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Posttranslational processing of recombinant human interferon-γ in animal expression systems

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Abstract

We have characterized the heterogeneity of recombinant human interferon-γ (IFN-γ) produced by three expression systems: Chinese hamster ovary cells, the mammary gland of transgenic mice, and baculovirus-infected *Spodoptera frugiperda* (Sf9) insect cells. Analyses of whole IFN-γ proteins by electrospray ionization–mass spectrometry (ESI-MS) from each recombinant source revealed heterogeneous populations of IFN-γ molecules resulting from variations in *N*-glycosylation and C-terminal polypeptide cleavages. A series of more specific analyses assisted interpretation of maximum entropy deconvoluted ESI-mass spectra of whole IFN-γ proteins; MALDI-MS analyses of released, desialylated *N*-glycans and of deglycosylated IFN-γ polypeptides were combined with analyses of 2-aminobenzamide labeled sialylated *N*-glycans by cation-exchange high-performance liquid chromatography. These analyses enabled identification of specific polypeptide cleavage sites and characterization of associated *N*-glycans. Production of recombinant IFN-γ in the mammalian expression systems yielded polypeptides C-terminally truncated at dibasic amino acid sites. Mammalian cell derived IFN-γ molecules displayed oligosaccharides with monosaccharide compositions equivalent to complex, sialylated, or high-mannose type *N*-glycans. In contrast, IFN-γ derived from baculovirus-infected Sf9 insect cells was truncated further toward the C-terminus and was associated with neutral (nonsialylated) *N*-glycans. These data demonstrate the profound influence of host cell type on posttranslational processing of recombinant proteins produced in eukaryotic systems.

Keywords: baculovirus; Chinese hamster ovary; glycosylation; interferon; mass spectrometry; proteolysis; recombinant protein; transgenic

In eukaryotic recombinant protein production systems there is currently a choice of yeast, insect cells, mammalian cells, and the mammary gland of transgenic animals (Hodgson, 1993). The choice of an expression system for production of a recombinant protein is dependent on process factors such as scale of operation, product recovery, product functionality, and product quality (Lubiniecki, 1994), and the influence of the host cell is of particular significance. Different cell types are known to vary in their ability to confer specific posttranslational modifications, such as glycosylation, which can affect the bioactivity, receptor binding, susceptibility to proteolysis, immunogenicity, and clearance rate of a therapeutic recombinant protein in vivo (Jenkins & Curling, 1994). Thus, the necessity of assessing the heterogeneity of a recombinant protein accurately during all stages of bioprocess development is of paramount importance.

In this study, we have examined the heterogeneity of the same recombinant protein, human interferon-γ, when produced by three commercially significant animal expression systems: Chinese hamster ovary cells, baculovirus-infected *Spodoptera frugiperda* (Sf9) insect cells, and the mammary gland of transgenic mice. Human IFN-γ is a pleiotropic immunomodulatory factor synthesized by antigen-sensitized T-lymphocytes. The

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Abbreviations: 2-AB, 2-aminobenzamide; CHO, Chinese hamster ovary; DHB, 2,5-dihydroxybenzoic acid; DHFR, dihydrofolate reductase; DMB, 1,2-diamino-4,5-methylene-dioxybenzene; ESI, electrospray ionization; Hex, hexose; HexNAc, *N*-acetylhexosamine; IFN-γ, interferon-γ; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry (spectrometer); Neu5Ac, *N*-acetylneuraminic acid; PBS, phosphate-buffered saline; PNGase F, peptide-*N*-glycosidase F; Pyr, pyroglutamic acid; RP-HPLC, reverse-phase high-performance liquid chromatography; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid.

cloned gene has been expressed in a variety of cell types as a noncovalently associated homodimer, and the crystal structure of recombinant human IFN-γ has been determined (Ealick et al., 1991). Previous studies have established that recombinant IFN-γ produced by CHO cells has three sources of variation: variable *N*-glycosylation site occupancy (nonglycosylated, glycosylated at Asn^{25} only, or glycosylated at Asn^{25} and Asn^{97}), site-specific *N*-glycan microheterogeneity, and C-terminal polypeptide truncations (Curling et al., 1990). Sareneva et al. (1994) have shown that glycosylation of Asn^{25} is necessary for efficient dimerization and secretion of IFN-γ. In this laboratory, we have previously shown that *N*-glycosylation of recombinant human IFN-γ is heterogeneous, Asn site specific, and host cell specific (James et al., 1995).

The emergence of "soft" ionization methods for biomolecule characterization by mass spectrometry such as electrospray ionization and matrix assisted laser desorption/ionization has provided a formidable weapon in the armory of the analytical protein chemist (Siuzdak, 1994). These techniques have been independently employed to identify unknown proteins, sequence polypeptides, confirm primary protein structure, characterize posttranslational modifications, and probe protein conformation (reviewed by Aebersold, 1993; Wang & Chait, 1994).

In this investigation, we demonstrate that ESI-MS and MALDI-MS analyses of intact or minimally fragmented protein molecules can be integrated to describe complex, heterogeneous recombinant protein populations in detail. ESI-MS provides the single most detailed source of data on glycoprotein heterogeneity; together with more specific information from MALDI-MS analysis of polypeptide and glycan fragments generated in vitro, it can yield detailed structural conclusions.

Using these methods, we show here that both glycosylation and proteolytic processing of the same recombinant protein, human IFN-γ, are host cell dependent.

Results and discussion

Recombinant human IFN-γ proteins are heterogeneous populations

Recombinant IFN-γ proteins derived from three expression systems—CHO cells, the mammary gland of transgenic mice, and baculovirus-infected Sf9 insect cells—were analyzed directly by ESI-MS. The zero-charge spectra obtained after initial processing of raw data and subsequent resolution enhancement by maximum-entropy analysis are presented in Figures 1A, 2A, and 3A. These analyses reveal that each IFN-γ preparation exists as a distinct population of multiple individual components and imply significant differences in posttranslational processing events with each expression system.

Whilst no protease inhibitors were included in either CHO or Sf9 insect cell cultures, so as to permit a direct comparison with transgenic mouse derived IFN-γ, extracellular degradation during sample processing was minimized by (1) immediate storage of filtered cell culture supernatants at −20 °C and lyophilization of mouse milk samples and (2) rapid purification (<1 h) of recombinant IFN-γ by one-step immunoaffinity chromatography. Separate batches of recombinant human IFN-γ prepared and purified as described yielded ESI-MS mass analyses very similar to those shown in this study, presenting no significant differences in C-terminal proteolysis or monosaccharide composition.

It was possible to tentatively identify nonglycosylated components after ESI-MS analysis on the basis of simple mass correlations (e.g., components A, B, and C in Fig. 1). These mass analyses suggested that the N-terminal Gln of recombinant IFN-γ from each source had been converted to pyroglutamic acid, with a resultant mass shift of −17 Da. In fact, no other mass correlations were possible. Accordingly, each IFN-γ preparation was found to be resistant to automated N-terminal sequencing. In each case, removal of the Pyr residue with pyroglutamate aminopeptidase (1:10 protein:protease; 18 h at 4 °C, 4 h at 25 °C; Boehringer) permitted unambiguous N-terminal sequence data to be obtained, starting with Asp^2 in each case (data not shown).

