



Characterisation of recombinant human IgE-Fc fragments expressed in baculovirus-infected insect cells

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Abstract

IgE mediates its effector functions through the Fc region and it has been demonstrated that structures in the Cε3-domain are crucial for FcεR-binding. In order to further study structures of importance for the function of IgE, such as the carbohydrates, fragments with unmodified amino acid sequence were blunt-end cloned and expressed in baculovirus-infected Sf9 cells. Two fragments of human IgE, one encompassing the entire Fc-region (rCε2-4) and a smaller one comprising the second and third domain (rCε2-3), were produced and characterised with respect to epitope expression, glycosylation and FcεR-binding. N-terminal analysis showed the expected VCSRDF-sequence of the Cε2-domain, confirming correct cleavage of the secretion signal. Immunoblotting and gel permeation chromatography demonstrated that rCε2-4 mainly formed a dimer, whereas rCε2-3 also existed as monomers and oligomers. Endoglycosidase-treatment revealed that both fragments were N-glycosylated. In inhibition ELISA, rCε2-4 and myeloma protein IgE(DES) reacted in a near equimolar way with monoclonal antibodies against the Cε2-, Cε3- and Cε4-domains, whereas rCε2-3 only reacted with anti-Cε2 mAbs. Moreover, in FACS analysis rCε2-4 interacted with two cell-lines constitutively expressing FcεRI or FcεRII, whereas rCε2-3 lacked reactivity. A substantial reduction in the ability of rCε2-4, following endoglycosidase treatment, to react with recombinant α-chain of the high affinity receptor for IgE in sandwich ELISA, indicated a role of N-linked oligosaccharides in stabilising receptor binding structures. Taken together, our results show that rCε2-4, but not rCε2-3, will be useful in studies of structure–function relationships of IgE, including the role of N-glycosylation, since it demonstrated appropriate epitope expression, conformation and ability to bind Fcε-receptors. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

IgE antibodies (Ab) are key molecules in mediating allergic symptoms and their biological activities are mainly governed by the classical high-affinity receptor

(FcεRI) and the so-called low-affinity receptor (FcεRII/CD23) (Sutton and Gould, 1993). The effector functions are mediated through the Fc region of IgE, which comprises the Cε2–Cε3–Cε4 domains (Fig. 1).

In order to study structures of importance for the function of IgE, numerous recombinant Fcε-fragments and human IgE antibodies have been produced (Helm et al., 1997). Although, high expression levels are obtained in bacteria (Ishizaka et al., 1986; Helm et al., 1996), they lack post-translational machinery and the recombinant proteins form insoluble inclusion bodies that are difficult to renature. To overcome these problems and to provide N-glycosylation various mammalian cells, including mouse L cells (Ikeyama, 1987a),

Abbreviations: Ab, antibody; Endo H, endoglycosidase H; FcεRI, high-affinity receptor for IgE; FcεRII, low-affinity receptor for IgE; mAb, monoclonal antibody; Mr, relative molecular mass; PBS, 50 mM phosphate buffered saline, pH 7.4; PNGF, N-glycosidase F; rFcεRIα, recombinant α-chain of the high affinity receptor for IgE.

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COS cells (Basu et al., 1993; Kamiya et al., 1996), NS-O and CHO cells (Young et al., 1995), have been used. However, many of the IgE-Fc fragments have been expressed as fusion proteins, or have had amino acid extensions, substitutions or truncations. Although, such modifications may have structural implications, the use of both prokaryotic and eukaryotic fragments has demonstrated that mainly structures in the C ϵ 3-domain are crucial for both Fc ϵ RI- and Fc ϵ RII-binding (Helm et al., 1997).

Baculovirus expression systems are frequently used because they offer relatively high levels of protein expression as well as eukaryotic protein processing capabilities including N-glycosylation (Kidd and Emery, 1993; Jarvis and Finn, 1995). Several functional Ig molecules (Potter et al., 1993) have been expressed but to our knowledge there are no baculovirus-produced human Fc ϵ -fragments reported, although recently a few C ϵ 3-domain restricted fragments were produced (Vangelista et al., 1999).

The aim of the present study was to use a baculovirus system to express two fragments of human IgE and to characterise their epitope expression, N-glycosylation and receptor binding properties as a basis for further structural and functional studies. Blunt-end cloning of the entire domains was used to avoid substitutions or additions in the amino acid sequence.

