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Structural analysis and localization of the carbohydrate moieties of a soluble human interferon γ receptor produced in baculovirus-infected insect cells

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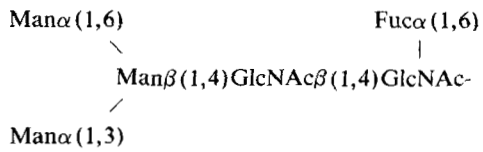
Abstract

A soluble form of the human interferon γ receptor that is required for the identification of interferon γ antagonists was expressed in baculovirus-infected insect cells. The protein carried N-linked carbohydrate and showed a heterogeneity on denaturing polyacrylamide gels. We investigated the utilization of the potential sites for N-linked glycosylation and the structure of the carbohydrate moieties of this soluble receptor. Amino acid sequence analysis and ion spray mass spectrometry revealed that of the five potential sites for N-linked glycosylation, Asn^{17} and Asn^{69} were always utilized, whereas Asn^{62} and Asn^{162} were utilized in approximately one-third of the protein population. Asn^{223} was never found to be glycosylated. The soluble receptor was treated with *N*-glycosidase F and the oligosaccharides released were analyzed by matrix-assisted laser desorption mass spectrometry, which showed that the protein carried six types of short carbohydrate chains. The predominant species was a hexasaccharide of molecular mass 1,039, containing a fucose subunit linked to the proximal *N*-acetylglucosamine residue:



Keywords: glycoprotein; glycosylation sites; insect cell-type glycosylation; soluble interferon γ receptor

Most native proteins carry carbohydrate moieties that fulfill important functions, some of which are still not well understood. Protein glycosylation shows a high structural diversity, and this might be responsible for the diverse and complex roles carbohydrates play in biological and clinical aspects (Paulson, 1989; Goochee et al., 1991; Kobata, 1992; Sharon & Lis, 1993). Development of carbohydrate-based drugs and glycotechnology products in general relies on information derived from recent advances in glycobiology that contributed to elucidation of important carbohydrate functions, like cell–cell interaction, protein turnover, protein transport, and others. A decisive step toward understanding the glycosylation functions is the investigation of the oligosaccharide structure and the determination of the glycosylation sites of the glycoproteins.

Recombinant proteins produced in eukaryotic expression systems are usually glycosylated, carrying characteristic carbohydrate types of the engineered cells from which they have been isolated. The availability of recombinant proteins opened new possibilities in studying the glycosylation function of glycoproteins that were impossible with native proteins available only in small amounts. Furthermore, selective digestion and chromatography, as well as sophisticated analytical techniques like mass spectrometry and high-resolution NMR spectroscopy, helped to elucidate the structures of the oligosaccharide moieties of certain recombinant proteins expressed in different eukaryotic systems (Townsend et al., 1988; Baldwin et al., 1990; Lee, 1990; Aeed et al., 1992; Edge et al., 1992; Fu & van Halbeek, 1992; Carr et al., 1993). In addition, the function and the effect of carbohydrate on physicochemical properties of several recombinant proteins, such as tissue-type plasminogen activator (Hotchkiss et al., 1988), erythropoietin (Takeuchi et al., 1990; Narhi et al., 1991), stem cell factors (Arakawa et al., 1991), and others, have been reported.

Soluble forms of human and mouse interferon γ (IFNγ) receptors were produced in *Escherichia coli*, in baculovirus-

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infected insect Sf9 cells, and in Chinese hamster ovary (CHO) cells (Fountoulakis et al., 1990, 1991; Gentz et al., 1992). The soluble receptors are important tools in the search for IFNγ antagonists that are potential pharmaceuticals against several disorders (Garotta et al., 1989). The soluble receptors were purified and characterized, and the effects of their carbohydrate moieties in certain physicochemical properties of the proteins, like resistance to thermal denaturation and proteolysis, were investigated (Fountoulakis & Gentz, 1992). The *E. coli*-derived protein was not glycosylated (Fountoulakis et al., 1990). The receptor produced in insect cells was glycosylated, carrying N-linked oligosaccharides. The receptor expressed in CHO cells was also glycosylated, carrying N- and O-linked carbohydrates and sialic acid residues as part of the N-linked glycosylation (Gentz et al., 1992). Here we report on the structure and site occupation of the carbohydrate moieties of the soluble IFNγ receptor produced in insect cells.

Results

Soluble IFNγ receptor produced in insect cells

Figure 1 shows a Coomassie blue-stained SDS-PAGE analysis of glycosylated and deglycosylated forms of the soluble human IFNγ receptor produced in insect Sf9 cells. Under nonreducing conditions, the glycosylated protein migrated as four bands with apparent molecular masses of approximately 28, 29, 30, and 32 kDa (lane 1). After removal of the N-linked glycosylation, the protein migrated at 26 kDa (lane 2), like the receptor produced in insect cells grown in the presence of tunicamicine (Gentz et al., 1992). The bands at approximately 27 kDa represent species with incomplete removal of the carbohydrate (lane 2). Under reducing conditions, the glycosylated and deglycosylated receptor forms showed a shift in mobility, migrating at about 33 and 30 kDa, respectively (not shown; Gentz et al., 1992). This shift is due to the presence of essential disulfide bonds responsible for the compactness of the protein (Stüber et al., 1993). In previous studies we showed that the insect cell-derived receptor carries only N-linked carbohydrate moieties and that glycosylation is not required for ligand binding (Fountoulakis & Gentz, 1992; Gentz et al., 1992).