Most components were, however, the result of a number of variable processing events and thus could not be directly assigned. Therefore, in order to determine more rigorously for individual components (1) polypeptide cleavage site, (2) number of *N*-glycosylation sites occupied, and (3) overall *N*-glycan monosaccharide composition (as summarized in Figs. 1B, 2B, and 3B), we employed simple analyses of released *N*-glycans to provide an integrated analysis of the different IFN-γ populations.

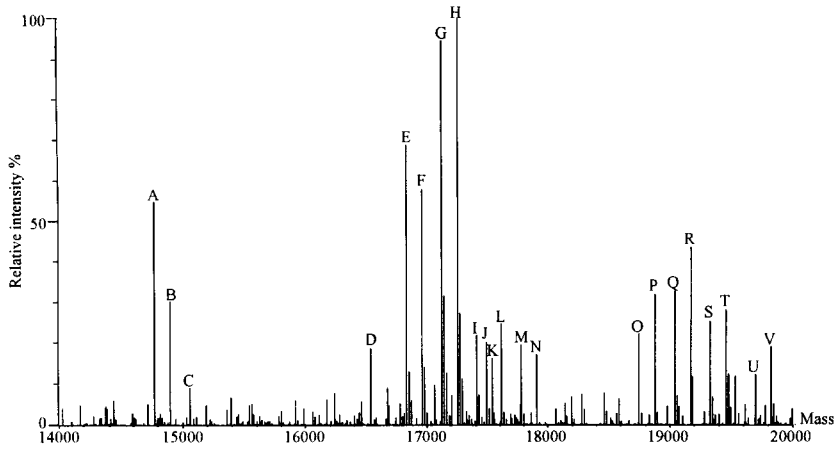
Complex ESI-MS analyses of recombinant IFN-γ glycoproteins can be interpreted using MALDI-MS and HPLC analyses of N-glycans

Sialylated and desialylated *N*-glycan pools were released from recombinant human IFN-γ proteins by digestion with PNGase F, with or without prior treatment with neuraminidase. Desialylated oligosaccharides were analyzed by MALDI-MS (Fig. 4) using DHB as matrix, yielding $[M+Na]^+$ ions (Harvey, 1993). The mass accuracy of this technique (±0.1%, ±1 Da/1 kDa) permitted the observed mass of a particular desialylated *N*-glycan to be assigned a single monosaccharide composition, as listed in Table 1. These assignments assume that *N*-glycan biosynthesis in the host cells has conformed with the basic eukaryotic biosynthetic pathway (Kornfeld & Kornfeld, 1985) and that a chitobiose core ($HexNAc_2$) is therefore present.

The mass and concomitant monosaccharide composition of CHO cell derived IFN-γ *N*-glycans were consistent with those of bi-, tri-, and tetra-antennary complex oligosaccharides with or without a deoxyhexose (fucose) residue. In comparison, *N*-glycans derived from the Sf9 insect cells were truncated, with monosaccharide compositions consistent with tri-mannosyl core and oligomannose oligosaccharides. Transgenic mouse derived *N*-glycans were a mixture of the above. No *N*-glycan mass was consistent with the presence of pentose monosaccharides, sulfated groups, or uronic acids.

These structural assignments were confirmed by cation-exchange HPLC analysis of 2-AB labeled, sialylated *N*-glycans (Fig. 5). CHO cell derived oligosaccharides were neutral (asialo) molecules or were mono, di-, tri-, or tetra-sialylated. Transgenic mouse derived oligosaccharides were neutral, mono-, or di-sialylated, whereas all Sf9 insect cell derived oligosaccharides were neutral. These analyses confirm previous site-specific analyses of CHO, transgenic mouse, and Sf9 IFN-γ glycosylation in this laboratory by exoglycosidase array sequencing of glycopeptides using MALDI-MS (James et al., 1995). Furthermore, we have previously determined, by derivatization of released sialic acids with the fluorophore DMB, followed by HPLC analysis (method of Hara et al., 1989), that >96% of sialic acids associated with the various recombinant IFN-γ species are *N*-acetylneuraminic

A



B

Component	Observed mass (Da)[a]	Correlating mass (Da)[b]	Polypeptide length	Asn sites occupied	Monosaccharide composition[c]
A	14772.5	14771.8	1-127	0	
B	14900.9	14900.0	1-128	0	
C	15056.0	15056.1	1-129	0	
D	16539.9	16541.4	1-127	1	H5 N4 D1
E	16833.0	16832.7	1-127	1	H5 N4 S1 D1
F	16960.8	16960.9	1-128	1	H5 N4 S1 D1
G	17123.7	17123.9	1-127	1	H5 N4 S2 D1
H	17251.4	17252.1	1-128	1	H5 N4 S2 D1
I	17408.3	17408.3	1-129	1	H5 N4 S2 D1
J	17489.2	17488.5	1-132	1	H5 N4 S1 D1
		17489.3*	1-127	1	H6 N5 S2 D1
K	17536.9	17536.5	1-130	1	H5 N4 S2 D1
		17538.5	1-130	1	H7 N6
L	17617.9	17616.6	1-133	1	H5 N4 S1 D1
		17617.3	1-124	2	H10 N6 S1
		17617.4*	1-128	1	H6 N5 S2 D1
M	17780.3	17779.7	1-132	1	H5 N4 S2 D1
		17780.5*	1-127	1	H6 N5 S3 D1
N	17906.9	17907.9*	1-133	1	H5 N4 S2 D1
		17908.7	1-128	1	H6 N5 S3 D1
O	18747.9	18747.4*	1-127	2	H10 N8 S2 D1
		18749.4	1-127	2	H12 N10
P	18874.9	18875.6	1-128	2	H10 N8 S2 D1
Q	19038.4	19038.7*	1-127	2	H10 N8 S3 D1
		19039.0	1-134	2	H11 N7 D1
		19039.9	1-132	2	H11 N9
R	19166.6	19166.9*	1-128	2	H10 N8 S3 D1
		19168.0	1-133	2	H11 N9
S	19328.2	19329.9	1-127	2	H10 N8 S4 D1
T	19456.1	19454.3*	1-130	2	H12 N10 D2
		19458.1	1-128	2	H10 N8 S4 D1
U	19696.2	19695.3	1-127	2	H11 N9 S4 D1
		19695.6	1-134	2	H12 N8 S1 D1
		19696.5*	1-132	2	H12 N10 S1
		19697.3	1-127	2	H13 N11 S2
V	19823.3	19822.6	1-133	2	H10 N8 S3 D1
		19823.5*	1-128	2	H11 N9 S4 D1
		19824.6	1-133	2	H12 N10 S1

