

High-level expression of the truncated α chain of human high-affinity receptor for IgE as a soluble form by baculovirus-infected insect cells

Biochemical characterization of the recombinant product

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The binding subunit of human high-affinity receptor for IgE (Fc ϵ RI α) was efficiently expressed as a truncated form in insect cells. The soluble (s)Fc ϵ RI α purified from culture medium by affinity chromatography with an anti-(α chain) mAb was nearly homogeneous and had an IgE-binding activity. The amino acid composition and the revealed N-terminal amino acid sequence of sFc ϵ RI α suggested that it was properly processed in insect cells. The apparent molecular mass (35 kDa) of purified sFc ϵ RI α was smaller than that of sFc ϵ RI α produced by CHO transfectants. The reduction of the apparent molecular mass after *N*-glycanase treatment showed the recombinant product was *N*-glycosylated. Peptide mapping of native and deglycosylated sFc ϵ RI α indicated that three Asn residues (Asn21, Asn42 and Asn166) should be almost fully glycosylated, and that two Asn residues (Asn74 and Asn135) were partially glycosylated.

The allergic reaction is initiated by activation of the high-affinity receptor for IgE (Fc ϵ RI) on mast cells. Following the activation of Fc ϵ RI, a series of biochemical events leading to cellular degranulation is induced. Fc ϵ RI on mast cells and basophils consists of non-covalently associated subunits: one α chain, one β chain and two disulfide-linked γ chains (reviewed in Metzger et al., 1986). Although the exact roles of these subunits are not yet fully known, the binding activity for the IgE has been localized on the α subunit by using recombinant DNA techniques (Ra et al., 1989, Blank et al., 1989, Hakimi et al., 1990). The activation of Fc ϵ RI is initiated by the binding of multivalent antigen to IgE on Fc ϵ RI α subunit.

The α subunit is a membrane-anchoring glycosylated protein of 57 kDa, and was revealed to belong to the immunoglobulin super-family by comparative sequence analysis of the cloned gene structures (Kinet et al., 1987, Shimizu et al., 1988). We recently established Chinese hamster ovary cell (CHO) transfectants producing the truncated human Fc ϵ RI α chain as a secreted form. The truncated soluble human Fc ϵ RI α chain (sFc ϵ RI α) was fully functional in binding IgE with high affinity (Blank et al., 1991, Ra et al., 1993), and had an ability to prevent the IgE-mediated allergic reaction (Ra et al., 1993). This truncated α subunit will enable us to analyze

the interaction between IgE and the α chain at molecular level.

To obtain sufficient amounts of material for further molecular-level analysis, such as crystallographic analysis and NMR, we tried a baculovirus gene expression system for expression of sFc ϵ RI α . The baculovirus expression system with the polyhedrin promoter is one of the strongest expression systems in eukaryotic cells and many secreted proteins have been efficiently expressed in this system as modified and correctly processed forms with their native biological activities (reviews in Luckow and Summers, 1988).

In this report, we describe the high-level expression of sFc ϵ RI α by baculovirus expression system and its biochemical characterization. The produced sFc ϵ RI α from *Spodoptera frugiperda* (Sf-9) cells infected with recombinant baculovirus was properly processed and had an equivalent molar activity for binding IgE to that from CHO transfectants. The peptide mapping of the recombinant products revealed where N-linked oligosaccharides attached. These identified features might give us the useful clues for further biophysical characterization of this receptor.

MATERIALS AND METHODS

Plasmid construction

The DNA fragment encoding the exodomain of the human Fc ϵ RI α chain with a stop codon was generated by polymerase chain reaction (PCR). The amplified fragment was sequenced and shown to have the same sequence as the corresponding regions in the previous reports (Kochan et al., 1988, Shimizu et al., 1988). The amplified fragment was then cloned into a *Bam*HI site of pAcYM1 (Matsuura et al., 1987),

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Abbreviations. CHO, Chinese hamster ovary; EndoH, endoglycosidase H; Fc ϵ RI α , α subunit of human high-affinity receptor for IgE; sFc ϵ RI α , a soluble form of Fc ϵ RI α ; PCR, polymerase chain reaction; MOI, multiplicity of infection; BSA, bovine serum albumin; Pth-, phenylthiohydantoin.

and the resultant expression plasmid was named pAcYM1-NTV.