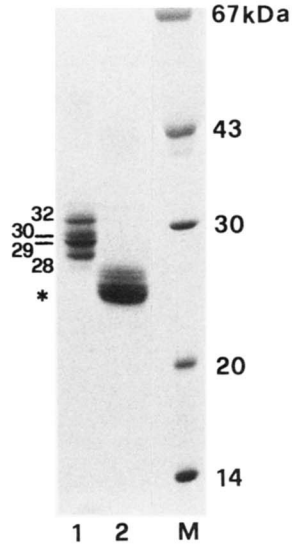


Fig. 1. SDS-PAGE analysis of the glycosylated and deglycosylated soluble human IFNγ receptor from insect Sf9 cells. The receptor was deglycosylated as described under Materials and methods. The proteins were electrophoresed under nonreducing conditions on a 12% SDS gel stained with Coomassie blue R250. Lane 1, glycosylated receptor. The four bands of the glycosylated protein migrating at approximately 28, 29, 30, and 32 kDa are indicated. Lane 2, receptor protein after treatment with *N*-glycosidase F. *, Deglycosylated receptor. The slower migrating weak bands represent partially deglycosylated receptor species. M, molecular mass markers.

N-terminal amino acid sequence analysis showed that the protein was properly processed, starting with Glu-Met-Gly-Thr-Ala-Asp-, as expected from the DNA sequence (Fountoulakis et al., 1992b; Gentz et al., 1992). A small population of the protein (15%) started with Gly-Thr-Ala-Asp-. Amino acid composition analysis revealed that the receptor has the predicted composition (data not shown). Ion spray mass spectrometric analysis of the glycosylated protein yielded two molecular masses of 28,986 ± 3 and 27,948 ± 5 Da, differing by approximately 1,039 Da. For the deglycosylated form a molecular mass of 25,869 ± 3 Da was determined, corresponding to the size calculated from the amino acid composition (25,868 Da) within experimental error. The molecular mass differences between glycosylated and deglycosylated receptor forms were 3,117 Da (1,039 × 3) or 2,079 Da (1,039 × 2). In some cases another species with a mass of 26,216 ± 2 Da was found, differing from the predicted one by 347 Da and representing a partially deglycosylated product corresponding to a receptor carrying a GlcNAc-Fuc disaccharide. This disaccharide would add 349.3 Da to the receptor mass, which is in good agreement with the observed mass of 26,216 Da. Thus, the protein was intact and the multiple bands observed on SDS-PAGE were not the result of proteolytic degradation. The heterogeneity could be associated with either different glycosylation patterns or unequal utilization of the potential glycosylation sites or both.

Utilization of the potential sites for N-linked glycosylation of the insect cell-derived receptor

Soluble IFNγ has five potential sites for N-linked glycosylation, at asparagine residues 17, 62, 69, 162, and 223. We first investigated whether a preferential utilization of the potential glycosylation sites occurred. The N-linked carbohydrate of the receptor was removed by incubation with *N*-glycosidase F. Following enzymatic cleavage, utilized asparagines from which glycosylation was completely released, were converted to aspartic acid residues. The deglycosylated protein was digested with endoproteinase Lys-C or endoproteinase Glu-C and proteinase K. The proteolytic fragments were separated by reversed-phase HPLC and were subjected to N-terminal amino acid sequence analysis. The molecular masses of the fragments were determined by ion spray mass spectrometry. The assignment of glycosylated asparagine residues was made on the basis of the absence of the phenylthiohydantoin derivative of asparagine in the corresponding Edman cycles and by comparison of the sequence found with the predicted sequence of the receptor. For utilized glycosylation sites from which the carbohydrate had not been removed during the enzymatic treatment, no asparagine or aspartic acid signal was observed; those from which the carbohydrate had been removed were identified as aspartic acid resi-

dues in the sequence analysis (Table 1). As shown in Table 1, the carbohydrate moieties were not completely removed in all cases. When carbohydrate remained linked to a proteolytic fragment, a molecular mass difference of approximately 1,039 Da, or a multiple of that value, was observed between the determined and calculated mass of the fragment.

In all fragments of the deglycosylated receptor analyzed, asparagine residues 17 and 69 were either absent or identified as aspartic acid (Table 2). In about one-third of the polypeptides sequenced, Asn^{62} and Asp^{162} were identified as aspartic acid and in two-thirds of the fragments as asparagine, indicating that in about two-thirds of the protein molecules these glycosylation sites were not utilized. In no case was Asn^{223} identified as aspartic acid, suggesting that this potential glycosylation site was not utilized. Although a representative number of fragments including each of the potential glycosylation sites was sequenced (about 10 in each case; Table 2), it is possible that some fragments carrying glycosylated asparagine residues were not detected, therefore some deviation from the percentages found may not be excluded.

Structural analysis of the carbohydrate

We further studied which monosaccharides were included in the carbohydrate moieties of the soluble receptor. The N-linked oligosaccharides were enzymatically removed and separated from the proteins by ultrafiltration, hydrolyzed in the presence of trifluoroacetic acid, and the hydrolysate was analyzed. The monosaccharide analysis was performed by high-performance anion-exchange chromatography (Hardy & Townsend, 1988) and by comparing the elution positions with the ones of commercially available monosaccharide standards (Fig. 2A). On account of the presence of glycerol in the receptor-derived monosaccharide sample, originating from the preparation of *N*-glycosidase F, a large flow-through peak was present (Fig. 2B). Only the monosaccharides fucose, glucosamine, and mannose could be detected in the receptor-derived carbohydrate (Fig. 2B).