2. Material and methods

2.1. Subcloning

Two PCR-products corresponding to the entire Fc region (rC ϵ 2-4) and a smaller one comprising the second and third domain (rC ϵ 2-3), were amplified from a vector (kindly provided by G. Buell, Phage, CA, USA) containing an insert of the constant part of the human ϵ -chain. The amplification was done in a standard

reaction mix with Pfu DNA polymerase (Stratagene, LaJolla, USA) using 5'-GTATGTTCAAGAGACTT-TACA-3' as forward primer and 5'-GAGGTCGACT-CATTTACCGGGATTACAGA-3' or 5'-GAGGTCGACTCAGCTGGTCTTGTCGTGGACC-3' as reverse primers. A *Sal*I restriction site was incorporated in the 5'-end of the reverse primers. The PCR was initiated with a denaturation step at 94°C for 8 min, whereafter Pfu was added at 80°C. The reaction was continued for 25 cycles at 94°C (1 min), 60°C (2 min), and 72°C (2.5 min), and ended with an elongation at 72°C (10 min). The PCR-products were digested with *Sal*I and ligated into the baculovirus transfer vector pMelBac B (Invitrogen, NV Leek, the Netherlands), that had sequentially been pretreated with *Bam*HI, mungbean nuclease and *Sal*I (Pharmacia Biotech AB, Uppsala, Sweden). After transformation into competent XL2 Blue bacteria (Stratagene), recombinant transfer plasmids were confirmed by automated DNA sequencing using dRhodamine terminator cycle sequencing kit (ABI PRISM 373 or 377; Perkin-Elmer, Foster City, USA).

2.2. Expression in insect cells

Sf9 cells (Invitrogen) derived from *Spodoptera frugiperda* were cotransfected with transfer plasmids containing insert and linearised viral DNA (Bac-N-Blue; Invitrogen) as outlined by the manufacturer. Supernatant from transfected cells was harvested after 72 h and recombinant virus isolated in a plaque purification assay. Briefly, 10-fold dilutions of medium were added to Sf9 cells in a 100 mm culture plate and incubated for 1 h at room temperature. Media were removed and the cells overlaid with 0.6% SeaPlaque agarose (InVivo AB, Stockholm, Sweden). After 3–4 days of culture at 27°C in a humid environment, 2 mg/ml Bluo-gal (Duchefa, Saveentech, Malmö, Sweden) in 1% agarose was added. Blue spots representing positive clones were picked, expanded and verified to be recombinant by PCR. To ensure clonality at least 3 rounds of plaque purifications were performed and the production of IgE-fragments from different clones was monitored by sandwich ELISA (see below). High-titer stocks were produced from selected recombinant virus clones by transfecting Sf9 cells growing in suspension (500 ml; 2×10^6 cells/ml) with Grace media containing 10% heat inactivated fetal calf serum and 30 μ g/ml gentamycin (Life Technologies AB, Täby, Sweden). After 10 days of culture the high-titer stocks were harvested and used to infect Sf9 cells (500 ml; 2×10^6 cells/ml), growing in serum-free medium Ex-Cell 400 (AMS Biotechnology, Täby, Sweden), at a multiplicity of infection of 4. The protease inhibitor phenylmethylsulfonyl fluoride (Sigma, Stockholm, Sweden) prepared in pro-

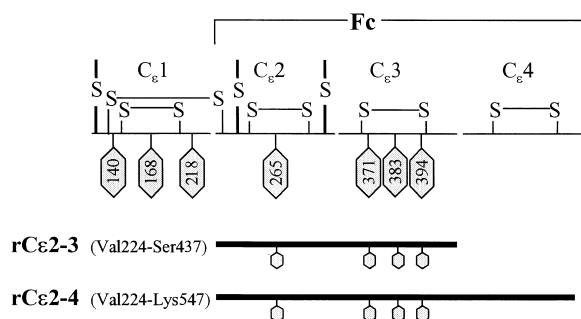


Fig. 1. Schematic model of the four constant domains of the ϵ -chain and the recombinant IgE-fragments, rC ϵ 2-3 and rC ϵ 2-4. The intra- and inter-chain disulfides are shown as well as the location of potential N-glycosylation sites (SWISS-PROT, AC:PO1854). Numbering is modified to comply with an ϵ -chain of 547 amino acids.

panol was added (80 µg/ml) and the supernatants kept at –20°C until use.

2.3. Protein purification and sequencing

Supernatants containing rCε2-3 and rCε2-4 fragments were concentrated 8–10 times by ultrafiltration on a PM10 membrane (Amicon, Beverly, MA, USA) and dialysed into 50 mM phosphate buffered saline, pH 7.4 (PBS) and used as such, or further purified by immunoaffinity chromatography using domain-specific anti-Cε2 and anti-Cε3 monoclonal antibody (mAb), respectively (Magnusson et al., in prep.). The fragments were eluted at pH 2.7 (0.1 M Glycine–HCl) and fractions (250 µl) immediately neutralised with 2 M Tris. Fractions containing fragments were pooled following identification by sandwich ELISA and SDS-PAGE with silver-staining on the Phastsystem (Pharmacia Biotech AB).