Generating recombinant baculovirus

10 μ g pAcYM1-NTV was co-transfected into Sf-9 cells with 1 μ g AcNPV DNA by means of the calcium phosphate precipitation method. After 3 days incubation, the culture medium was recovered. The recombinant baculovirus, named AcNTV, was purified by three sequences of visual plaque screening (Summers and Smith, 1987). To monitor the ability to produce sFc ϵ RI α of recombinant baculovirus, the culture supernatant was analyzed by Western blotting.

Production and purification of sFc ϵ RI α

3.5×10^8 cells of Sf-9 were infected by AcNTV at the multiplicity of infection (MOI) of 5, and cultured in 350 ml Grace medium containing 10% fetal bovine serum in a spinner flask. After 72 h cultivation at 27°C, the culture medium was recovered and proteins were precipitated with saturated ammonium sulfate (60% at the final concentration). After dialysis against NaCl/Tris (150 mM NaCl, 20 mM Tris/HCl, pH 7.5), precipitates were subjected to gel-permeation chromatography on a Sephacryl S200 column (2.6 cm $\times$ 65 cm) at a flow rate of 30 ml/h with NaCl/Tris. Fractions containing sFc ϵ RI α were collected, dialyzed against binding buffer (0.5 M NaCl, 20 mM Tris/HCl, pH 7.6), then applied to an affinity column constructed with BrCN-activated Sepharose beads coupled with an anti-(human Fc ϵ RI α chain) mAb. 1 ml of the resin was coupled with 5 mg of the purified mouse monoclonal IgG. The specifically bound materials were eluted from the column with the buffer containing 3 M NaSCN. The pooled eluates were immediately dialyzed against 0.1 M NaCl, 20 mM Tris/HCl, pH 7.6.

Western blotting

SDS/PAGE was performed as described previously (Laemmli, 1970) by using the precast-gradient gel (Daiichi chemical or Wakamori) and the resolved proteins were transferred to Immobilon-poly(vinylidene difluoride) membrane (Millipore) in semi-dry conditions. The membrane was treated in NaCl/Tris, 0.05% Tween 20 containing 3% bovine serum albumin (BSA) for blocking. sFc ϵ RI α immobilized on the membrane was probed with the anti-(Fc ϵ RI α chain) mAb as a first antibody and a goat anti-(mouse immunoglobulin) IgG conjugated with alkaline phosphatase (Bio-Rad) as a second antibody. The enzymic activity on the membrane was detected by using 4-nitroblue tetrazolium chloride and X-phosphate (Boehringer Mannheim) as substrates.

Endoglycosidase treatment

100 ng SDS-denatured sFc ϵ RI α was digested by 0.3 U *N*-glycanase (Genzyme) or 0.02 U endoglycosidase H (Endo H, Genzyme) following the supplier's instruction, subjected to SDS/PAGE and analyzed by means of silver staining.

Amino acid composition analysis and sequence analysis

The purified sFc ϵ RI α was hydrolyzed in 6 M HCl at 110°C for 24 h and analyzed by means of an amino-acid analyzer (Nihon Denshi: model JLC-300). N-terminal sequence analysis of the sFc ϵ RI α was carried out by automated