The receptor carbohydrates were analyzed by high-performance anion-exchange chromatography, and the elution profile was compared to the profile of commercial oligosaccharide standards. The unequal utilization of the glycosylation sites and the relatively small difference of molecular mass between the glycosylated and deglycosylated forms of the proteolytic fragments (Table 1) suggested that the receptor probably carried short carbohydrate chains. The standards used had the structures of Manα1,6(Manα1,3)Manβ1,4GlcNAcβ1,4(Fucα1,6)GlcNAc (*a*) and Manα1,6(Manα1,3)Manβ1,4GlcNAcβ1,4GlcNAc (*b*), differing by the presence of one fucose residue linked to the proximal *N*-acetylglucosamine subunit. The two standards were efficiently separated by the elution gradient (Fig. 3A). To make sure that the standards indeed differed by one linked fucose residue, oligosaccharide *a* was digested with fucosidase and the re-

Table 1. *Amino acid sequence analysis of representative proteolytic fragments of the soluble IFNγ receptor*[a]

Sequence determined[b]	Potential glycosylation sites included	Molecular mass of fragment: Determined (Da)	Molecular mass of fragment: Calculated (Da)	Glycosylation site utilized	Molecular mass of oligosaccharide (Da)
EMGTADLGPSSVPTPTDVTI[c]	Asn^{17}	5,271.5	5,271.6[d]	Asn^{17}	–
EMGTADLGPSsVPTpTXV[e]	Asn^{17}	6,312.4	5,271.6[d]	Asn^{17}	1,040.8
NSEXIDAXINISHHYXDISDH[c]	Asn^{62}, Asn^{69}	3,908.8	3,908.8[f]	Asn^{69}	–
NSEXIDAXIDISHHYXDIS[c]	Asn^{62}, Asn^{69}	3,908.8	3,908.8[f]	Asn^{62}, Asn^{69}	–
NsEXIDAXIXIshhYXXI[e]	Asn^{62}, Asn^{69}	5,985.8	3,908.8[f]	Asn^{62}, Asn^{69}	2,077.0[g]
NsEXIDAXINISHHY[e]	Asn^{62}, Asn^{69}	4,945.8	3,908.8[f]	Asn^{62}	1,037.0
VRMXGS[e]	Asn^{162}	1,701.4	663.4[h]	Asn^{162}	1,037.7
VRMNGS	Asn^{162}	663.4	663.4[h]	–	–
IQYKILTQKEDDXDEIQXQL VXITIFNSSIKGS	Asn^{223}	5,441.2	5,441.2[i]	–	–

[a] The N-linked carbohydrates of the soluble IFNγ receptor were removed by incubation with *N*-glycosidase F. Complete release of the carbohydrate moieties from the utilized glycosylation sites converted the corresponding asparagines to aspartic acid residues. The deglycosylated protein was digested with either endoproteinase Lys-C or with endoproteinase Glu-C and proteinase K. The fragments were separated by reversed-phase HPLC and subjected to N-terminal amino acid sequence analysis. The molecular masses were determined by ion spray mass spectrometry, as described under Materials and methods.

[b] The expected positions of the potential glycosylation sites (asparagine or aspartic acid residues) are shown in boldface type; X denotes unidentified residue; lowercase letters indicate residues not identified with certainty.

[c] In these fragments, the glycosylation of the utilized site was completely removed during the enzymatic treatment with *N*-glycosidase F.

[d] Corresponds to the molecular mass of the fragment Glu^{1}–Lys^{47}.

[e] In these fragments, the carbohydrate of the utilized sites was not removed; the assignment of glycosylation was made on the absence of phenylthiohydantoin derivatives for asparagine in the corresponding Edman cycles and the difference between determined and expected values of molecular mass.

[f] Corresponds to the molecular mass of the fragment Asn^{53}–Lys^{86}.

[g] The molecular mass of 2,077 Da is the sum of the masses of two oligosaccharides of approximately 1,039 Da each, located at Asn^{62} and Asn^{69}.

[h] Corresponds to the molecular mass of the fragment Val^{159}–Ser^{164}.

[i] Corresponds to the molecular mass of the fragments Ile^{166}–Glu^{201} and Val^{217}–Ser^{229}, which are connected by the disulfide bond Cys^{197}–Cys^{218}. The molecular mass is reduced by an additional 2 Da because of the presence of a second disulfide bond between Cys^{178} and Cys^{183}.

Table 2. *Utilization of the potential N-glycosylation sites of the soluble IFNγ receptor*[a]

Potential glycosylation site	Number of proteolytic fragments sequenced	Glycosylation sites identified	Protein population with glycosylation site utilized (%)
Asn^{17}	7	7	100
Asn^{62}	11	4	40
Asn^{69}	10	10	100
Asn^{162}	9	3	30
Asn^{223}	10	0	0

[a] The proteolytic fragments of the soluble IFNγ receptor were analyzed as described for Table 1.

action products were analyzed on the same system. The peak corresponding to oligosaccharide *a* was converted to the peak corresponding to oligosaccharide *b* (data not shown).