The purified IgE-fragments were run on 10% Bis–Tris NuPAGE precast gel (Novex, San Diego, USA) in 3-(*N*-morpholino) propane sulfonic acid running buffer under reducing conditions and thereafter electroblotted onto polyvinylidene fluoride membrane (0.2 µm; Biorad, Stockholm, Sweden) in an Xcell II minicell (Novex). The bands were visualised by Coomassie Brilliant blue staining and thereafter excised for N-terminal amino acid sequencing (Hewlett-Packard G1005A Protein sequencing system; Protein Analysis Center, Karolinska Institute, Stockholm, Sweden).

2.4. Immunoblotting

Proteins and molecular mass standards (SeeBlue; Novex) were separated by SDS-PAGE on 10% or 4–12% Bis–Tris NuPAGE precast gels in 3-(*N*-morpholino) propane sulfonic acid running buffer and thereafter electroblotted onto 0.45 µm nitrocellulose membrane (Novex). Samples were treated in Novex sample buffer, with or without NuPAGE reducing agent, for 5 min at 80°C. Following blocking with PBS containing 4% milkpowder, 2% BSA and 0.05% Tween20, the membranes were incubated overnight at 4°C with biotinylated anti-Cε2 mAbs. Alkaline phosphatase-conjugated streptavidin (Dakopatts AB, Stockholm, Sweden) was added for 2 h and the membranes washed with 9‰ NaCl prior development with AP color buffer (Biorad). All washing steps between incubations were done with blocking solution diluted 10 times with PBS containing 0.05% Tween20.

2.5. ELISA

The reactivity of domain-specific anti-IgE mAbs and baculovirus produced recombinant α-chain of the high affinity receptor for IgE (rFcεRIα) (a kind gift from

Dr. F. Kricek) with the IgE-Fc fragments and IgE(DES) myeloma protein (Magnusson and Vaerman, 1986) was tested in sandwich and inhibition ELISA as outlined below.

1. In sandwich ELISA, microtiter plate wells (Maxisorb, NUNC, Roskilde, Denmark) were coated overnight with glycosylation-independent mAbs (2 µg/ml) (Björklund et al., 1999), incubated overnight with three-fold dilutions of the IgE-samples followed by a 2 h incubation with biotinylated anti-IgE mAbs or rFcεRIα. In the assay of rCε2-3, one anti-Cε2 mAb was used as capture Ab and another anti-Cε2 mAb, with a different specificity, as secondary Ab. For rCε2-4, either anti-Cε2 or anti-Cε3 mAbs were used as capture Ab, and biotinylated mAbs against the Cε2-, Cε3- or Cε4-domain as secondary Ab (Magnusson et al., 1986; Magnusson et al., in preparation).
2. Inhibition ELISA was performed by preincubating, overnight, three-fold dilutions of the IgE-samples with dilutions of biotinylated anti-IgE mAbs chosen from the linear part of the corresponding titration curves. The residual activity of the mixture was then determined on microtiter plate wells coated with 2 µg/ml IgE(DES) myeloma protein (Magnusson and Vaerman, 1986). The last step in both ELISA involved a 2 h incubation with streptavidin conjugated to alkaline phosphatase before colour development with *p*-nitrophenyl phosphate and recording of optical density at 405 nm in an Anthos 2001 reader (Ekoscann, Upplands-Väsby, Sweden).

The samples and reagents were diluted in PBS containing 0.2% BSA. All coating and incubation steps (100 µl) were followed by four washings with PBS containing 0.05% Tween20 in a Delfia Plate-wash (Wallac, Upplands-Väsby, Sweden).

2.6. Gel permeation chromatography

Standard proteins and the IgE-Fc fragments were fractionated by gel permeation chromatography (FPLC-system; Pharmacia AB) using either two Superose-6 columns arranged in tandem or one Superdex 200 column, equilibrated in PBS. Absorbance at 280 nm of standard proteins was monitored and fractions (150 or 250 µl) were collected for IgE-fragment analysis by sandwich ELISA.

2.7. Endoglycosidase treatment

N-linked oligosaccharides were removed by N-glycosidase F (PNGF) and Endoglycosidase H (Endo H) purchased from Boehringer Mannheim Scandinavia AB, Stockholm, Sweden. PNGF has a general specificity for all N-linked oligosaccharides, whereas Endo

H selectively removes high-mannose and hybrid oligosaccharides. The IgE-Fc fragments (2 nM) were treated with 5 U/ml PNGF in 0.125 M phosphate-buffer (pH 7.5) containing 5 mM EDTA, or with 0.15 U/ml Endo H in 0.1 M phosphate-buffer (pH 6.0) at 37°C for 18 h, with buffer alone as controls. The preparations were stored at –20°C until use.

