pVL1392-D2-H₆. The resulting pUT-SEC-D2-H₆ (cloned for mammalian expression of D2-H₆; L. V., M. C.-G. & O. B. unpublished data) vector was *HindIII/StuI*-digested and the annealed *HindIII/StuI* sticky oligonucleotides (5'-AGCTTTCTG GCTCCGGAG-GCTCTGGGGACTGGCTGCTCCTTCAGG-3' and 5'-CCTGAAGGAGCA GCCAGTCCCCAGAGCCTCCGG-AGCCAGAA-3'), containing an internal *BspEI* site, were ligated in the linearised plasmid. A *HindIII/KpnI* fragment was released from the resulting plasmid and ligated into the corresponding sites of pcDNA3. The resulting plasmid was *HindIII/BspEI*-digested and the scFv-containing *HindIII/BspEI* fragment, excised from pBCL1,¹⁹ was ligated therein, leading to the final pcDNA3-scFv-D2-H₆ construct.

Cells transfection and selection

CHO (5 × 10⁵) cells were resuspended in cold PBS (phosphate-buffered saline: 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 137 mM NaCl, 3 mM KCl (pH 7.2)) and electroporated with pcDNA3 constructs (10 µg for pcDNA3-D1D2-H₆, pcDNA3-SV5-D1, pcDNA3-D2-γCH3, pcDNA3-SV5-D1-IRES-D2-γCH3, and a mix of 10 µg of pcDNA3-SV5-D1 and 1 µg of pcDNA3-D2-γCH3) previously linearised by *PvuI* digestion, with a single pulse at 25 µF and 1000 V in a Bio-Rad Gene Pulser (Bio-Rad, Hercules, CA, USA). After electroporation, cells were kept five minutes 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, USA). Selection of positive clones was carried out in the presence of 400 µg/ml Geneticin (G-418 sulphate; Life Technologies). Sp2/0 cells (1 × 10⁶) were electroporated as described above with 10 µg of *PvuI*-linearised pcDNA3-D2-γCH3, with a single pulse at 960 µF and 250 V. Screening of D2-γCH3-secreting cells was executed by coating a 96-wells ELISA plate (Nunc Maxisorp, Roskilde, Denmark) with goat polyclonal anti-human IgG (Fc) antibodies (Pierce, Rockford, IL, USA), 1 µg/well in 50 mM Na₂CO₃/NaHCO₃ (pH 9.5) overnight at 4 °C. Cells supernatants were added and incubated for one hour at room temperature, followed by horse radish peroxidase (HRP)-conjugated goat anti-human IgG (Fc) antibodies (Pierce) for one hour at room temperature.

Detection was performed using the ImmunoPure TMB substrate kit (Pierce) followed by plate reading with a Bio-Rad 550 microplate reader. Screening of D1D2-H₆-secreting cells was executed by ELISA (monitoring IgE-D1D2 binding), coating wells with commercial IgE (Chemicon, Temecula, CA, USA), detecting by 9E1, a mouse mAb anti-human FcεRIα D1¹⁴ and revealing with HRP-conjugated goat anti-mouse IgG (Fc) antibodies (Pierce). Selection of SV5-D1-secreting cells was executed by Western blot analysis (see below).

Metabolic labelling, immunoprecipitation, deglycosylation and electrophoresis

Metabolic labelling of D2-γCH3-secreting CHO cells was performed as described for sdα.¹⁴ Briefly, 80% confluent cells were cultured in methionine-free medium for 20 minutes at 37 °C. L-[³⁵S]methionine (Amersham Pharmacia Biotech, Uppsala, Sweden) was then added to a final concentration of 100 µCi/ml and, after four hours incubation at 37 °C, supernatants were collected and cells were lysed, centrifuged and lysate supernatants collected. Extracellular and intracellular fractions were immunoprecipitated with rabbit anti-human IgG (Dako, Glostrup, Denmark) and agarose-immobilised Protein A (Repligen, Cambridge, MA, USA). Agarose beads were then washed and proteins were eluted and analysed by SDS-PAGE under reducing (2.5% (v/v) β-mercaptoethanol) and non-reducing conditions. Eluted proteins were treated with endoglycosidase H (Endo H) and peptide-N-glycosidase F (PNGase F) (New England Biolabs, Beverly, MA, USA) under reducing conditions and analysed by SDS-PAGE. CHO supernatant containing ³⁵S-labelled scFv-D2-H₆ was immunoprecipitated as described above, using mouse polyclonal anti-idiotypic sera, obtained by protein (serum 1) or DNA (serum 2) immunisation, as described.²¹