High-performance anion-exchange chromatography analysis of the receptor-derived carbohydrate showed that the protein carried mainly one kind of oligosaccharide (Fig. 3B). There were also approximately five small peaks present with retention times longer than that of peak *c*, corresponding probably to larger carbohydrate chains. Very small peaks, eluted before peak *c*, were observed as well, most likely corresponding to trimmed oligosaccharides (the rest of the carbohydrate remained linked to the receptor, as revealed by mass spectrometry analysis). The major peak comigrated with the peak corresponding to oligosaccharide standard *a* (compare Fig. 3A with B). When the receptor-derived carbohydrate was added to the mixture of oligosaccharide standards *a* and *b* and chromatographed on the same system, the intensity of peak *a*, corresponding to oligosaccharide with one fucose residue linked, increased in comparison with that of the *b*, corresponding to the carbohydrate lacking the fucose residue (Fig. 3C). The molecular mass of the predominant oligosaccharide chain detected is 1,039 Da, which is in agreement with the mass difference between glycosylated and deglycosylated fragments (Table 1). Thus, the receptor carries one major type of carbohydrate chain of the structure

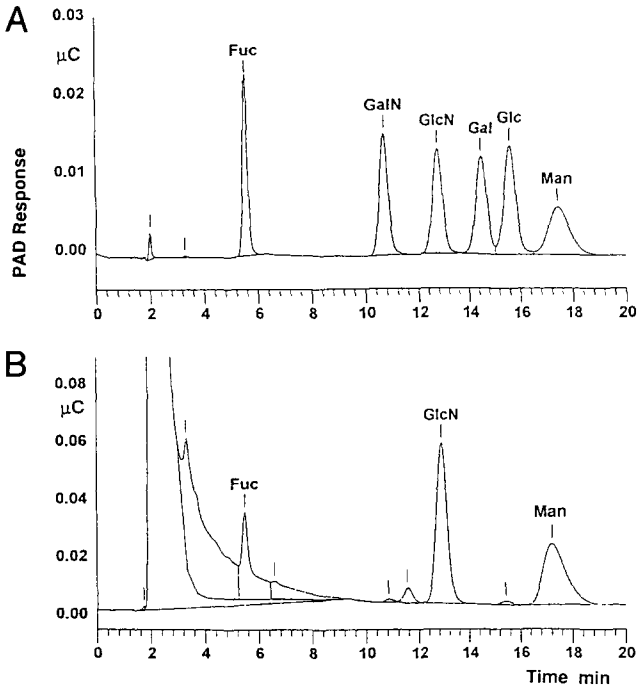


Fig. 2. Analysis of the monosaccharide composition of the soluble IFNγ receptor-derived carbohydrate. Mixture of commercial monosaccharide standards (**A**) and monosaccharides derived from hydrolysis of the receptor carbohydrate (**B**) were analyzed on a CarboPac PA1 column (4.5 × 250 mm) eluted with 16 mM NaOH. The protein carbohydrate was enzymatically removed from the receptor and hydrolyzed as stated under Materials and methods. Fuc, fucose; Gal, galactose; GalN, galactosamine; Glc, glucose; GlcN, glucosamine; Man, mannose; PAD, pulsed amperometric detection.

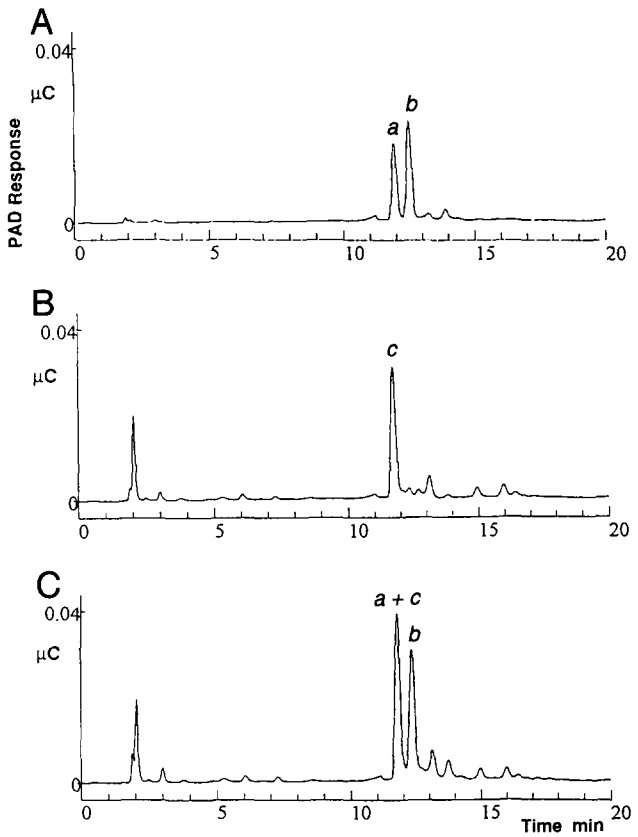
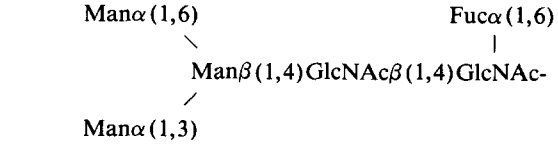


Fig. 3. Analysis of the carbohydrate moieties of the soluble IFNγ receptor. Commercial oligosaccharide standards (**A**), receptor-derived carbohydrate (**B**), and a mixture of the oligosaccharide standards with the receptor carbohydrate (**C**) were analyzed on a CarboPac PA-100 column (4.5 × 250 mm) eluted with a linear gradient of 0–100 mM NaOAc in 100 mM NaOH. The protein was deglycosylated by incubation with *N*-glycosidase F and the carbohydrate was separated from the enzyme by ultrafiltration. *a*, oligosaccharide standard Manα1,6(Manα1,3)Manβ1,4GlcNAcβ1,4(Fucα1,6)GlcNAc; *b*, oligosaccharide standard Manα1,6(Manα1,3)Manβ1,4GlcNAcβ1,4GlcNAc; *c*, oligosaccharides derived from enzymatic deglycosylation of the soluble IFNγ receptor; PAD, pulsed amperometric detection.