Fig. 1. ESI-MS analysis of recombinant human IFN-γ produced by CHO cells. Approximately 50 pmol (1 μg) of protein was used for analysis. **A:** Deconvoluted (zero-charge, centroided data) spectrum obtained after data processing by maximum-entropy analysis. **B:** Integrated analysis of individual IFN-γ components using the data presented in Table 1 and Figure 5. [a]Components with a relative intensity of less than 10% were not included in this analysis. [b]Correlating mass refers to the average molecular mass of an IFN-γ species having the specified polypeptide length and monosaccharide composition calculated as described in the text. Correlating masses outside a mass accuracy of ±0.01% (±2.0 Da/20 kDa) are indicated by the extent of the difference in parentheses. NA indicates no possible correlating mass. Where there is more than one correlating mass for an observed mass, closest mass matches are indicated by an asterisk. [c]Monosaccharide symbols: H, hexose; N, *N*-acetylhexosamine; S, *N*-acetylneuraminic acid; D, deoxyhexose. The number of residues of each monosaccharide is indicated after each one-letter symbol.

acid, with only small proportions of *N*-glycolylneuraminic acid (unpublished data).

We can therefore infer that an individual *N*-glycan associated with a recombinant IFN-γ polypeptide, shown to be present by MALDI-MS, may be nonsialylated or may be variably sialylated to a degree determined by the core monosaccharide composition of that oligosaccharide. For example, a desialylated oligosaccharide of composition Hex_5 $HexNAc_4$ can be either a biantennary or hybrid core structure and may thus be associated with either zero, one, or two sialic acid molecules. Larger glycans could be neutral or associated with up to four Neu5Ac residues.

Consequently, for the recombinant IFN-γ populations derived from each expression system, a list of neutral and/or variably sialylated *N*-glycan molecules was devised, based on the MALDI-MS (Table 1) and 2-AB labeled oligosaccharide analyses (Fig. 5). As IFN-γ may exist as 0*N*, 1*N*, or 2*N* site-occupancy variants, all possible combinations of *N*-glycan masses were calculated (for IFN-γ from a single expression system) from the experimental data above using a simple two-dimensional matrix spreadsheet (matrix 1). These data were employed to assign polypeptide cleavage site, *N*-glycosylation site occupancy, and overall monosaccharide composition for individual major components of the maximum entropy processed ESI-MS spectra of intact IFN-γ proteins. Components with an intensity of less than 10% relative to the most intense component were not included in these analyses. The sum of the relative intensities of these minor components did not exceed 30% of the total in any of the deconvoluted ESI-MS analyses. Furthermore, no minor component exceeded 1.0% of the total component intensity in any of these analyses.

A simple two-dimensional matrix spreadsheet (matrix 2) was used to calculate mass differences between the observed mass of each component of a spectrum and the theoretical masses of

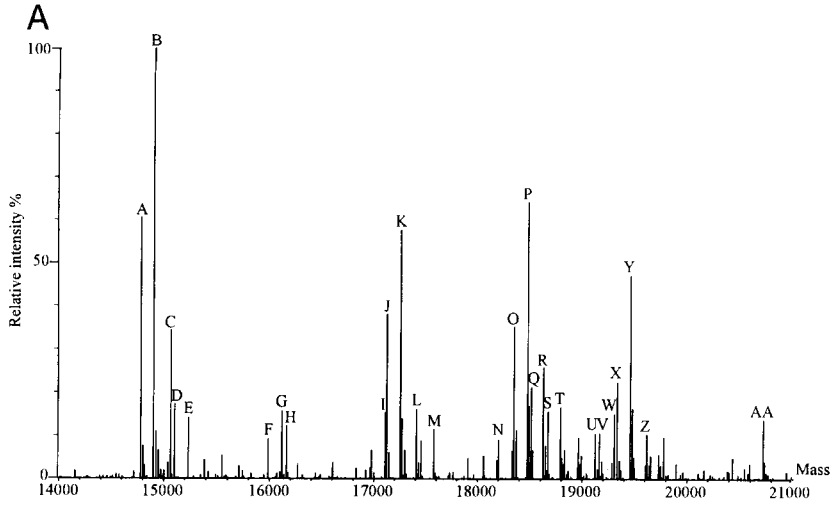


Fig. 2. ESI-MS analysis of recombinant human IFN-γ produced by the mammary gland of transgenic mice. Details as for Figure 1.

B

Component	Observed mass (Da)	Correlating mass (Da)	Polypeptide length	Asn sites occupied	Monosaccharide composition
A	14772.5	14771.8	1-127	0	
B	14900.4	14900.0	1-128	0	
C	15056.8	15056.1	1-129	0	
D	15096.9	NA			
E	15224.3	NA			
F	15988.3	15988.9	1-127	1	H5 N2
G	16116.5	16117.1	1-128	1	H5 N2
H	16160.4	16160.5	1-138	0	
I	17107.0	17106.0	1-128	1	H5 N4 S2
J	17124.4	17123.9	1-127	1	H5 N4 S2 D1
K	17253.0	17252.1	1-128	1	H5 N4 S2 D1
L	17409.1	17408.3	1-129	1	H5 N4 S2 D1
M	17576.0	NA			
N	18195.6	18194.9	1-127	2	H10 N6 S2
O	18341.0	18341.0	1-127	2	H10 N6 S2 D1
P	18470.0	18469.2	1-128	2	H10 N6 S2 D1
Q	18511.9	18512.6	1-138	1	H5 N4 S2 D1
R	18627.2	18625.4	1-129	2	H10 N6 S2 D1
S	18668.8	18668.8	1-139	1	H5 N4 S2 D1
T	18793.8	NA			
U	19124.3	19125.0	1-133	2	H10 N6 S2 D1
V	19165.5	19166.9	1-128	2	H10 N8 S3 D1
W	19312.8	19312.0	1-128	2	H10 N8 S4
		19313.0*	1-128	2	H10 N8 S3 D2
X	19329.2	19329.9	1-127	2	H10 N8 S4 D1
Y	19458.6	19458.1	1-128	2	H10 N8 S4 D1
Z	19614.7	19614.3	1-129	2	H10 N8 S4 D1
AA	20719.0	20718.6	1-138	2	H10 N8 S4 D1