Western blot analysis

Cells supernatants containing SV5-D1 and D2-γCH3 were separated under reducing and non-reducing SDS-PAGE and transferred to a nitrocellulose membrane (Sartorius, Göttingen, Germany). SV5-D1 was detected either using 9E1 or a mouse monoclonal anti-SV5¹⁸ and revealed by HRP-conjugated goat anti-mouse IgG (Fc) antibodies (Pierce). Detection of D2-γCH3 was performed with HRP-conjugated goat anti-human IgG (Fc) antibodies (Pierce). Supernatants containing D1D2-H₆ were detected using 9E1, while D2-H₆ was detected with an HRP-conjugated mouse monoclonal antibody anti-C-terminal hexahistidine (Invitrogen). Bound antibodies were revealed by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech) autoradiography.

Secretory IgE binding assays

sdα, D2-γCH3 and an irrelevant protein, a γCH3 fusion to a scFv from a murine lymphoma,¹⁹ were captured on an ELISA plate coated with goat anti-human IgG (Fc) (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA), as described.¹⁴ Following, supernatant from IgE_{SI}-secreting J558L cells was incubated for one hour at room temperature and bound IgE antibodies were detected using HRP-conjugated goat anti-human IgE (Fc) (Kirkegaard & Perry Laboratories). Plate colorimetry was performed using 2 mg/ml *o*-phenylenediamine (Sigma, St. Louis, MO, USA) and 0.03% (v/v) H₂O₂ as

peroxidase substrate, in 100 mM K₂HPO₄/KH₂PO₄ (pH 6.0).

Modelling

The coordinates for the crystal structure of human FcεRIα,⁴ PDB entry 1F2Q, were used to construct a model of the human FcεRIα D2 intertwined dimer. To model the angle between the two pseudo-domains we analysed both the available crystal structure of the dimeric form of rat CD2 N-terminal D1 domain,²⁵ (PDB entry 1CDC) and the crystal structures of the dimeric form of rat CD2 D1 deletion mutant (ΔMet46,ΔLys47)^{26,27} (PDB entries 1A64, 1A6P and 1A7B).

In order to use CD2 D1 as a template for the FcεRIα D2 dimer model, we analysed the structural details of the Ig fold of the two modules. FcεRIα D2 belongs to the C2 set in the Ig superfamily (IgSF) classification,²⁸ while CD2 D1 falls in the V set. The differences in the β-strand topology of these two IgSF sets are the absence of the C' and D strands in the C2 set, while V domains lack the A' strand. These account for the smaller size of C2 domains compared to V domains (specifically, human FcεRIα D2 is 87 residues long while the rat CD2 D1 length is 99 residues).