The receptor-derived oligosaccharides were analyzed by matrix-assisted laser desorption ionization mass spectrometry, which revealed six masses for the carbohydrates (spectrum not shown). The major carbohydrate species *1* constituted approximately 60% of total oligosaccharides, as deduced from comparison of the area corresponding to this peak with the sum of the areas of all peaks. A molecular mass of approximately 1,080 Da was determined for carbohydrate species *1*. The directly determined mass of 1,080 Da corresponds to the calculated and indirectly found mass of 1,039 Da, allowing for the addition of a water molecule (18 Da), following enzymatic cleavage, and of a sodium ion (23 Da) as an oligosaccharide adduct (a molecular mass of approximately 1,080 was determined for oligosaccharide standard *a* as well). For carbohydrate species *2* and *3*, constituting approximately 13% each, and for species *4–6*, constituting approximately 4% each of total oligosaccharides, masses of 1,096, 1,283, 1,300, 1,652, and 1,797 Da, respectively, were determined. Peaks of 500–700 Da molecular mass were also present, which, however, were not resolved well because of disturbing signals of the matrix (data not shown). The latter signals most likely correspond to trimmed carbohydrates. From the determined molecular masses, potential structures could be assigned to the oligosaccharides of peaks *2–4* (Fig. 4). As indicated, carbohydrates *2* and *4* would lack the fucose linked to the proximal *N*-acetylglucosamine and contain additional mannose and *N*-acetylglucosamine residues. For the oligosaccharides of peaks *5* and *6*, no structures are given. The distribution of the oligosaccharide species at the potential glycosylation sites was not investigated in this study.

The purification scheme for the receptor protein included a Concanavalin A chromatographic step (Fountoulakis et al., 1991), and receptor species carrying carbohydrate moieties that do not bind to this lectin would have been missed. Consequently, the corresponding carbohydrates would have not been detected in this analysis. However, this is highly unlikely because tri- and tetraantennary complex-type sugars, which do not bind to Concanavalin A and would have not been detected, are uncommon in insect cell-type glycosylation (Goochee et al., 1991; Grabenhorst et al., 1993). Unlike tri- and tetraantennary complex-type sugars, biantennary complex-, tri- and tetraantennary high mannose-, and hybrid-type carbohydrates bind to the lectin (Kobata, 1992). Furthermore, we investigated whether the signal of the receptor in the original preparation consisted of more than the four bands found in the purified protein (Fig. 1, lane 1). By ligand blot analysis of the secreted nonpurified proteins, the receptor appeared as four bands and showed the same migration pattern as the purified protein (data not shown).

Discussion

We investigated the apparent heterogeneity of a soluble human IFNγ receptor produced in baculovirus-infected insect cells. The heterogeneity was found to be mainly due to unequal utilization of the five potential sites for N-linked glycosylation and to a lesser extent to linkage of different carbohydrate moieties. The native IFNγ receptor is a 90-kDa glycoprotein that carries N-linked carbohydrates contributing approximately 20 kDa to its apparent molecular mass and sialic acid residues as part of the N-linked glycosylation, contributing approximately 4 kDa; it is not clear whether it carries O-linked oligosaccharides (Fountoulakis et al., 1989). The native IFNγ receptor also showed a heterogeneity that was partially due to proteolytic fragmentation (Fountoulakis et al., 1989) and partially due to the different N-linked glycosylation patterns depending on the cell line from which the protein was isolated (Fischer et al., 1990; van Loon et al., 1991). However, there has been no report yet about the structure of the carbohydrate moieties of the native IFNγ receptor or about the mode of utilization of its potential glycosylation sites. Therefore, we cannot correlate the results found by using the insect cell-derived soluble receptor with the situation in the native protein. Comparison with other recombinant soluble IFNγ receptors is not feasible at present.

The soluble receptor studied here carries a glycosylation pattern typical of proteins produced in insect cells (Goochee et al., 1991; Grabenhorst et al., 1992, 1993), i.e., short chains consisting of the core pentasaccharide structure of N-linked carbohydrates (Kobata, 1992) with additional fucose, mannose, and *N*-acetylglucosamine residues (Fig. 4). The contribution of glycosylation to the apparent molecular mass of this protein is smaller in comparison with other forms of the IFNγ receptor. Thus, the N-linked oligosaccharides of the insect cell-derived soluble receptor contribute 4–5 kDa to the apparent mass of the protein (if all glycosylation sites are utilized), whereas the carbohydrates of the soluble receptor produced in CHO cells contribute 10–12 kDa (Gentz et al., 1992), of the native IFNγ receptor approximately 20 kDa (Fountoulakis et al., 1989), and of a hybrid protein, consisting of the IFNγ receptor extracellular domain and parts of the human immunoglobulin G (IgG) heavy chain, expressed in CHO cells approximately 60 kDa (unpubl. results).