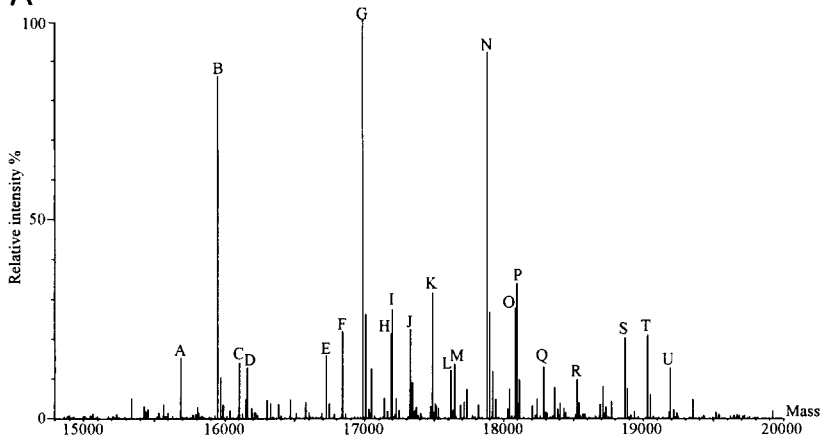
IFN-γ polypeptide variants progressively C-terminally truncated, starting with the full-length polypeptide (1-143) to a polypeptide consisting of amino acids 1-120. Thus, for each component, a sequence of potential carbohydrate masses was generated, each differing by the mass of one extra amino acid from the C-terminus of IFN-γ. By comparison of each potential carbohydrate mass calculated for a component in matrix 2 with the possible combinations of actual oligosaccharide masses in matrix 1, it was possible to identify oligosaccharide mass values consistent with cleavage of the polypeptide backbone at a particular site. A mass accuracy of ±0.01% (±2 Da/20 kDa) was assumed. Observed masses either correlated with a polypeptide mass alone (indicating no glycosylation), correlated with the mass of a polypeptide plus one *N*-glycan (indicating occupation of one Asn site), or correlated with a polypeptide plus a combination of two *N*-glycan masses (indicating occupation of both Asn sites). For each oligosaccharide mass there was an associated monosaccharide composition, permitting assignment of an overall monosaccharide composition. In some cases there was more than one possible combination of cleavage site and monosaccharide composition for a given observed mass. These mass degeneracies are included in the interpretations of the ESI-MS analyses of CHO cell, transgenic mouse, and Sf9 insect cell derived IFN-γ populations, as summarized in Figures 1B, 2B, and 3B, respectively.

Recombinant human IFN-γ proteins produced by different host cells differ in glycosylation and proteolytic processing

IFN-γ produced by Chinese hamster ovary cells

CHO cell derived IFN-γ components could be separated into three major groups differing in number of Asn sites occupied (Fig. 1). Fifty percent of analyzed components could be assigned a single monosaccharide composition and polypeptide cleavage site unambiguously. Where only one Asn site was occupied (known to be Asn^{25} from previous work; James et al., 1995), nearly all possible bi-antennary (Hex_5 $HexNAc_4$) and possible tri-antennary (Hex_6 $HexNAc_5$) *N*-glycans were sialylated and associated with one deoxyhexose monosaccharide (presumably fucose). However, where two Asn sites were occupied (Asn^{25} and Asn^{97}), possible monosaccharide compositions indicated variably sialylated and fucosylated *N*-glycans, confirming differences in glycan processing events at Asn^{97} and Asn^{25} sites, as indicated by previous site-specific analyses in this laboratory

A



B

Component	Observed mass (Da)	Correlating mass (Da)	Polypeptide length	Asn sites occupied	Monosaccharide composition
A	15687.9	15686.9	1-134	0	
B	15948.1	15947.3	1-136	0	
C	16103.9	16103.4	1-137	0	
D	16161.5	16160.5	1-138	0	
E	16726.0	16725.7	1-130	1	H7 N2
		16725.9*	1-134	1	H3 N2 D1
F	16840.5	16839.0	1-135	1	H3 N2 D1
		16840.1*	1-136	1	H3 N2
G	16986.9	16986.2	1-136	1	H3 N2 D1
H	17190.8	17191.1*	1-129	2	H6 N5 D1
		17189.4	1-136	1	H3 N3 D1
I	17200.3	17199.5*	1-137	1	H3 N3
		17199.5	1-138	1	H3 N2 D1
J	17327.6	17326.5	1-136	1	H6 N2
		17329.3	1-131	2	H6 N5
K	17487.8	17487.5*	1-133	2	H6 N4 D1
		17488.6	1-136	1	H7 N2
L	17619.3	17618.5	1-130	2	H10 N4
		17618.7	1-134	2	H6 N4 D1
		17619.6*	1-132	2	H6 N6
M	17647.1	17646.8	1-137	1	H6 N2
N	17879.4	17878.0	1-135	2	H6 N4 D2
		17878.9	1-134	2	H6 N6
		17879.0*	1-136	2	H6 N4 D1
O	18083.7	18082.2	1-136	2	H6 N5 D1
		18083.0*	1-131	2	H11 N4 D1
P	18092.6	18092.3	1-137	2	H6 N5
		18092.3	1-138	2	H6 N4 D1
Q	18286.5	18286.2*	1-131	2	H11 N5 D1
		18285.4	1-136	2	H6 N6 D1
R	18526.7	18527.6	1-136	2	H10 N4 D1
S	18867.6	18867.9*	1-136	2	H13 N4
T	19028.1	19028.2*	1-141	2	H6 N6 D2
		19030.0	1-136	2	H14 N4
U	19189.3	19192.2 (2.9)	1-136	2	H15 N4

Fig. 3. ESI-MS analysis of recombinant human IFN-γ produced by baculovirus-infected Sf9 insect cells. Details as for Figure 1.

by exoglycosidase array sequencing (James et al., 1995). No components appeared to be associated with oligomannose type oligosaccharides, where the ratio of Hex:HexNAc would exceed 1.5:1. *N*-glycan heterogeneity may not have been entirely due to intracellular processing alone. Gramer and Goochee (1993) observed sialidase and other glycosidase activities in CHO cell lysate and cell culture supernatant. Extracellular digestion may therefore account for some variations in glycosylation.

The ESI-MS data indicate that the majority of CHO cell derived IFN-γ polypeptides terminate between Gly^{127} and Gln^{133} (Fig. 6); no full-length molecules were observed. Proteolytic cleavages in this region of the molecule were confirmed by MALDI-MS analysis of the polypeptide backbone deglycosylated by PNGase F (Fig. 7). However, there is a significant difference between the observed masses of the nonglycosylated polypeptides in Figure 1B (polypeptides with molecular masses of 14,772.5, 14,900.9, and 15,056.0 Da; components A–C) and the masses of the major polypeptides resulting from deglycosylation of CHO derived IFN-γ by PNGase F, shown in Figure 7 (polypeptides with an *m/z* for singly protonated molecular ions of 15,058, 15,186, and 15,343). Furthermore, IFN-γ species with only one site occupied, Asn^{25} (components D–I), were all truncated between Gly^{127} and Arg^{129}, similar to the nonglycosylated components. IFN-γ molecules with two Asn sites occupied (components O–V) may be less susceptible to proteolysis at Gly^{127}-Arg^{129} (there are multiple mass correlations with components Q, R, T, U, V). However, this discrepancy, which did not arise with either the transgenic mouse or Sf9 insect cell data sets, cannot be adequately explained. Indeed, recent evidence suggests that *N*-glycans at Asn^{25}, and not Asn^{97}, of recombinant human IFN-γ are critical for protease resistance (Sareneva et al., 1995).