In the crystal structures of intertwined rat CD2 D1 and of its deletion mutant (ΔMet46,ΔLys47), the C' β-strand unfolds and shapes itself into the so-called hinge region²⁵ between the two Ig pseudo-domains (each domain is composed by two different polypeptide chain halves). Since human FcεRIα D2 is 12 residues shorter than rat CD2 D1, we used CD2 D1 domain deletion mutant (ΔMet46,ΔLys47) (PDB entry 1A64) as the most appropriate three-dimensional structural template to model the orientation of the two FcεRIα D2 pseudo-domains. The superposition of the 3D structures of FcεRIα D2 and CD2 D1, showed that a possible hinge region in FcεRIα D2 should include the C' strand. Therefore, we disrupted strand C' to allow interdigitation of FcεRIα D2, forming a similar arrangement to the C'' strand in the intertwined CD2 D1. We have manually rebuilt, using the program O,⁴⁰ the main-chain hinge region of the FcεRIα D2 dimer that spans residues 126–134 and includes the C' strand. The reciprocal orientation of the two pseudo-domains in the intertwined dimer of FcεRIα D2 was superimposed on that of CD2 D1 deletion mutant ΔMet46,ΔLys47.^{26,27} The side-chains orientations of the residues encompassing the hinge region were adjusted manually. Energy minimisation of residues Ala126–His134 was then performed using the GRO-MOS96 force field as implemented in the program Swiss-PdbViewer.⁴¹ The criteria for judging the quality of the model included the preservation of an approximate 2-fold symmetry between the two pseudo-domains and the absence of side-chain steric clashes.

A ribbon representation (Figure 8(b)) of the final model of intertwined human FcεRIα D2 has been produced with MOLMOL⁴² and the molecular surface (Figure 8(c) and (d)) has been calculated with the program GRASP.⁴³

Acknowledgements

This work is dedicated to the memory of Roberto Lorenzi, an esteemed former collaborator. We

thank Oliviero Carugo for critically reading the manuscript, Federico Odreman Macchioli and Alessandro Marcello for fruitful discussion on the IRES-dependent expression and Alessandro Pintar for help in preparing the MOLMOL Figures. L. V. was supported by an ICGEB Postdoctoral Fellowship, M. C.-G. was supported by a Pre-doctoral Fellowship from the International School for Advanced Studies (SISSA), Trieste, Italy.

References

1. Kinet, J. P. (1999). The high-affinity IgE receptor (FcεRI): from physiology to pathology. *Annu. Rev. Immunol.* **17**, 931–972.
2. Letourneur, O., Sechi, S., Willette-Brown, J., Robertson, M. W. & Kinet, J. P. (1995). Glycosylation of human truncated FcεRI α chain is necessary for efficient folding in the endoplasmic reticulum. *J. Biol. Chem.* **270**, 8249–8256.
3. Albrecht, B., Woisetschlager, M. & Robertson, M. W. (2000). Export of the high affinity IgE receptor from the endoplasmic reticulum depends on a glycosylation-mediated quality control mechanism. *J. Immunol.* **165**, 5686–5694.
4. Garman, S. C., Kinet, J. P. & Jardetzky, T. S. (1998). Crystal structure of the human high-affinity IgE receptor. *Cell*, **95**, 951–961.
5. Sondermann, P., Huber, R. & Jacob, U. (1999). Crystal structure of the soluble form of the human Fcγ-receptor IIb: a new member of the immunoglobulin superfamily at 1.7 Å resolution. *EMBO J.* **18**, 1095–1103.
6. Maxwell, K. F., Powell, M. S., Hulett, M. D., Barton, P. A., McKenzie, I. F., Garrett, T. P. & Hogarth, P. M. (1999). Crystal structure of the human leukocyte Fcε receptor, FcεRIα. *Nature Struct. Biol.* **6**, 437–442.
7. Zhang, Y., Boesen, C. C., Radaev, S., Brooks, A. G., Fridman, W. H., Sautes-Fridman, C. & Sun, P. D. (2000). Crystal structure of the extracellular domain of a human FcγRIII. *Immunity*, **13**, 387–395.
8. Garman, S. C., Wurzburg, B. A., Tarchevskaya, S. S., Kinet, J. P. & Jardetzky, T. S. (2000). Structure of the FcεRI fragment of human IgE bound to its high-affinity receptor FcεRIα. *Nature*, **406**, 259–266.
9. Sondermann, P., Huber, R., Oosthuizen, V. & Jacob, U. (2000). The 3.2 Å crystal structure of the human IgG1 Fc fragment–FcγRIII complex. *Nature*, **406**, 267–273.
10. Radaev, S., Motyka, S., Fridman, W. H., Sautes-Fridman, C. & Sun, P. D. (2001). The structure of a human type III Fcγ receptor in complex with Fc. *J. Biol. Chem.* **276**, 16469–16477.
11. Rigby, L. J., Epa, V. C., Mackay, G. A., Hulett, M. D., Sutton, B. J., Gould, H. J. & Hogarth, P. M. (2000). Domain one of the high affinity IgE receptor, FcεRI, regulates binding to IgE through its interface with domain two. *J. Biol. Chem.* **275**, 9664–9672.
12. Ma, B., Tsai, C. J. & Nussinov, R. (2000). Binding and folding: in search of intramolecular chaperone-like building block fragments. *Protein Eng.* **13**, 617–627.
13. Vangelista, L., Laffer, S., Turek, R., Grönlund, H., Sperr, W. R., Valent, P. *et al.* (1999). The Ig-like modules Cε3 and α2 are the minimal units necessary for human IgE–FcεRI interaction. *J. Clin. Invest.* **103**, 1571–1578.