2.8. Cells and flowcytometry

KU 812 and RPMI 8866 cells constitutively expressing FcεRI (Magnusson et al., 1995) and FcεRII (Peterson and Conrad, 1985), respectively, were cultured in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum, 2 mM L-glutamine and 100 µg/ml gentamycin (Life Technologies AB).

To study FcεR-interactions five-fold dilutions of IgE(DES), or the concentrated cell supernatants of IgE-Fc fragments (100 µl), were added to 500,000 cells (50 µl) followed by biotinylated anti-Cε2 mAb and phycoerythrin-conjugated streptavidin (Dakopatts AB). All incubations were performed at room temperature for 1 h and in between the cells were washed three times by centrifugation (5 min; 1000 × *g*) with 1 ml PBS containing 0.1% BSA. Finally, the cells were resuspended in PBS containing 0.1% BSA and kept at 4°C in the dark pending FACS-analysis. Flowcytometric analysis was performed on a FACScan (Becton-Dickinson, Mountain View, CA, USA) analysing 5000–10,000 cells in each experiment. The fluorescence intensity was recorded in the FL2 channel, after gating on homogeneous cell populations. Background consisted of cells incubated with all reagents except IgE samples.

3. Results

3.1. Characterisation of recombinant IgE-Fc fragments

Supernatants from the virus-infected Sf9 cells, containing rCε2-3 (Val224-Ser437) and rCε2-4 (Val224-Lys547), were harvested after 72 h of culture, as this in preliminary studies had been shown to be a suitable time-point for highest protein production. The expression levels were estimated to be ≈15 nM for rCε2-4 and ≈3 nM for rCε2-3, as measured in the cell supernatants by sandwich ELISA using purified IgE(DES) as standard and assuming fragments in the form of dimers.

Analysis of the N-terminal amino acid sequence of the fragments revealed the sequence VCSRDF. This corresponds to the expected N-terminal amino acids of the Cε2-domain and confirmed correct cleavage of the secretion signal.

Immunoblotting, following SDS-PAGE under redu-

cing conditions, demonstrated higher relative molecular mass (Mr) for both rCε2-3 (30–34 kDa) and rCε2-4 (40–44 kDa) (Fig. 2) than the predicted ones (23.7 and 36.1 kDa, respectively). These apparent heterogeneities in size are probably caused by differences in glycosylation (see below). Analysis, under nonreducing conditions, indicated that rCε2-4 mainly formed a dimer whereas rCε2-3 existed as monomers and oligomers as well (Fig. 2). Gel permeation chromatography of rCε2-3 and rCε2-4, followed by sandwich ELISA on the fractions, confirmed the results for rCε2-4, but indicated that also rCε2-3 was mainly in the form of a dimer (data not shown). These divergent results for rCε2-3 may be caused by an exchange of disulfide bonds during heat treatment in SDS-buffer (Ikeyama, 1987b).

3.2. Epitope expression

Inhibition ELISA, with biotinylated mAbs to all three domains of IgE-Fc, was used to compare epitope expression between the recombinant fragments and IgE(DES). The concentrations of the fragments were calibrated using the mean values obtained with two anti-Cε2 mAbs. The results showed that IgE(DES) and

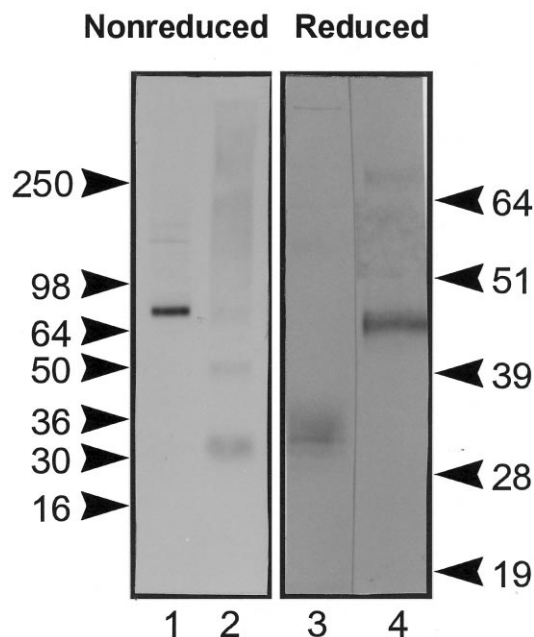


Fig. 2. Immunoblotting of recombinant IgE-fragments. rCε2-3 (1 nM) and rCε2-4 (1 nM) were separated by SDS-PAGE on 4–20% and 10% gels, under non-reducing and reducing conditions, respectively. rCε2-3 (Lanes 2 and 3) and rCε2-4 (Lanes 1 and 4) were detected with an anti-Cε2 mAb. The positions of molecular mass standards (kDa) are indicated. No bands were observed with an isotype-matched control mAb directed against an idiotype of IgE(DES) (Magnusson et al., 1986), neither was any bands detected in supernatants from uninfected Sf9 cells with the anti-Cε2 mAb (data not shown).

rCε2-4 reacted in a parallel and near equimolar way with mAbs against the Cε2-, Cε3- and Cε4-domains, whereas rCε2-3 only reacted with anti-Cε2 mAbs (Fig. 3).