Curling et al. (1990) have demonstrated that proteolysis of IFN-γ occurs prior to secretion from CHO cells. The observed polypeptides may be a result of initial cleavage at the dibasic C-terminal sequence Lys^{128}-Arg^{129}-Lys^{130}-Arg^{131} of human IFN-γ by the subtilisin-related endoprotease furin (PACE). Furin cleaves C-terminal of the recognition sequence (Lys/Arg)-X-(Lys/Arg)-Arg (Watanabe et al., 1992) and is known to be present in the constitutive secretory pathway of mammalian cells (Smeekens, 1993). The endoprotease is active in the Golgi com-

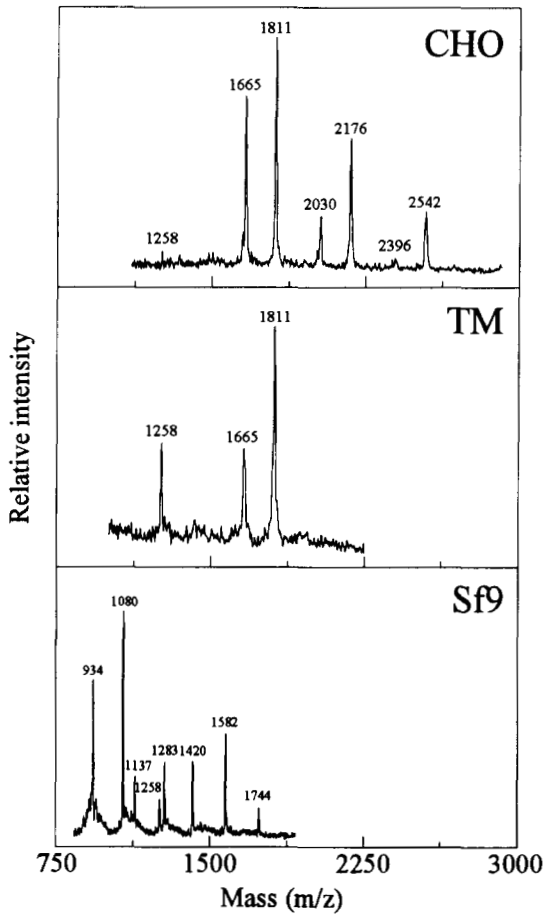


Fig. 4. MALDI-MS analysis of desialylated *N*-glycans from recombinant human IFN-γ proteins. CHO, *N*-glycans derived from Chinese hamster ovary cells; TM, *N*-glycans derived from the mammary gland of transgenic mice; Sf9, *N*-glycans derived from baculovirus-infected insect cells. *N*-glycans are $[M+Na]^+$ ions; *m/z* values are indicated.

Table 1. *Monosaccharide composition of desialylated N-glycans identified by MALDI-MS (Fig. 4)*[a]

IFN-γ expression system	Observed mass (Da), $[M+Na]^+$	Theoretical mass (Da), $[M+Na]^+$	Monosaccharide composition[b]
CHO	1,258	1,258.1	H5 N2
	1,665	1,664.5	H5 N4
	1,811	1,810.6	H5 N4 D1
	2,030	2,029.8	H6 N5
	2,176	2,175.9	H6 N5 D1
	2,396	2,395.1	H7 N6
	2,542	2,541.3	H7 N6 D1
TM	1,258	1,258.1	H5 N2
	1,665	1,664.5	H5 N4
	1,811	1,810.6	H5 N4 D1
Sf9	934	933.8	H3 N2
	1,080	1,079.9	H3 N2 D1
	1,137	1,137.0	H3 N3
	1,258	1,258.1	H5 N2
	1,283	1,283.1	H3 N3 D1
	1,420	1,420.2	H6 N2
	1,582	1,582.4	H7 N2
	1,744	1,744.5	H8 N2

[a] CHO, Chinese hamster ovary cells; TM, mammary gland of transgenic mice; Sf9, baculovirus-infected Sf9 insect cells. Monosaccharide compositions were deduced using an algorithm prepared by Dr. David Harvey, Glycobiology Institute, University of Oxford, Oxford, UK.

[b] Monosaccharide symbols: H, hexose; N, *N*-acetylhexosamine; D, deoxyhexose. The number of residues of each monosaccharide is indicated after each one-letter symbol.

plex (Rehemtulla et al., 1992) and has been identified in CHO cells (Hatsuzawa et al., 1992). Further removal of basic arginine and lysine residues may be the result of intra- or extracellular digestion by serine endoproteases and/or carboxypeptidases.

IFN-γ produced by the mammary gland of transgenic mice

The limited variation in carbohydrates associated with the transgenic mouse derived IFN-γ (Fig. 4; Table 1) permitted assignment of a single monosaccharide composition and polypeptide cleavage site for nearly all components (Fig. 2). Modifications of the transgenic mouse derived IFN-γ were generally similar to those of the CHO cell derived IFN-γ. A number of minor components (D, E, M, T) could not be assigned; the reason for this is unclear. However, while this analysis can only be regarded as semiquantitative, these data suggest that most IFN-γ molecules expressed in this system are either nonglycosylated or glycosylated at two Asn sites. In addition, some *N*-glycans associated with major components (O, P) had a relatively high Hex:HexNAc ratio (1.67:1), suggesting the presence of oligomannose or hybrid *N*-oligosaccharides. Other major components (J, K, Y) were likely to be associated with complex *N*-glycans.

The majority of transgenic mouse derived IFN-γ polypeptides were truncated to approximately the same extent as CHO cell derived IFN-γ (terminating at Gly^{127}, Lys^{128}, or Arg^{129}), indicating that a similar proteolytic mechanism may be operating. However, there was no major difference between the molecular weights of IFN-γ polypeptides calculated using the ESI-MS data (Fig. 2) and those determined by the MALDI-MS analysis of PNGase F deglycosylated polypeptides (Fig. 7), indicating that glycosylation does not appear to affect proteolytic processing of the transgenic mouse derived IFN-γ. Some minor components were truncated at Gly^{138} (H, Q, AA); the reason for this is again unclear.

IFN-γ produced by baculovirus-infected Sf9 insect cells

Processing of recombinant IFN-γ in the Sf9 insect cells was relatively restricted. As for CHO cell derived IFN-γ, Sf9 insect cell derived IFN-γ components exhibited variable Asn site occupancy. Where present, possible *N*-glycans were combinations of the neutral, truncated oligosaccharides listed in Table 1. These data confirm other studies that demonstrate that the glycosylation of recombinant proteins in Sf9 insect cells results in oligomannose type *N*-glycans (Voss et al., 1993; Manneberg et al., 1994). However, the ability of insect cells to perform complex glycosylation may be dependent on the stage of the baculovirus infection cycle (Davidson & Castellino, 1991) and the insect cell type. Licari et al. (1993) have also found exoglycosidase activities in insect cell lysates and cell-free supernatants, including those from Sf9 cell cultures.