14. Vangelista, L., Cesco-Gaspere, M., Lorenzi, R. & Burrone, O. (2002). A minimal receptor-Ig chimera of human FcεRI α-chain efficiently binds secretory and membrane IgE. *Protein Eng.* **15**, 51–57.
15. Blank, U., Ra, C. S. & Kinet, J. P. (1991). Characterization of truncated alpha chain products from human, rat, and mouse high affinity receptor for immunoglobulin E. *J. Biol. Chem.* **266**, 2639–2646.
16. Sayers, I., Cain, S. A., Swan, J. R., Pickett, M. A., Watt, P. J., Holgate, S. T. *et al.* (1998). Amino acid residues that influence Fc epsilon RI-mediated effector functions of human immunoglobulin E. *Biochemistry*, **37**, 16152–16164.
17. Li, E., Pedraza, A., Bestagno, M., Mancardi, S., Sanchez, R. & Burrone, O. (1997). Mammalian cell expression of dimeric small immune proteins (SIP). *Protein Eng.* **10**, 731–736.
18. Hanke, T., Szawlowski, P. & Randall, R. E. (1992). Construction of solid matrix-antibody-antigen complexes containing simian immunodeficiency virus p27 using tag-specific monoclonal antibody and tag-linked antigen. *J. Gen. Virol.* **73**, 653–660.
19. Benvenuti, F., Burrone, O. R. & Efremov, D. G. (2000). Anti-idiotypic DNA vaccines for lymphoma immunotherapy require the presence of both variable region genes for tumor protection. *Gene Ther.* **7**, 605–611.
20. Martinez-Salas, E. (1999). Internal ribosome entry site biology and its use in expression vectors. *Curr. Opin. Biotechnol.* **10**, 458–464.
21. Benvenuti, F. & Burrone, O. R. (2001). Anti-idiotypic antibodies induced by genetic immunisation are directed exclusively against combined V(L)/V(H) determinants. *Gene Ther.* **8**, 1555–1561.
22. Bennett, M. J., Schlunegger, M. P. & Eisenberg, D. (1995). 3D domain swapping: a mechanism for oligomer assembly. *Protein Sci.* **4**, 2455–2468.
23. Heringa, J. & Taylor, W. R. (1997). Three-dimensional domain duplication, swapping and stealing. *Curr. Opin. Struct. Biol.* **7**, 416–421.
24. Bork, P., Holm, L. & Sander, C. (1994). The immunoglobulin fold. Structural classification, sequence patterns and common core. *J. Mol. Biol.* **242**, 309–320.
25. Murray, A. J., Lewis, S. J., Barclay, A. N. & Brady, R. L. (1995). One sequence, two folds: a metastable structure of CD2. *Proc. Natl Acad. Sci. USA*, **92**, 7337–7341.
26. Murray, A. J., Head, J. G., Barker, J. J. & Brady, R. L. (1998). Engineering an intertwined form of CD2 for stability and assembly. *Nature Struct. Biol.* **5**, 778–782.
27. Hayes, M. V., Sessions, R. B., Brady, R. L. & Clarke, A. R. (1999). Engineered assembly of intertwined oligomers of an immunoglobulin chain. *J. Mol. Biol.* **285**, 1857–1867.
28. Harpaz, Y. & Chothia, C. (1994). Many of the immunoglobulin superfamily domains in cell adhesion molecules and surface receptors belong to a new structural set which is close to that containing variable domains. *J. Mol. Biol.* **238**, 528–539.