3.3. N-glycosylation

The IgE-Fc fragments were analysed by immuno-

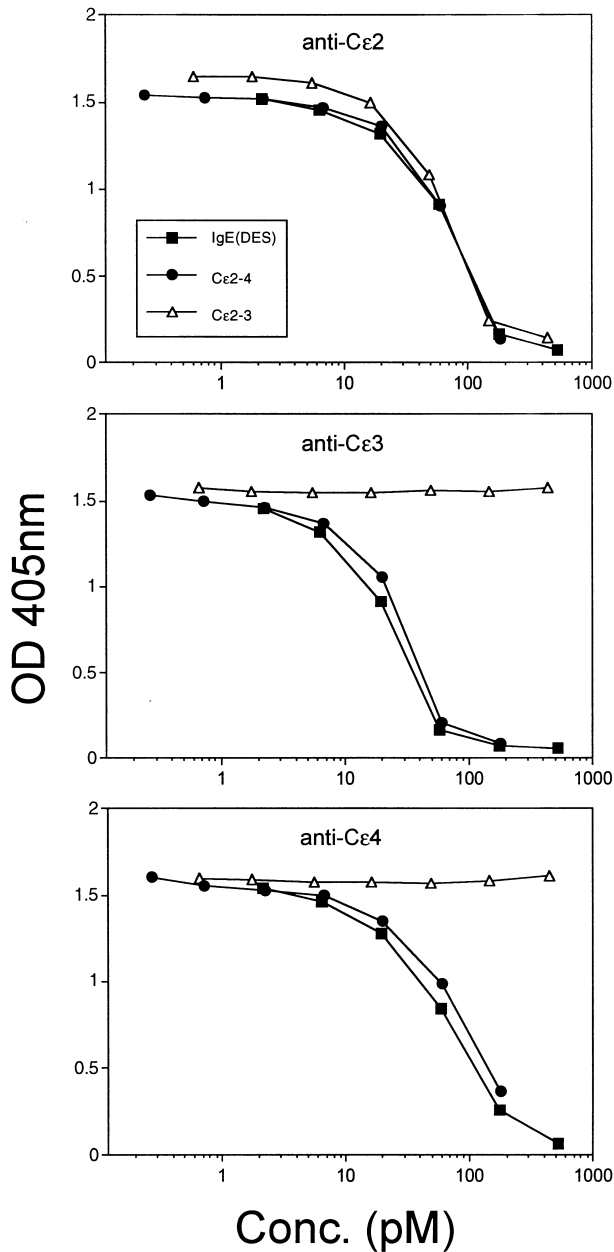


Fig. 3. Epitope expression of rCε2-3 and rCε2-4. The inhibitory activities of rCε2-3, rCε2-4 and IgE(DES) myeloma protein with mAbs against the Cε2-, Cε3- and Cε4-domains were compared in competitive ELISA. The concentrations of rCε2-3 and rCε2-4 were calibrated by taking the mean values obtained with two different anti-Cε2 mAbs using IgE(DES) as standard and assuming dimeric fragments.

blotting under reducing conditions following treatment with Endo H or PNGF. Several bands were detected in all test samples and the strongest one was used to calculate differences in Mr (Fig. 4). Endo H-treatment of rCε2-4 caused a decrease in Mr of the major band of approximately 2 kDa. PNGF-treatment seemed to be more efficient with a decrease of approximately 5 kDa (Fig. 4). A 4 kDa decrease was obtained following PNGF-treatment of rCε2-3 (data not shown). These effects of endoglycosidase treatment show that the fragments are N-glycosylated and suggest that the banding patterns reflect fragments with different amount of carbohydrates.