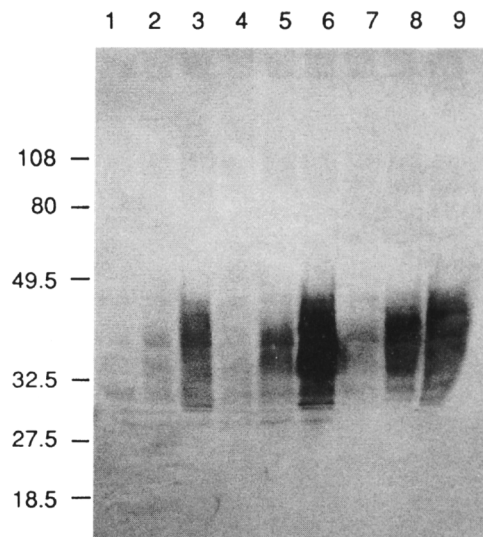


Fig. 1. Expression of the sFc ϵ RI α . Sf-9 cells were infected at MOI of 0.05 (lanes 1, 2, 3), 0.5 (lanes 4, 5, 6) and 5 (lanes 7, 8, 9). 10 μ l culture medium recovered at 24 h (lanes 1, 4, 7), 48 h (2, 5, 8) and 72 h (3, 6, 9) post-infection was subjected to SDS/PAGE (10–20% gradient gel), and electro-transferred to Immobilon-poly(vinylidene difluoride) membrane. The sFc ϵ RI α was detected with an anti-(α chain) mAb (detailed in Materials and Methods). On the left side of the figure, the positions of prestained molecular-mass markers (Bio-Rad) is shown.

Edman degradation method using an Applied Biosystems 477A protein sequencer equipped with a model 120A on-line phenylthiohydantoin (Pth)-Xaa analyzer.

Lysyl endopeptidase digestion and peptide fractionation

3 nmol sFc ϵ RI α was reduced and carboxymethylated as described previously (Hirs, 1967). The carboxymethylated sFc ϵ RI α was then digested with lysyl endopeptidase (Wako pure chemical), and 800 pmol of the digested peptides were fractionated on a C8 reverse-phase column (Waters μ Bondasphere C8-300A, 3.9 mm $\times$ 150 mm) with a linear gradient of acetonitrile (0 to 70%) over 90 min at a flow rate of 0.5 ml/min. For deglycosylated peptide analysis, 800 pmol of the endopeptidase-treated peptides were digested by *N*-glycanase and fractionated under the same conditions as described above.

Competitive inhibitor assay of IgE binding with the sFc ϵ RI α

The plate (Nunc; Maxisorp F8) was coated with 300 ng/ml of the standard sFc ϵ RI α produced by CHO transfectants (Ra et al., 1993). After blocking with NaCl/Tris containing 1% BSA, test samples, which were diluted with NaCl/Tris containing 0.05% Tween 20, 0.2% BSA, were applied to wells after addition of human IgE (10 ng, Yamasa Shoyu). After 1 h incubation at 25°C, the bound IgE was detected by a goat anti-(human IgE) serum conjugated with peroxidase (Cappel). The enzyme activities were measured by using tetramethylbenzidine as a substrate.

RESULTS

Production of sFc ϵ RI α by baculovirus

The truncated human α chain cDNA fragment that encodes the signal peptide of 23 amino acids and the mature