29. Hebert, D. N., Foellmer, B. & Helenius, A. (1995). Glucose trimming and reglucosylation determine glycoprotein association with calnexin in the endoplasmic reticulum. *Cell*, **81**, 425–433.
30. Trombetta, E. S. & Helenius, A. (1998). Lectins as chaperones in glycoprotein folding. *Curr. Opin. Struct. Biol.* **8**, 587–592.
31. Mallamaci, M. A., Chizzonite, R., Griffin, M., Nettleton, M., Hakimi, J., Tsien, W. H. & Kochan, J. P. (1993). Identification of sites on the human Fc epsilon RI alpha subunit which are involved in binding human and rat IgE. *J. Biol. Chem.* **268**, 22076–22083.
32. Robertson, M. W. (1993). Phage and *Escherichia coli* expression of the human high affinity immunoglobulin E receptor alpha-subunit ectodomain. Domain localization of the IgE-binding site. *J. Biol. Chem.* **268**, 12736–12743.
33. Scarselli, E., Esposito, G. & Traboni, C. (1993). Receptor phage. Display of functional domains of the human high affinity IgE receptor on the M13 phage surface. *FEBS Letters*, **329**, 223–226.
34. Garman, S. C., Sechi, S., Kinet, J. P. & Jardetzky, T. S. (2001). The analysis of the human high affinity IgE receptor Fc epsilon RI alpha from multiple crystal forms. *J. Mol. Biol.* **311**, 1049–1062.
35. Davis, R. S., Wang, Y. H., Kubagawa, H. & Cooper, M. D. (2001). Identification of a family of Fc receptor homologs with preferential B cell expression. *Proc. Natl Acad. Sci. USA*, **98**, 9772–9777.
36. Hulet, M. D., Brinkworth, R. I., McKenzie, I. F. & Hogarth, P. M. (1999). Fine structure analysis of interaction of FcεRI with IgE. *J. Biol. Chem.* **274**, 13345–13352.
37. Batista, F. D., Efremov, D. G. & Burrone, O. R. (1995). Characterization and expression of alternatively spliced IgE heavy chain transcripts produced by peripheral blood lymphocytes. *J. Immunol.* **154**, 209–218.
38. Batista, F. D., Efremov, D. G. & Burrone, O. R. (1996). Characterization of a second secreted IgE isoform and identification of an asymmetric pathway of IgE assembly. *Proc. Natl Acad. Sci. USA*, **93**, 3399–3404.
39. Ghattas, I. R., Sanes, J. R. & Majors, J. E. (1991). The encephalomyocarditis virus internal ribosome entry site allows efficient co-expression of two genes from a recombinant provirus in cultured cells and in embryos. *Mol. Cell Biol.* **11**, 5848–5859.
40. Jones, T. A., Zou, J. Y., Cowan, S. W. & Kjeldgaard, M. (1991). Improved methods for binding protein models in electron density maps and the location of errors in these models. *Acta Crystallog. sect. A*, **47**, 110–119.
41. Guex, N. & Peitsch, M. C. (1997). SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis*, **18**, 2714–2723.
42. Koradi, R., Billeter, M. & Wuthrich, K. (1996). MOL-MOL: a program for display and analysis of macromolecular structures. *J. Mol. Graph.* **14**, 29–32.
43. Nicholls, A., Bharadwaj, R. & Honig, B. (1993). GRASP a graphical representation and analysis of surface properties. *Biophys. J.* **64**, A166.

Edited by A. R. Fersht

(Received 14 May 2002; received in revised form 29 July 2002; accepted 12 August 2002)