3.4. FcεRI- and FcεRII-interaction

The receptorbinding ability of the recombinant fragments was investigated at several five-fold dilutions of the concentrated cell supernatants by FACS analysis using two cell-lines, KU 812 and RPMI 8866, constitutively expressing FcεRI and FcεRII, respectively. The results demonstrated that rCε2-4 (3 nM) clearly reacted with both receptors, whereas in sharp contrast, rCε2-3 (15 nM) did not significantly react with either receptor (Fig. 5). Interestingly, on a molar basis, the reactivity of rCε2-4 (3 nM) was better with both FcεRI and FcεRII than that of IgE(DES) (10 nM) (Fig. 5). The reason for this is unclear, but may be re-

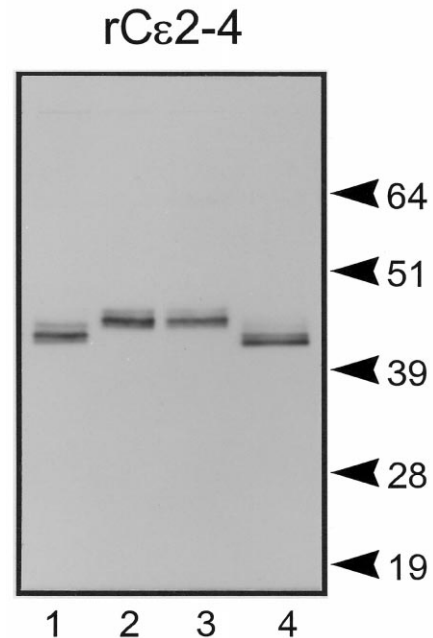


Fig. 4. Deglycosylation of rCε2-4. Endoglycosidase-treated rCε2-4 (1 nM) was separated by SDS-PAGE on 10% gels under reducing conditions, electroblotted and detected with an anti-Cε2 mAb. Lane 1. Endo H, 2. Buffer-control (Endo H), 3. Buffer-control (PNGF), 4. PNGF. The positions of molecular mass standards (kDa) are indicated.

lated to differences in glycosylation, molecular size and/or accessibility of the mAb used for detection.

3.5. Interaction of deglycosylated rCε2-4 with rFcεRIα

To evaluate the importance of N-glycosylation for FcεRI-binding structures, the reactivity of deglycosylated rCε2-4 with biotinylated rFcεRIα was examined by sandwich ELISA. No such studies were performed with rCε2-3 since it did not react in neither inhibition nor sandwich ELISA with rFcεRIα (data not shown). Compared to buffer-control PNGF-treated rCε2-4 demonstrated a considerable loss in reactivity with rFcεRIα. Endo H-treatment had a similar, but less pronounced effect (Fig. 6). To investigate if these findings could be a consequence of aggregation, buffer-control and endoglycosidase-treated rCε2-4 were separated by gel permeation chromatography. The fractions were evaluated in two different sandwich ELISA, both of which used an anti-Cε3 mAb as capture Ab. There was no sign of aggregated rCε2-4 following treatment with either PNGF or Endo H, and the main peak of activity was found in elution volumes corresponding to dimeric rCε2-4. These results were obtained with a biotinylated glycosylation-independent anti-Cε2 mAb (Björklund et al., 1999) as secondary reagent, and they were also used to calibrate the concentrations in the fractions for comparative purposes. When biotinylated rFcεRIα was used as secondary reagent, a significant

loss in reactivity was observed for endoglycosidase-treated rCε2-4 as compared to buffer-control (data not shown). These results indicate that the reduced reactivity of PNGF- and Endo H-treated rCε2-4 with rFcεRIα is caused by deglycosylation and not aggregation.

4. Discussion

In this study, we have subcloned, expressed and characterised two human IgE-Fc fragments, rCε2-3 and rCε2-4. These fragments encompassed entire domains and were blunt-end cloned directly after the mellitin secretion signal to avoid amino acid extensions, substitutions or truncations. Both fragments were secreted into the culture medium in the nM range. Despite these relatively low expression levels both fragments could be characterised after 8–10-fold concentration of cell supernatants, or purification by anti-IgE affinity chromatography. Characterisation of size and form demonstrated that rCε2-4 mainly formed a disulfide-linked dimer, whereas rCε2-3 also existed as monomers and several oligomers, depending on method of analysis. Study of epitope expression with rFcεRIα and domain-specific mAbs by inhibition ELISA, and FcεR-binding properties by FACS, suggested that rCε2-4, but not rCε2-3, had a correct overall conformation. Moreover, endoglycosidase treatment with PNGF and/or Endo H revealed that both fragments were N-glycosylated.