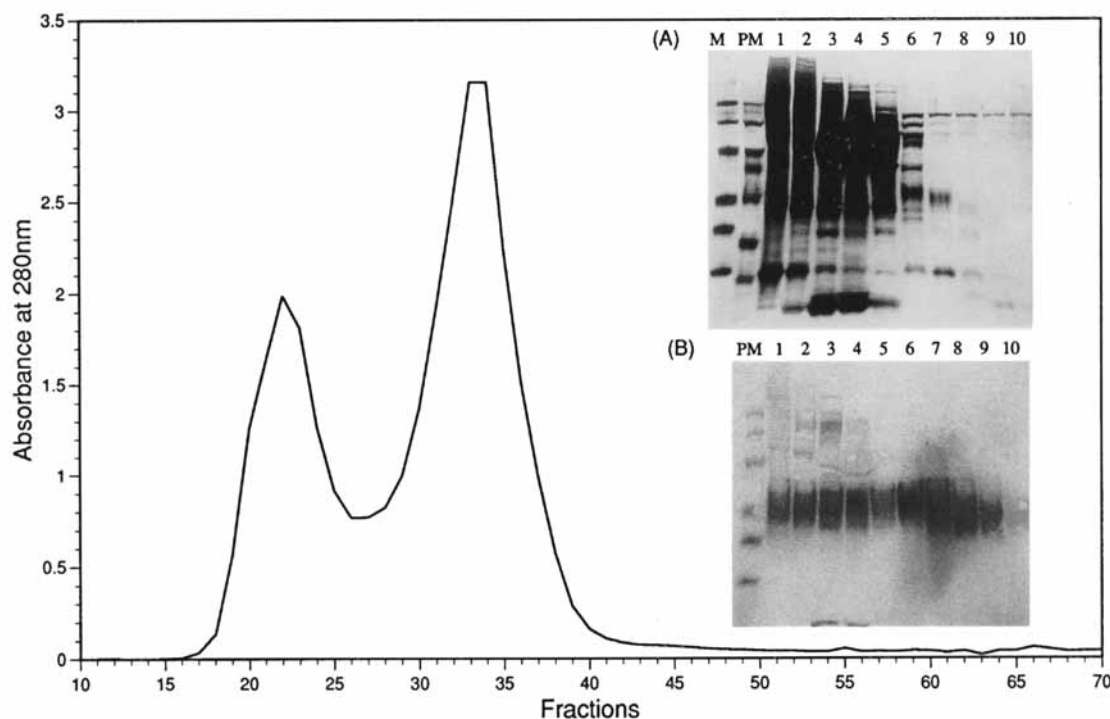


Fig. 2. Gel-permeation chromatography of the sFcεRI α. 33% of the ammonium sulfate precipitate was fractionated by a Sephacryl S200 column. 10 μl (A) or 5 μl (B) of every four fractions from 22–58 (lanes 1–10) was subjected to SDS/PAGE. (A) and (B) show the silver-stained gel and the Western blot, respectively. Lane M, low-molecular-mass standards (phosphorylase b, 97.4 kDa; BSA, 66.2 kDa; ovalbumin, 42.7 kDa; carbonic anhydrase, 31.0 kDa; trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa); lane PM, prestained molecular-mass standards (see Fig. 1).

peptides of 172 amino acid residues was located just downstream of a polyhedrin promoter on pAcYM1 (Matsuura, et al., 1987). The recombinant baculovirus (AcNTV) carrying the truncated α chain cDNA was purified and the levels of the sFcεRI α expression from Sf-9 cells infected with the purified virus were monitored by Western blotting. The blot indicated that the maximum level of the expression was observed 72 h after infection at a MOI of 5 (Fig. 1). The sFcεRI α appeared to be glycosylated like that produced by the stable CHO transfectants (Ra et al., 1993), since the broad immunostained band was detected at the position around 35 kDa instead of 20 kDa, the deduced molecular mass of sFcεRI α based on the amino acid sequence.

Purification of the recombinant sFcεRI α

We purified the sFcεRI α from the culture medium 72 h post infection at a MOI of 5. On Sephacryl S200 gel-permeation chromatography, the materials that were immunoreactive to the monoclonal antibody were detected in broad range of fractions (Fig. 2), indicating that a part of the secreted sFcεRI α may be aggregated with other components in the medium, or be incorrectly folded. Since the majority of the sFcεRI α was eluted in fractions corresponding to 30–50 kDa, we applied these fractions to affinity chromatography with the monoclonal antibody covalently linked to Sepharose 4B as a final purification step. From 350 ml of the culture medium, we obtained 1.89 mg of the sFcεRI α.

Characterization of the purified sFcεRI α

We determined the amino acid composition of the purified product and its amino acid sequence from its N-termini.

Table 1. Amino acid composition of the purified sFcεRI α. Theoretical amino acid composition is based on the published nucleotide sequence (Shimizu et al., 1988).