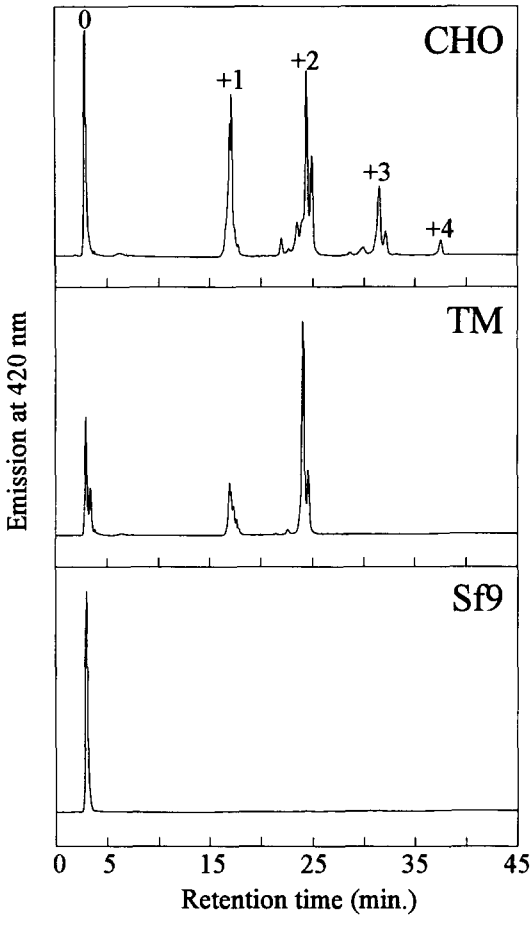


Fig. 5. Analysis of 2-aminobenzamide labeled sialylated *N*-glycans from recombinant human IFN-γ proteins by cation-exchange HPLC. Labeled *N*-glycans separate into peak groups designated as neutral (0), mono-sialylated (+1), di-sialylated (+2), tri-sialylated (+3), and tetra-sialylated (+4). CHO, *N*-glycans derived from Chinese hamster ovary cells; TM, *N*-glycans derived from the mammary gland of transgenic mice; Sf9, *N*-glycans derived from baculovirus-infected insect cells.

In contrast to the recombinant IFN-γ polypeptides produced by the mammalian cells, IFN-γ polypeptides from Sf9 insect cells terminated between Met^{134} and Gly^{138}. The nonglycosylated components (A–D) were all truncated in this region, and the main components (B, G, N) all exhibited possible C-terminal cleavages in this part of the molecule. This was confirmed by the MALDI-MS analysis of deglycosylated polypeptide shown in Figure 7, which showed a single major signal with an *m/z* for the singly protonated molecular ion of 15,989, approximately corresponding to a polypeptide terminating at Phe^{136}. It is therefore unlikely that polypeptides outside this range exist. There may be two explanations for proteolysis in this part of the IFN-γ molecule: (1) cleavage by endoproteases and/or carboxypeptidases released upon lysis of insect cells as a result of the baculovirus infection cycle, and (2) cleavage by a specific proprotein endoprotease during secretion. The C-terminal sequence Arg^{137}-Gly^{138}-Arg^{139}-Arg^{140} may be recognized by an insect homologue of mammalian furin, such as that identified in *Drosophila* (Roebroek et al., 1992). The high concentration of basic residues in this part of the IFN-γ sequence suggests that a trypsin-like endoprotease may be responsible, as suggested for C-terminal cleavages of mouse interleukin-3 expressed in baculovirus infected silkworm larvae (Knepper et al., 1992). However, this mechanism does not explain truncations C-terminal of Phe^{136} and the absence of cleavages in the region Lys^{128} to Arg^{131}.

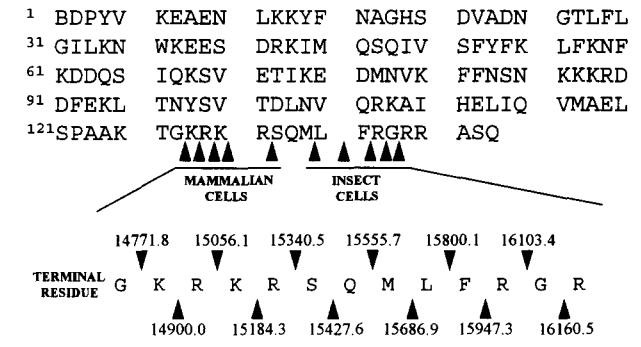


Fig. 6. Schematic representation of the human IFN-γ amino acid sequence with an expanded C-terminal region illustrating cleavage of the recombinant protein produced in mammalian and insect cells. The calculated average masses of polypeptides terminating in this region are shown. B, pyroglutamic acid.

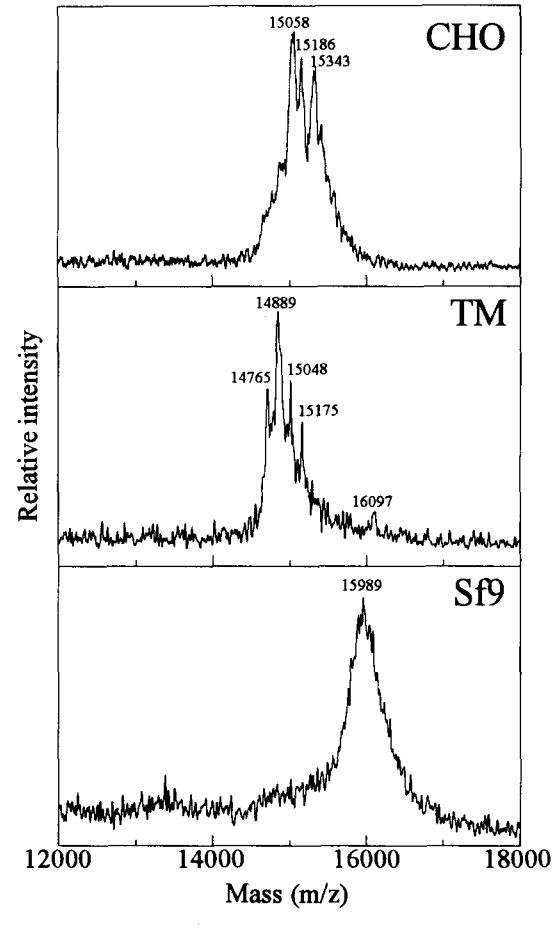


Fig. 7. Analysis of deglycosylated recombinant human IFN-γ proteins by MALDI-MS. CHO, IFN-γ produced by Chinese hamster ovary cells; TM, IFN-γ produced by the mammary gland of transgenic mice; Sf9, IFN-γ produced by baculovirus-infected Sf9 insect cells. Spectra were obtained using ±10 pmol of polypeptide in each case. IFN-γ polypeptides are $[M+H]^+$ ions; *m/z* values are indicated.