The carbohydrate structure *1* (Fig. 4) of the insect cell-derived receptor is identical with the major glycosylation species of human IFNβ and human interleukin-2, both produced in insect Sf21 cells. Three carbohydrate structures were determined for recombinant IFNβ, one identical with oligosaccharide *1* and two others, each lacking either Manα(1,3) or Manα(1,6) (Grabenhorst et al., 1992). Oligosaccharide structures of type *1* were found in proteins expressed in baculovirus-infected insect cells, like interleukin-2 (Grabenhorst et al., 1993), interleukin-3 (Knepper et al., 1992), influenza virus hemagglutinin (Kuroda et al., 1990; Klenk et al., 1992), and others. According to the data of the mass spectrometric analysis, the minor species *3* and *4* should include additional *N*-acetylglucosamine subunits most likely linked to the α-mannosyl(1,3) residue (Fig. 4) and may represent intermediate oligosaccharide processing products.

The glycosylation machinery of insect cells has not yet been thoroughly investigated. Insect cells are expected to possess the capability of (at least) minimal oligosaccharide processing. It seems that they can add both N- and O-linked glycosylation, whereas there is a controversy concerning their capacity to add sialic acid residues (Fountoulakis et al., 1991; Goochee et al., 1991; Grabenhorst et al., 1992, 1993). The existence of processing machinery is also suggested by the addition of fucose to the proximal *N*-acetylglucosamine residue, as has been shown for many proteins (Kuroda et al., 1990; Grabenhorst et al., 1992, 1993; Klenk et al., 1992; Knepper et al., 1992) and as we found with the soluble IFNγ receptor. Recent reports suggest that insect cells are capable of trimming *N*-glycans to trimannosyl cores (Kuroda et al., 1990) and that they contain the necessary glycosidases to assemble complex-type oligosaccharides on proteins (Velardo et al., 1993). Insect cells likely possess silent genes encoding necessary complex-type glycosylation processing en-

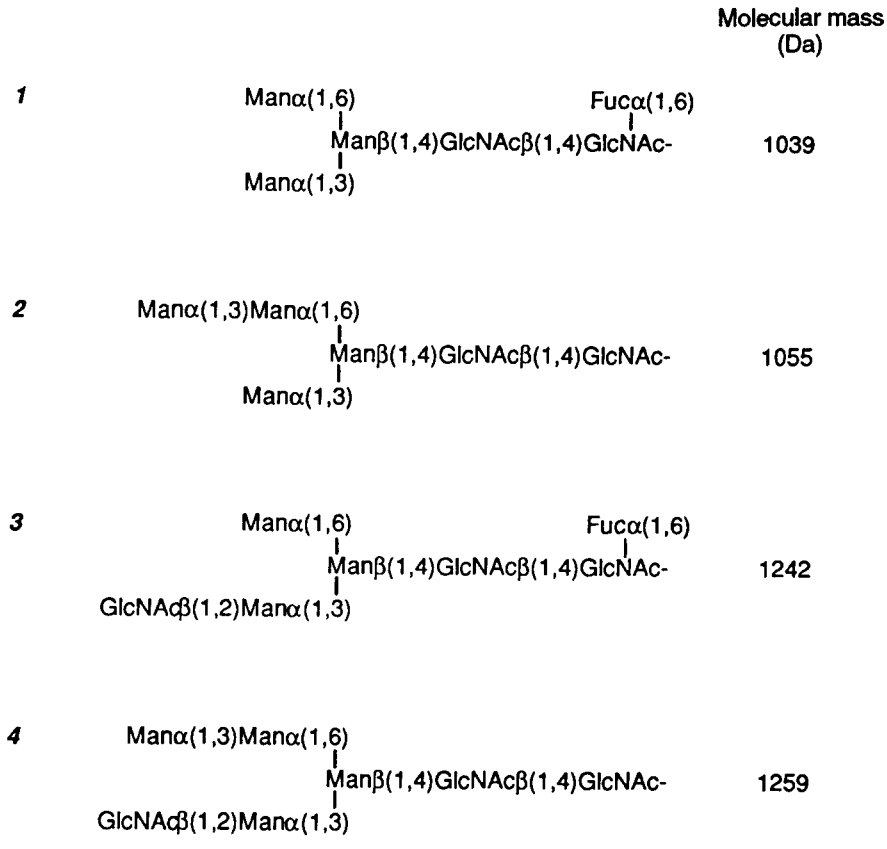


Fig. 4. Structures of the oligosaccharides of peaks *1–4* of mass spectrometric analysis. Carbohydrates were enzymatically removed from the receptor and analyzed by matrix-assisted laser desorption ionization mass spectrometry as stated under Materials and methods. The structure of oligosaccharide *1* was verified. The structures of oligosaccharides *2–4* are hypothetical, deduced from the determined molecular masses. The observed molecular masses of the oligosaccharides are indicated. They are corrected for a sodium ion adduct (23 Da) and the mass of a water molecule (18 Da) following enzymatic cleavage of the carbohydrates. Structures of isomers or for the oligosaccharides of peaks *5* and *6* of the laser desorption mass spectral analysis are not given.

zymes, including sialyltransferases. The glycosylation machinery is activated by external stimuli, like viral infection, in the presence of appropriate substrates (Velardo et al., 1993). The carbohydrate analysis of the soluble IFNγ receptor showed that insect cells can produce short unsialylated carbohydrate chains of mannose and hybrid types (Fig. 4).

Utilization of the potential glycosylation sites seems to proceed stepwise, as we found with a soluble mouse IFNγ receptor produced in the same expression system (Fountoulakis et al., 1991). According to ligand blot analysis, the intact protein was detected as 28- and 30-kDa bands in the culture medium 3 days postinfection, whereas 1 day later, additional protein bands migrating at 26 and 27 kDa, most likely carrying less carbohydrate, were also visible. The utilization of the glycosylation sites of the mouse protein has not yet been investigated. However, the similarity of the migration pattern on SDS-polyacrylamide gels in comparison with the human counterpart suggests that in the receptor of mouse origin not every one of the five potential glycosylation sites has been equally utilized.