Amino acid	Composition	
	sFcεRI α	theoretical
	residues/molecule	
Asx	21.21	22
Thr	8.68	9
Ser	12.41	14
Glx	23.97	23
Gly	9.79	9
Ala	6.64	6
Cys-Cys	1.81	2
Val	13.25	15
Met	1.06	1
Ile	6.09	7
Leu	12.57	13
Tyr	12.48	10
Phe	8.09	7
His	4.03	4
Lys	10.78	11
Trp	0	0
Arg	2.91	3
Pro	7.23	7

The amino acid composition of the purified sFcεRI α was nearly identical to that of the predicted one from its nucleotide sequence (Table 1). The N-termini of the purified rat FcεRI α from RBL-2H3 cells had been reported to be blocked (Shimizu et al., 1988), but we could determine the N-terminal amino acid without any deblocking procedure.

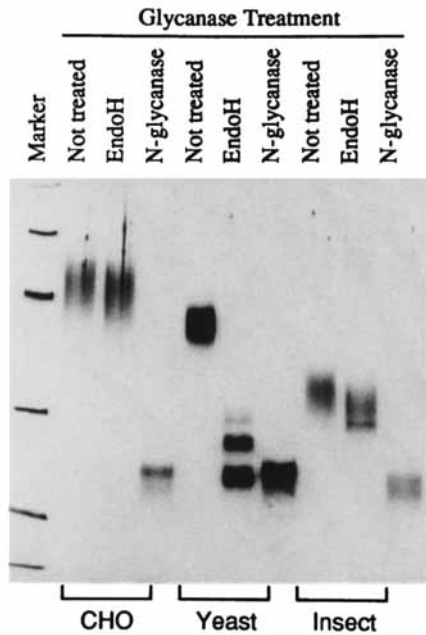


Fig. 3. Comparison of the apparent molecular masses and glycosylation patterns of sFcεRI α produced by various hosts. 100 ng sFcεRI α purified from culture medium of CHO transfectants (CHO), yeast transformants (Yeast), or baculovirus infected Sf-9 cells (Insect) were analyzed by SDS/PAGE after treatment with EndoH or *N*-glycanase. The gel was stained by using a silver staining kit. The faint bands observed at 29 kDa in lanes of EndoH treated samples represent EndoH. Marker, low-molecular-mass standards (see legend to Fig. 3).

The determined N-terminal amino acid was valine, and 29 of the 30 amino acids from its N-termini were identical to those of the predicted sequence (data not shown). These data indicated that the recombinant human sFcεRI α was correctly processed in Sf-9 cells and that nearly homogeneous fractions were purified. The residue predicted in the 21st cycle of Edman degradation should be assigned to Asn, but no Pth-Asn peak was detected. As a Pth-Asn peak was clearly detected at cycle 25, it seems that the Asn at the 21st residue is modified.

We compared, by means of SDS/PAGE, the molecular masses of the sFcεRI α from infected Sf-9 cells with those produced by CHO transfectants and yeast transformants. The sFcεRI α used in the comparison experiment were purified, from the culture medium of CHO transfectants by affinity chromatography with mouse IgE (Ra et al., 1993), and from yeast transformants by affinity chromatography with the monoclonal antibody (unpublished results). The silver-stained