For structural and functional reasons numerous recombinant fragments of the human ε-chain have

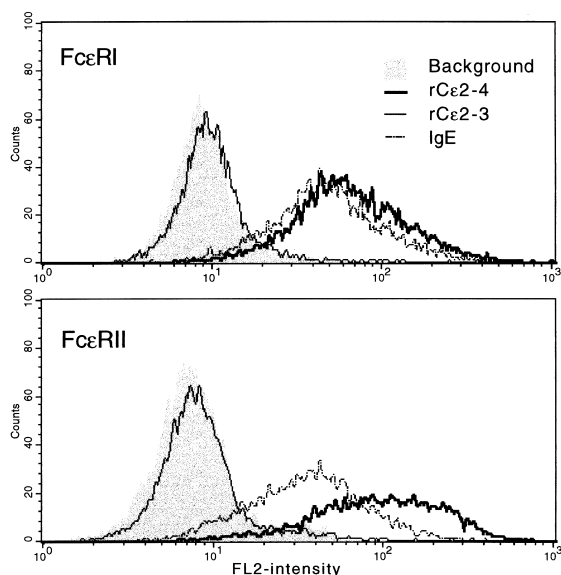


Fig. 5. Interaction of rCε2-3 and rCε2-4 with FcεR as analysed by FACS. rCε2-3 (15 nM) and rCε2-4 (3 nM) from concentrated cell supernatants, and IgE(DES) myeloma protein (10 nM) were incubated with KU 812 and RPMI 8866 cells expressing FcεRI and FcεRII, respectively. The histograms represent the intensity of fluorescence in the FL2 channel after staining with biotinylated anti-Cε2 mAb and phycoerythrin-conjugated streptavidin.

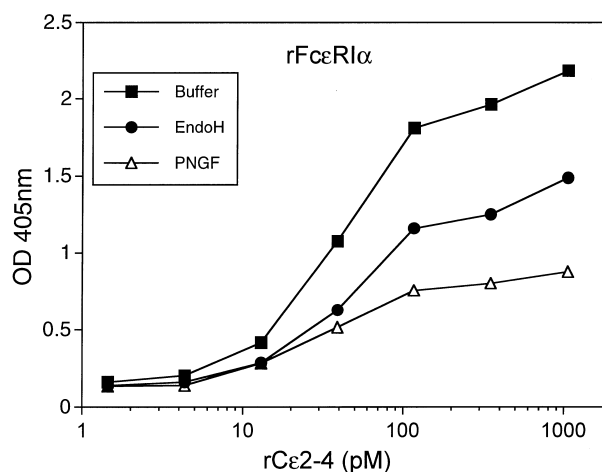


Fig. 6. Interaction of deglycosylated rCε2-4 with rFcεRIα in sandwich ELISA. rCε2-4 was treated with PNGF, Endo H or buffer alone. The reactivity patterns were analysed by ELISA, using a glycosylation-independent anti-Cε3 mAb as capture Ab and biotinylated rFcεRIα as detecting reagent. The concentrations of rCε2-4 in the different preparations were calibrated with a glycosylation-independent anti-Cε2 mAb as secondary Ab (Björklund et al., 1999).

previously been expressed in *E. coli* and various mammalian cells (Sutton and Gould, 1993; Helm et al., 1997). These studies have pinpointed major FcεR-binding structures to the Cε3-domain, despite that many fragments have had some kind of alterations in the Fcε-sequences. Although, many of these modifications seem of little importance, even single amino acid substitutions in Fcε-sequences may have dramatic effects on FcεRI-interaction (Nettleton and Kochan, 1995; Helm et al., 1997; Sayers et al., 1998). We showed here baculovirus expression and characterisation of the first entire unmodified human Fcε-fragment, rCε2-4. Previously, a substitution of Cys225 was of particular concern, since this cysteine in native IgE forms an intra-chain disulfide bond with a cysteine in the Cε1-domain which could cause aggregation (Young et al., 1995). However, our results suggested that rCε2-4 was correctly assembled as mainly a dimer. It is unclear though whether the two Cys225 remain free or form a stabilising inter-chain disulfide bond, or whether they cause an oligomerization restricted to rCε2-3. Nevertheless, our studies with domain-specific anti-IgE mAbs, rFcεRIα and with cells expressing FcεRI or FcεRII, gave parallel results, and clearly indicated that only rCε2-4 was correctly folded, whereas rCε2-3 seemed to have major structural changes in the Cε3-domain. Also in previous studies with several truncated eukaryotic IgE-Fc fragments, Cε2-3 fragments failed to react with FcεRI (Basu et al., 1993), whereas fragment encompassing the Cε3- and Cε4-domains interacted with both FcεRI and FcεRII (Basu et al., 1993; Kamiya et al., 1996; Shi et al., 1997). These findings and ours suggest that the Cε4-domain, although not directly involved in FcεR-binding (Helm et al., 1996; Helm et al., 1997) is of importance for maintaining the conformation of the Cε3-domain (Basu et al., 1993). Intriguingly though, Vangelista et al. (1999) recently demonstrated that baculovirus-produced Cε3-fragments having a few N-terminal amino acid extensions reacted with rFcεRIα. However, their results were based on qualitative data without any knowledge about form (monomer vs. dimer), actual concentration or epitope expression of the fragments as compared to IgE. However, if confirmed their results suggest that the Cε4-domain is of minor importance, implicating that the Cε2-domain has a negative influence on the conformation of the Cε3-domain in the absence of any Cε4-domain. Further studies are necessary to clarify on a kinetic and quantitative basis, whether the Cε4-domain has any stabilising effect or not, e.g. by promoting dimerisation.