Conclusions

The mass accuracy and resolution of ESI-MS in combination with maximum-entropy data analysis confirmed a correct primary structure for each recombinant IFN-γ, including an N-terminal pyroglutamic acid residue. However, all recombinant IFN-γ populations consisted of molecules with C-terminal "ragged ends." Natural human IFN-γ isolated from peripheral blood lymphocytes has also been shown to have an N-terminal pyroglutamic acid residue and six different C-termini (Gly^{127}, Lys^{128}, Arg^{129}, Lys^{130}, Ser^{132}, Met^{134}; Rinderknecht et al., 1984). Thus, both natural IFN-γ and recombinant IFN-γ from mammalian cells are subject to similar polypeptide processing events.

Insect cell derived IFN-γ also had an intact N-terminus (Pyr), despite reports of N-terminally cleaved recombinant proteins produced by baculovirus-infected insect cell expression systems (e.g., Grabenhorst et al., 1993), possibly due to the activity of a baculovirus-encoded cathepsin L-like cysteine protease (Slack et al., 1995). However, as shown in the present study, other reports also describe C-terminal truncations of insect cell derived recombinant proteins, such as interferon-α2 (Sugiyama et al., 1993), ascribed to "tryptic activity."

It should be stated, however, that proteolytic cleavage events should be considered to be recombinant protein specific. Whereas IFN-γ may be particularly susceptible to proteolysis, other recombinant proteins produced by CHO cells or by the mammary gland of transgenic animals, such as tissue plasminogen activator (Chloupek et al., 1989) or α1-antitrypsin (Wright et al., 1991) are recovered as intact, bioactive molecules similar to their natural counterparts.

We have shown that both glycosylation and proteolysis of the same recombinant protein produced in different animal expression systems is not uniform and is dependent on the host cell type. In fact, all three recombinant IFN-γ populations differed in proteolysis, Asn site occupancy, and *N*-glycan processing. However, it is the latter modification that can best be described as host cell specific. This study therefore emphasizes the need for rigorous analysis of potential recombinant protein products during bioprocess development.

In general, this phenomenon would have implications for the potential use of recombinant IFN-γ as a therapeutic agent. For example, based on our understanding of the molecular requirements for IFN-γ bioactivity, we can predict the behavior of our recombinant preparations in vivo. An intact C- and N-terminus is known to be required for full IFN-γ bioactivity (reviewed by Farrar & Schreiber, 1993). In addition, work with IFN-γ C-terminal deletion mutants has demonstrated that residues Lys^{130}-Arg^{131}-Ser^{132} are crucial for receptor binding (Lundell et al., 1991). Thus, we can predict that the recombinant product derived from mammalian cells is likely to be 10–1,000-fold less active than the full-length protein (Arakawa et al., 1986). The insect cell IFN-γ is likely, however, to be more active than its mammalian counterparts. Indeed, recombinant human IFN-γ produced by baculovirus infected insect cells has previously been shown to be as active as natural human leukocyte IFN-γ in antiviral assays (Sareneva et al., 1994). The molecular interactions between IFN-γ and its receptor have recently been comprehensively examined (Walter et al., 1995) by crystallization of the receptor-ligand complex.

Furthermore, the half-life of the IFN-γ proteins in vivo may differ markedly as a result of different *N*-glycan processing. IFN-γ molecules carrying *N*-glycans with terminal mannose or galactose residues are likely to be cleared more rapidly by macrophage mannose receptors and liver asialoglycoprotein receptors (Drickamer, 1991). Thus, we may expect the insect cell derived IFN-γ to be cleared more rapidly than the IFN-γ proteins produced by mammalian cells. Sareneva et al. (1993) have demonstrated that insect cell derived recombinant human IFN-γ is cleared from the rabbit circulation more rapidly than either natural IFN-γ (complex type *N*-glycans) or a mutated nonglycosylated recombinant IFN-γ.

In conclusion, the minimal bioactivity of mammalian cell derived recombinant IFN-γ and the suboptimal pharmacokinetic behavior of insect cell derived IFN-γ would likely preclude their clinical use. In fact, it is recombinant IFN-γ produced by *Escherichia coli* (Actimmune™) that is employed therapeutically.

Materials and methods

Expression systems

A mutant CHO-K1 cell line lacking dihydrofolate reductase ($DHFR^-$) was co-transfected with the genes for IFN-γ and DHFR by The Wellcome Foundation (Beckenham, Kent, UK). This cell line was adapted for growth in serum-free medium in this laboratory (Curling et al., 1990). IFN-γ and DHFR gene copy numbers were amplified using methotrexate (1 μM). Clarified culture supernatant from 5-day-old batch cultures of recombinant CHO cells containing approximately 1 μg/mL IFN-γ was used for subsequent purification.

A hybrid gene consisting of the sheep β-lactoglobulin promoter region (4.7 kb) and the structural part of the human IFN-γ genomic gene (5.5 kb) was used for the production of transgenic mice, as described by Dobrovolsky et al. (1993). The transgenic mouse milk used in this study contained approximately 400 μg/mL IFN-γ. Milk samples were diluted 1:1.5 with PBS and defatted by repeated centrifugation at 12,000 × *g* prior to purification.

Spodoptera frugiperda (Sf9) insect cells were infected by a recombinant baculovirus kindly provided by The Wellcome Foundation. The vector carried human IFN-γ cDNA under the control of the late baculovirus polyhedrin gene promoter. Sf9 cells at a density of 1×10^6 cells/mL were infected at a multiplicity of infection of 5. The infected cells were incubated at 26 °C for 3 days prior to clarification of the supernatant by centrifugation.

Purification of IFN-γ proteins

All IFN-γ-containing samples were adjusted to PBS, 0.02% (w/v) NaN_3, pH 7.2 (buffer 1), and loaded directly onto a 1 × 1-cm immunoaffinity column (Sepharose linked to MAb 20B8 raised against *E. coli* derived recombinant IFN-γ; provided by Celltech Ltd., Slough, UK) under gravity; then, unbound proteins were washed off with 20 mL of buffer 1. Bound IFN-γ was eluted with 3 mL of 0.1 M Gly-HCl, pH 2.5, immediately neutralized with 0.3 mL of 1 M Tris-HCl, pH 8.0, then concentrated and desalted by ultrafiltration with Centricon®-10 concentrators (Amicon Ltd., Stonehouse, UK) prior to lyophilization and storage at −20 °C. Monoclonal antibody 20B8 has been shown to be specific for the IFN-γ polypeptide by epitope mapping (data not shown); therefore, this immunoaffinity purification is independent of *N*-glycans associated with IFN-γ. Recombinant

human IFN-γ prepared by this method was >98% pure, as determined by analysis of silver-stained SDS-polyacrylamide gels (data not shown).

Protein determinations

Protein assays were performed with an assay kit supplied by Bio-Rad Laboratories Ltd. (Hemel Hempstead, UK). Bovine serum albumin was used as standard.