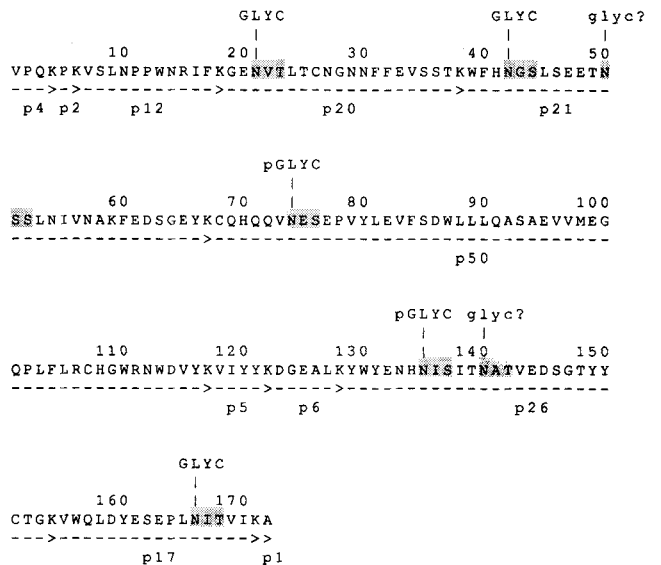


Fig. 4. The map of peptides generated by digestion with lysyl endopeptidase and positions attached with N-linked oligosaccharides. The predicted peptides, which should be generated by digestion of the sFcεRI α with lysyl endopeptidase, are designated as P with a number. The consensus sequences for N-glycosylation sites are shadowed. GLYC and pGLYC represent the positions of completely glycosylated and partially glycosylated Asn residues, respectively, that were identified by peptide sequencing. glyc? represents an Asn residue the glycosylation state of which could not be clearly defined.

gel indicated that the molecular mass of the sFcεRI α from the infected Sf-9 was smaller than those from the other hosts (Fig. 3).

Analysis of the glycosylation state of the sFcεRI α

To elucidate the glycosylation state, human sFcεRI α from different hosts were digested by glycosidases and analyzed by SDS/PAGE (Fig. 3). After EndoH digestion, sFcεRI α from yeasts was converted to two bands with the apparent molecular masses of 24 kDa and 27 kDa in contrast to those from insect cells and CHO cells that were partially deglycosylated. Since EndoH is known to selectively digest high-mannose type N-linked oligosaccharides, it was obvious that only the small portion of the N-linked oligosaccharides in sFcεRI α from insects and CHO cells were high-mannose type.

Polypeptides digested by *N*-glycanase formed two distinct bands (25 kDa and 22 kDa) with smearing on the silver-

Table 2. Summary of peptide mapping of the sFcεRI α. Peptide peak fractions, that were identified by sequence analysis, are shown. Peptides in parentheses are minor components. Nomenclature of peptides and peaks are shown in Fig. 4 and Fig. 5, respectively. n.d., not determined.

Treatment	Peptide composition of peak no.								
	1	2	3	4	5	6	7	8	9
Non-treated	p5	p21	p21 p26	p20	p20 p26	p17	p12	p50	p50
Deglycosylated	p5	p20 (p21)	p20 (p21)	p21 p26	(p26)	p12	p17	n.d.	p50

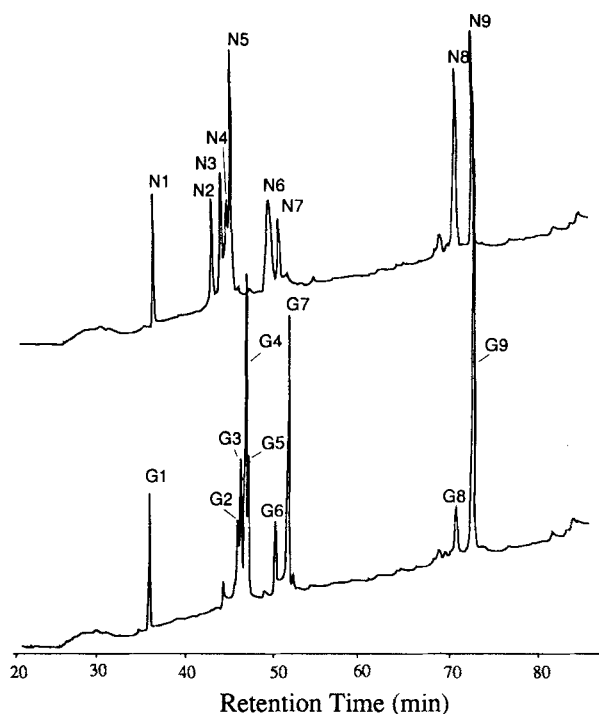


Fig. 5. Peptide mapping of the sFcεRI α by digestion with lysyl endopeptidase. Upper chart (non-treated): 800 pmol peptides digested with lysyl endopeptidase were subjected to reverse-phase HPLC. Lower chart (deglycosylated): 800 pmol of the peptidase-digested peptides were fractionated after *N*-glycanase treatment. The peak fractions, designated as shown in the figure, were collected and subjected to amino acid sequence analysis.