Surprisingly we found that rCε2-4 reacted better with both FcεR as compared to IgE on a molar basis.

Similar findings were previously reported for FcεRI (Young et al., 1995) and possible explanations might be related to kinetics, molecular size, glycosylation and/or accessibility of anti-IgE mAbs used in detection.

The limited knowledge on N-glycosylation of human IgE is derived from studies of a few myeloma proteins (Baenzinger et al., 1974a, b; Dorrington and Bennich, 1978). The human Fcε-region has 4 potential sites for N-glycosylation (Fig. 1) of which Asn383 is considered unglycosylated in both IgE myeloma proteins and recombinant fragments (Dorrington and Bennich, 1978; Young et al., 1995; Nettleton and Kochan, 1993). In addition, results with Asn/Gln-mutants of IgE-Fc, produced in CHO cells, have suggested underglycosylation of Asn371 (Young et al., 1995).

We and others have previously in different ways tried to investigate how N-linked oligosaccharides in human IgE affects binding to FcεRI (Basu et al., 1993; Nettleton and Kochan, 1995; Sayers et al., 1998; Björklund et al., 1999) and FcεRII (Vercelli et al., 1989; Young et al., 1995; Sayers et al., 1998). It has been shown with recombinant IgE-Fc fragments and chimeric IgE that deglycosylation with Endo F (Basu et al., 1993), or substitution of Asn394 (Nettleton and Kochan, 1995; Sayers et al., 1998) reduces FcεRI-binding. However, this effect has been attributed to aggregation or structural changes in the vicinity of Asn394 rather than to the lack of carbohydrates. On the contrary, the oligosaccharides on Asn265 have been suggested to shield the binding of IgE to FcεRII (Vercelli et al., 1989; Young et al., 1995). We recently described that PNGF-treated IgE proteins had a markedly reduced ability to bind rFcεRIα in sandwich ELISA, and that the deglycosylation modulated epitope expression in the Cε2-domain (Björklund et al., 1999). These results were obtained on monomeric IgE excluding any aggregation effect, but the glycosylation sites involved were not determined.

We here demonstrate N-glycosylation in both our fragments and the Mr-changes obtained by endoglycosidases suggest occupation of at least two of the four potential sites. The more pronounced effect by PNGF suggests the presence of complex oligosaccharides besides the high-mannose and/or hybrid ones expected from the Endo H-treatment. However, differences in substrate accessibility or enzyme efficacy cannot be ruled out. Moreover, we show that, in line with our previous findings on IgE proteins, deglycosylated rCε2-4 also had a reduced reactivity with rFcεRIα in ELISA. Basu et al. (1993) presented similar data after Endo F-treatment, but attributed the effect to aggregation. Our chromatographic results indicated that deglycosylated rCε2-4 was not aggregated and yet had a significant loss in reactivity with rFcεRIα following PNGF- or Endo H-treatment.

Thus, IgE-Fc fragments produced by the baculovirus

system seems suitable to further elucidate the functional importance of N-glycosylation. Although, insect cells have a post-translational machinery resembling that of mammalian cells, their N-glycosylation system is less advanced and produces mainly high-mannose oligosaccharides. However, some studies have demonstrated the presence of complex N-linked glycans (Jarvis and Finn, 1995) as also suggested from our data. Previously, a porcine IgE-Fc fragment (Vernersson et al., 1997) and three similar human Cε3-fragments (Vangelista et al., 1999) were produced in a baculovirus expressions system but no N-glycosylation studies were performed. Further studies are warranted both on the glycosylation pattern of Fcε-fragments produced in insect cells and the functional importance of the individual glycosylation sites.

In conclusion, we have produced two human IgE-Fc fragments without any amino acid substitutions or extensions in a baculovirus expression system. The rCε2-4 fragment demonstrated a correct overall conformation both in terms of epitope expression and interaction with FcεRI and FcεRII. On the contrary, the rCε2-3-fragment appeared to have major structural changes in the Cε3-domain, suggesting a role for the Cε4-domain to maintain a correct conformation of the Cε3-domain. In addition, N-glycosylation of IgE-Fc seems important in stabilising structures involved in FcεRI-binding. Our findings indicate that baculovirus-produced rCε2-4 will be useful to further elucidate structure-function relationships in human IgE including effects of N-glycosylation.

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