Preparation of released N-glycans and deglycosylated IFN-γ protein

To prepare desialylated *N*-glycans, approximately 500 μg of recombinant IFN-γ protein was reconstituted in 190 μL of 50 mM sodium acetate buffer, pH 5.0, containing 0.02% NaN_3. Terminal sialic acids were removed by addition of 0.2 units of *Arthrobacter ureafaciens* neuraminidase (Oxford Glycosystems Ltd., Abingdon, UK) in 10 μL of sodium acetate buffer, followed by digestion for 24 h at 37 °C. Desialylated IFN-γ protein was then concentrated to ~100 μL and buffer exchanged into 50 mM NH_4HCO_3, pH 8.1, by repeated ultrafiltration with Microcon™ centrifugal concentrators (Amicon Ltd., Stonehouse, UK).

Prior to digestion with PNGase F (recombinant *Flavobacterium meningosepticum* produced in *E. coli*; Oxford Glycosystems Ltd.), approximately 500 μg of sialylated or desialylated IFN-γ protein was denatured by addition of 8 μL of 10% (w/v) SDS and boiling for 2 min. Proteins were then digested at 30 °C for 18 h in a final volume of 200 μL, containing 0.5% (v/v) Nonidet P-40 (BDH, Poole, UK), 0.1% (w/v) SDS, 20 units of PNGase F (desalted by repeated ultrafiltration with Microcon™ centrifugal concentrators), and 50 mM NH_4HCO_3, pH 8.1.

Released sialylated or desialylated *N*-glycans were separated from deglycosylated IFN-γ polypeptide and detergents by RP-HPLC with a Waters 616 system (Waters Ltd., Watford, UK). The entire digestion mixture was loaded onto a Waters Delta-Pak™ C_{18} column (300 Å, 5 μm, 2 × 150 mm) at a flow rate of 0.2 mL/min. *N*-glycans were collected in the first 8 mL of column flow-through in solvent A (0.05% TFA in H_2O), then concentrated and desalted by lyophilization. Deglycosylated IFN-γ protein was eluted with a linear gradient of 100% solvent A to 100% solvent B (0.05% TFA, 80% acetonitrile in H_2O) over 30 min and monitored at 210 nm with a Waters 486 detector. By this procedure, IFN-γ polypeptide co-eluted with Nonidet-P40 detergent after 25 min, followed by elution of excess SDS after 40 min. A fraction of the deglycosylated IFN-γ and Nonidet P-40 mixture, containing ~100 μg of protein, was diluted with an equal volume of H_2O and subjected to further C_{18} RP-HPLC to separate the polypeptide from the Nonidet P-40 and trace SDS with a linear gradient of solvent A to solvent B over 45 min at a flow rate of 0.2 mL/min. Eluted IFN-γ polypeptide was then adjusted to 40% acetonitrile in H_2O and concentrated to ~100 μL (~1 mg/mL) with Microcon™ centrifugal concentrators.

Analysis of 2-AB labeled sialylated N-glycans by HPLC

Sialylated *N*-glycans released from recombinant IFN-γ proteins were reductively aminated at the reducing terminus with the fluorophore 2-aminobenzamide (excitation 330 nm, emission 420 nm) using a kit supplied by Oxford Glycosystems Ltd. (Abingdon, UK). All procedures were carried out according to the manufacturer's instructions. 2-AB labeled *N*-glycans were separated into neutral, mono-, di-, tri-, or tetra-sialylated structures by HPLC with a Glycosep™ C cation-exchange column (4.6 × 100 mm; Oxford Glycosystems Ltd.). By this procedure, neutral oligosaccharides elute in the void volume, and charged *N*-glycans are progressively eluted using a linear gradient of 100% solvent A (20% acetonitrile, 80% H_2O) to 100% solvent B (20% acetonitrile, 80% 250 mM ammonium formate, pH 4.5) at a flow rate of 0.3 mL/min over 35 min. Oligosaccharide elution was monitored with a Waters 474 scanning fluorescence detector.

Matrix-assisted laser desorption/ionization mass spectrometry

All MALDI-MS spectra were obtained with a VG TofSpec (VG Organic, Manchester, UK). Aqueous solutions of desialylated *N*-glycans released by PNGase F were analyzed using DHB (Aldrich Chemical Co. Ltd., Dorset, UK) as matrix by the method of Harvey (1993). Spectra were externally calibrated using an equimolar (25 mM) mixture of asialo, galactosylated triantennary ($[M+Na]^+$ = 2,029.8 Da) and β-mannosyl, α-fucosyl chitobiose ($[M+Na]^+$ = 755.7 Da) oligosaccharides (Oxford Glycosystems Ltd.). Deglycosylated IFN-γ polypeptides were analyzed using "super-DHB" as matrix by the method of Tsarbopoulos et al. (1994). Spectra were externally calibrated using horse heart myoglobin (16,951.5 Da; Zaia et al., 1992) supplied by Sigma Chemical Co. (Poole, UK). Polypeptides were adjusted to a concentration of ±20 pmol μL^{-1} and mixed 1:1 with matrix solution prior to analysis. In both cases, ions desorbed by pulses (1-Hz repetition rate) of light from a N_2 laser at 337 nm were accelerated either at 20,000 V potential (*N*-glycans) or 30,000 V potential (deglycosylated IFN-γ) in positive ion mode. After a linear flight path of 0.65 m, ions were detected by a micro-channel plate detector (2,000 V applied voltage) and digitized at 250 MHz. Observed signals were adjusted so as to obtain maximum peak resolution (optimum signal to noise ratio at minimum laser energy), and the spectra from 20 laser pulses were averaged.

Electrospray ionization mass spectrometry

Spectra were obtained with a VG Quattro II triple quadrupole mass spectrometer (VG Organic, Altrincham, UK) having a mass range for singly charged ions of 4,000 Da. Lyophilized proteins were redissolved in 50% aqueous acetonitrile, 0.2% formic acid to a concentration of 0.1 μg/μL and introduced into the electrospray source at 4 μL/min. The mass-to-charge (m/z) range 600–1,800 Da was scanned at 10 s/scan, and data were summed over 3–10 min, depending on the intensity and complexity of the spectra. During each scan, the sample orifice to skimmer potential (cone voltage) was scanned from 30 V at m/z 600 to 75 V at m/z 1,800. The capillary voltage was set to 3.5 kV. Mass scale calibration employed the multiply charged ion series from a separate introduction of horse heart myoglobin. Molecular weights are based on the following atomic weights of the elements: C = 12.011, H = 1.00794, N = 14.00674, O = 15.9994, S = 32.066. Background subtracted m/z data were processed by software employing a maximum-entropy (MaxEnt) based analysis in order to produce zero-charge protein molecular weight information with optimum signal to noise ratio, resolution, and

mass accuracy (Ferrige et al., 1992). Typical processing times were 30 min using a 60-MHz Pentium PC operating under Windows NT.

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