stained gel. These two bands appear with different intensity ratios among sFcεRI α from different hosts. The molecular masses of these bands were bigger than the deglycosylated form (20 kDa), and the same patterns of digested products were observed when they were treated with a 20-fold higher amount of *N*-glycanase (data not shown). Therefore, it is suggested that some N-linked oligosaccharides may be insensitive to *N*-glycanase or O-linked oligosaccharides may be attached to recombinant sFcεRI α. These results indicated that the differences in molecular masses observed in sFcεRI α

from different hosts were due to differential glycosylation states.

Peptide mapping of the purified sFcεRI α

Although it is clear that both native FcεRI α and recombinant sFcεRI α are *N*-glycosylated, it has not been defined where glycosylation occurs in the seven putative *N*-glycosylation sites (Asn-Xaa-Thr/Ser) of sFcεRI α (Fig. 4). Therefore we tried to identify glycosylation sites of the sFcεRI α from the infected Sf-9 by analyzing glycosylated and deglycosylated peptides digested with lysyl endopeptidase. If Asn is *N*-glycosylated, the Pth-Asn peak should not be detected on sequence analysis of the glycosylated peptide and Pth-Asp should be detected on the same sequencing cycle for the peptide deglycosylated by *N*-glycanase.

The lysyl-endopeptidase-digested peptides were isolated by HPLC and each amino acid sequence was determined (Fig. 5 and Table 2). As shown in Fig. 5, there may be five glycosylated peptides after digestion with lysyl endopeptidase. Peptide p50 was detected in peaks N8, N9 and G9. Though the recovery of N8 was insufficient to obtain clear Pth-Xaa peaks, the seventh cycle of N9 and G9 was Pth-Asn, and a mixture of Pth-Asn and Pth-Asp (molar ratio, 10:7), respectively. These data indicated that Asn74 (in p50) should be partially glycosylated. Peptide p17 was located in N6 and G7, and there was no Pth-Asn in the 12th cycle of N6, but Pth-Asp was detected in the same cycle of G7. Therefore Asn166 (in p17) should be completely glycosylated.

Although the other fragments, p20, p21 and p26 may be glycosylated they could not be completely separated from each other (Table 2); the locations of these fragments on chromatography were shifted after *N*-glycanase treatment indicating that all of these fragments were *N*-glycosylated. The amino acid sequence analysis of these peaks are summarized as follows. N2 contained p21 and Pth-Asn was not detected at the 4th and the 12th cycles of the Edman degradation of this peak. N3 contained p21 and p26, and Pth-Asn was not detected at the 4th and 7th cycles of the sequence analysis of this peak. N5 contained p20 and p26, and Pth-Asn was detected at the 7th cycle. Peak G4 also contained p21 and p26 peptides, and Pth-Asp was detected at the 4th, 7th and 12th, and Pth-Asn was also detected at the 7th cycle. These data indicated that Asn135 in p26 was partially glycosylated,

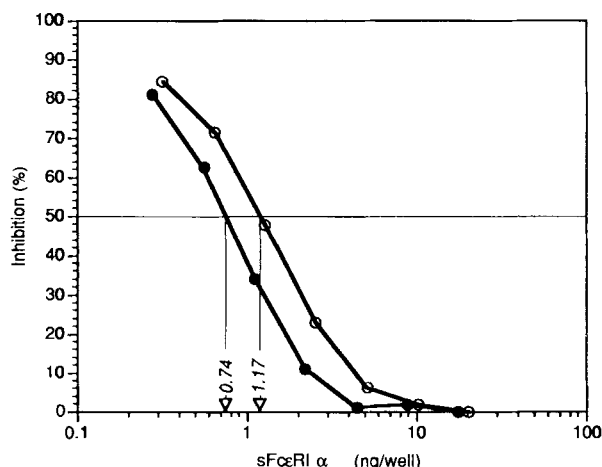


Fig. 6. IgE binding activity of the sFcεRI α. IgE-binding competition assay was performed as described in Materials and Methods. (●), sFcεRI α produced by AcNTV infected insect cells; (○), sFcεRI α produced by CHO transfectants.