The carbohydrate structure and the mass spectrometric analysis of the glycosylated protein revealed that the insect cell-derived soluble receptor usually carries two or three oligosaccharide chains. This finding helps to explain the observed heterogeneity on SDS gels (Fig. 1), assuming that all carbohydrate species can be found at all occupied sites. Thus, the 28-kDa species may be glycosylated at the two asparagine residues that were found to be always utilized. The 29-kDa species may represent a receptor protein glycosylated at the mentioned residues and, in addition, at one of the residues found to be glycosylated in about one-third of the protein population. The 32-kDa band may represent a protein glycosylated at all four utilized glycosylation sites. The 30-kDa species, which is represented by a weak, diffuse band, may represent receptor molecules with three utilized glycosylation sites carrying in addition to the basic hexasaccharide *1* also larger glycosylation patterns (species *2–4*, Fig. 4). Of the four bands of the glycosylated receptor, the one migrating at 29 kDa is the strongest, suggesting that in a large population of the protein three glycosylation sites are utilized (Fig. 1, lane 1; agreement of the calculated molecular masses of the different receptor forms with those observed for the protein bands on SDS gels may not be accurate, as SDS-PAGE does not reveal exact masses of glycoproteins).

A schematic representation of the IFNγ receptor is shown in Figure 5, where the approximate locations of the potential glycosylation sites of the protein are indicated. This model was drawn on the basis of crystallographic studies of the growth hormone–growth hormone receptor complex (de Vos et al., 1992) and of predicted structural similarity between the receptors for growth hormone and IFNγ (Bazan, 1990), as we previously described (Stüber et al., 1993). According to this model, the extracellular ligand binding domain of the IFNγ receptor consists of two Ig-like domains connected by one disulfide bond between Cys^{105} and Cys^{150}. Two receptor molecules bind one IFNγ dimer (Fountoulakis et al., 1992b). The ligand–receptor interaction domain is most likely located at the region surrounding the junction of the two Ig-like domains (Stüber et al., 1993). Three utilized glycosylation sites—Asn^{17}, Asn^{62}, and Asn^{69}—are located on the upper Ig-like domain of the IFNγ receptor protein. The fourth site, Asn^{162}, and the never-utilized site, Asn^{223}, are located on the bottom Ig-like domain, adjacent to the transmem-

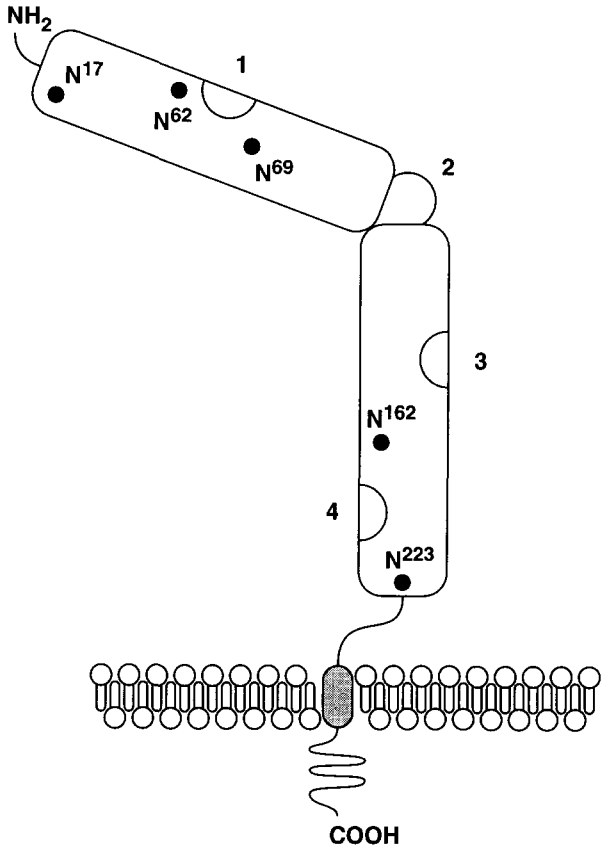


Fig. 5. Schematic representation of the extracellular domain of the IFNγ receptor showing the approximate location of the five potential sites for N-linked glycosylation. The model was drawn in analogy to the crystal structure of the growth hormone receptor, as explained in Stüber et al. (1993). 1–4, Approximate locations of the four disulfide bonds of the receptor, Cys^{60}-Cys^{68}, Cys^{105}-Cys^{150}, Cys^{178}-Cys^{183}, and Cys^{197}-Cys^{218}, respectively. ●, Approximate location of the five potential sites for N-linked carbohydrate. Asparagine residues 17 and 69 were always utilized, 62 and 162 were utilized in one-third of the protein population, and 223 was never utilized.

brane region of the receptor (Fig. 5). From the model it is obvious that the glycosylation sites utilized are located in exposed regions of the protein.

No function has been reported so far for the glycosylation of the native or of the recombinant IFNγ receptors. Glycosylation is not required for ligand binding, but it may be responsible for the protein conformation on the cell surface (Fountoulakis et al., 1989; Fischer et al., 1990; van Loon et al., 1991). The insect cell-type glycosylation of the soluble IFNγ receptor produced in this system does not seem to influence the physicochemical properties of the protein (Fountoulakis & Gentz, 1992). It also does not seem to exert typical functions ascribed for oligosaccharides of glycoproteins, like protection against proteolysis or increase of resistance against thermal denaturation. On the contrary, this type of glycosylation may increase the sensitivity of the protein to certain proteolytic attacks (Fountoulakis & Gentz, 1992). The carbohydrate pattern of the soluble receptor lacks sialic acid residues that could be responsible for the relatively short half-life (about 2 h) of the insect cell-derived mouse IFNγ receptor when injected into animals (Gentz et al., 1992; Ozmen et al., 1993).