and almost all Asn42 in p21 was glycosylated. Deglycosylated p20 was eluted at G2 and G3, and Pth-Asp was detected at the 3rd cycle, and Pth-Asn was not detected at the 3rd cycle of N4 and N5 which contained p20. Therefore almost all Asn21 was glycosylated. The glycosylation of Asn50 and Asn140 was not clearly defined, because there were the other Asn residues of the contaminating peptides in the same cycle.

IgE-binding activity of the sFcεRI α

The binding of IgE to sFcεRI α was monitored by IgE-binding competition assay described in Materials and Methods. In this system, the samples containing sFcεRI α are expected to decrease, in a dose-dependent manner, IgE binding to the standard sFcεRI α from the CHO transfectants attached on the plate (Fig. 6).

The purified sFcεRI α produced by insect cells actually inhibited IgE binding to the standard sFcεRI α fixed on the plate in a similar manner to the sFcεRI α produced by CHO transformants (Fig. 6). 50% inhibition of IgE binding was observed at 0.74 ng sFcεRI α (insect cells) and 1.17 ng sFcεRI α (CHO). Taking into account that the molecular mass ratio of both sFcεRI α is 35:50, this result indicates that the sFcεRI α from insect cells has equivalent IgE-binding activity to that of the standard.

DISCUSSION

The expressed sFcεRI α of the infected Sf-9 cells has a lower molecular mass than those from CHO transfectants and yeast transformants; this is in agreement with previous reports that mammalian glycoproteins produced by insect cells frequently have lower molecular masses than those produced by mammalian hosts or native forms (Steiner et al., 1988, Jarvis and Summers, 1989, Kuroda et al., 1990, Graber et al., 1992). This difference in the molecular masses is caused by the less complicated oligosaccharides attached by insect cells (Kuroda et al., 1990).

There are seven possible N-linked glycosylation sites in sFcεRI α predicted from its cDNA sequence (Shimizu et al., 1988, Kochan et al., 1988). We defined the state of glycosylation of these sites by peptide mapping and amino acid sequence analysis. Fig. 4 summarizes the revealed glycosylation pattern. Five of seven predicted sites were completely or partially glycosylated and the other two sites were suggested to be glycosylated. The heterogeneity (on or off) of glycosylation was found in two Asn residues (Asn74 and Asn135). It is also obvious that heterogeneity resides in the oligosaccharides themselves attached to the sFcεRI α because there are EndoH-sensitive and EndoH-resistant N-linked oligosaccharides on the recombinant sFcεRI α like other recombinant products produced by insect cells (Jarvis and Summers, 1989, Kuroda et al., 1990, Davidson and Casrellino, 1991). Our findings indicate that both the partial glycosylation state and the heterogeneous forms in oligosaccharides contribute to generate the heterogeneity of molecular masses found for sFcεRI α.

We cannot conclude whether the defined occupation patterns of N-oligosaccharides on Asn residues are specific for recombinant products from insect cells because information on the manner of glycosylation of the native and recombinant

FcεRI α from mammalian cells has not been available. It has been reported that no difference in the glycosylation sites is found between influenza virus hemagglutinin A produced by insect cells and by chicken cells (Kuroda et al., 1990), therefore it is plausible that the same occupation pattern can be observed in native or recombinant products from mammalian cells.

Our aim in this work was to obtain sufficient amount of sFcεRI α for further analysis such as crystallographic and NMR analysis. This aim was partially accomplished because we could now easily obtain about 2 mg sFcεRI α from only 350 ml culture medium of the infected insect cells. The precise structural information in this work will help to further analyze the structure of the sFcεRI α, although the heterogeneous glycosylation state observed in the purified sFcεRI α might interfere with the formation of crystal structure, and the glycosylated sFcεRI α exceeds the maximum size that has been resolved by NMR analysis.

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