In conclusion, the soluble IFNγ receptor produced in insect cells shows an unequal utilization of its glycosylation sites, those located on exposed domains of the protein being preferentially utilized. The receptor carries one major type of oligosaccharide chain consisting of the core pentasaccharide structure common to all N-linked carbohydrates with one additional fucose linked to the proximal *N*-acetylglucosamine residue.

Materials and methods

Materials

Reagents for the preparation of SDS-polyacrylamide gels and low-molecular-mass protein size markers were from Bio-Rad. Coomassie brilliant blue R250 was from Serva. Sequencing-grade proteolytic enzymes, *N*-glycosidase F, and α-fucosidase were purchased from Boehringer Mannheim. Monosaccharide and oligosaccharide standards were purchased from Dionex Corporation.

Analytical methods

The soluble receptor was resolved on 12% SDS-polyacrylamide gels and revealed by staining with Coomassie blue. If not otherwise indicated, no reducing agent was present in the sample buffer. The protein concentration was determined by amino acid analysis (Fountoulakis et al., 1992a). Ligand blots were performed as previously described (Fountoulakis et al., 1989).

Soluble IFNγ receptor produced in Sf9 cells

The protein was secreted into the culture medium and purified from cell culture supernatants essentially as the soluble mouse IFNγ receptor produced in the same expression system (Fountoulakis et al., 1991; Fountoulakis & Juranville, 1993). The receptor comprises the extracellular domain of the native protein (residues 1–229) and does not include the signal peptide sequence.

Enzymatic removal of carbohydrate

The removal of carbohydrate was performed as described (Fountoulakis & Gentz, 1992). In short, 100 μg of receptor in 500 μL of phosphate-buffered saline, pH 7.4, was incubated with 5 U of *N*-glycosidase F at 37 °C for 2 days. The deglycosylation process was followed by SDS-PAGE. More than 90% of carbohydrate was usually removed. *N*-glycosidase F was separated by binding the protein mixture to a Polybuffer Exchanger column (Pharmacia LKB Biotechnology). The enzyme was recovered in the flow-through fraction. The deglycosylated receptor was eluted with 1 M NaCl and the eluate was dialyzed to remove the salt. The flow-through fraction was passed through an M_r 10,000 cut-off filter to remove the enzyme, and the filtrate was used for carbohydrate structure analysis.

Digestion of the IFNγ receptor

The deglycosylated receptor, in 0.1 M ammonium bicarbonate buffer, pH 7.8, was incubated with either endoproteinase Lys-C or endoproteinase Glu-C and proteinase K (receptor:enzyme ratio 25:1, each) at 37 °C for 24 h. The reaction was stopped by

addition of phenylmethylsulfonyl fluoride to a final concentration of 1 mM.

Amino acid sequence analysis

The proteolytic products were separated by reversed-phase HPLC on an Aquapore OD-300, 2.1 × 220-mm column (Brownlee-Labs), developed with a linear gradient of 0–65% acetonitrile containing 0.1% trifluoroacetic acid on a Hewlett-Packard 1090A system. The absorbance was recorded at 214 nm. Selected peptide-containing fractions were subjected to amino-terminal sequence analysis on an Applied Biosystems 477A sequenator equipped with an on-line phenylthiohydantoin-amino acid analyzer (Hewick et al., 1981).

Amino acid composition analysis

The proteins were hydrolyzed with 6 N HCl at 110 °C for 24 h, and analysis was performed by a modification of the method of Spackman et al. (1958) on a Kontron Liquimat III amino acid analyzer.

Mass spectrometry

Fragments of interest were analyzed by ion spray mass spectrometry on an API III system (SCIEX). The proteins were dissolved in 1 M acetic acid in water:methanol (50:50, v/v) and 10 μL was flow injected. Mass analysis was performed in the positive ion mode. Scans between *m/z* 400 and 2,000 in 0.2 mass to charge unit steps were recorded.

Receptor-derived carbohydrates were dissolved 1:10 in 2,5-dihydroxybenzoic acid and analyzed by matrix-assisted laser desorption ionization mass spectrometry on an LDI-1700 time-of-flight mass spectrometer lacking a reflectron (Biomolecular Separations Inc.). Calibration was external to the samples.

Hydrolysis of the receptor-derived carbohydrate

For monosaccharide analysis, the receptor-derived carbohydrates were hydrolyzed in 2 M trifluoroacetic acid at 100 °C for 4 h.

Carbohydrate analysis

Monosaccharide standards and monosaccharides derived from the receptor carbohydrate were analyzed on a CarboPac PA1 column (4.5 × 250 mm; Dionex) developed with 16 mM NaOH at 1 mL/min. Oligosaccharide standards and receptor-derived oligosaccharides were analyzed on a CarboPac PA-100 column (4.5 × 250 mm; Dionex) eluted with a linear gradient of 0–100 mM NaOAc in 100 mM NaOH at 1 mL/min. Chromatographic analysis was done on a DX-300 high performance anion exchange system equipped with a pulsed amperometric detector with post-column alkalinization for detection at low alkalinity (Dionex Corporation).

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