Role of *N*-glycosylation in the synthesis, dimerization and secretion of human interferon-γ

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Human interferon-γ (IFN-γ) is a secretory glycoprotein, which has two potential *N*-linked glycosylation sites at positions Asn-25 and Asn-97 of its 143 amino acid long mature polypeptide chain. In order to understand the role of glycan residues in the synthesis and secretion of human IFN-γ, both or either one of the potential *N*-linked glycosylation sites were mutated to Gln. The mutant and the wild-type (Wt) polypeptides were expressed in insect cells using a baculovirus vector. Elimination of the *N*-glycosylation site at position Asn-97 (N97Q) resulted in secreted protein yields of 70–90% as compared with the Wt production, whereas only 10–25% (N25Q) and 1–10% (N25Q,N97Q) levels of protein production was observed when the first or both sites were mutated, respectively. Although there was a difference between extracellular levels of produced protein, the kinetics of secretion was similar for all different IFN-γ molecules. The Wt and the *N*-glycosylation site mutants were all secreted as dimers. The formation of biologically active dimers was more efficient for IFN-γ polypeptides that had the intact glycosylation site at Asn-25 as compared with the other two mutant forms of IFN-γ. The extent of dimerization correlated well with the observed secretion. The specific antiviral activity was of the same order (1×10^7 i.u./mg of protein) for the glycosylated IFN-γ molecules, whereas it was slightly lower (0.5×10^7 i.u./mg of protein) for the unglycosylated mutant form.

INTRODUCTION

Interferon-γ (IFN-γ) is an important cytokine that has potent antimicrobial, antiproliferative and immunomodulatory functions (for reviews see Weissmann and Weber, 1986; Joklik 1990; Farrar and Schreiber, 1993). It is produced by T-lymphocytes and NK cells. Physiologically, IFN-γ is induced by specific antigens of $CD8^+$ and $CD4^+$ T-cell populations. Under experimental conditions, direct stimulation of T-cells by specific antibodies, certain mitogens and chemicals leads to the production of IFN-γ. IFN-γ mRNA is found at 6–8 h after stimulation and is followed by accumulation of secreted IFN-γ protein in extracellular fluid several hours later (Farrar et al., 1986). The events in the biogenesis of IFN-γ including the synthesis, folding, dimerization and kinetics of secretion of IFN-γ are poorly understood.

The primary translation product of human IFN-γ consists of 166 amino acids, 23 of which form the amino terminal signal peptide that is cleaved off in the endoplasmic (ER) reticulum (Devos et al., 1982; Gray et al., 1982). The carboxy-terminal end of the molecule is susceptible to posttranslational proteolysis. Several forms of human IFN-γ lacking 9–16 carboxy terminal amino acids have been found (Rinderknecht et al., 1984). The mature polypeptide chain has two potential *N*-linked glycosylation sites at positions 25 and 97. There is considerable heterogeneity in the core glycosylation of IFN-γ and the molecules may contain 0, 1 or 2 glycan residues (Yip et al., 1982; Cantell et al., 1992). The biologically active IFN-γ is a homodimer. There are no cysteine residues in the mature IFN-γ and thus the polypeptide lacks intramolecular disulphide bonds. Recently, the three-dimensional structures of human and rabbit IFN-γ have been resolved (Ealick et al., 1991; Samudzi et al., 1991). The subunits are associated to each other in an antiparallel fashion and the molecules have multiple interactions along the polypeptide chains, forming a compact, relatively globular symmetrical dimeric structure. IFN-γ dimer has two receptor-binding-sites and it can simultaneously bind to two receptor molecules (Greenlund et al., 1993). All four potential *N*-linked glycosylation sites are located on the surface of the IFN-γ dimer (Ealick et al., 1991; Samudzi et al., 1991). Glycosylation is apparently not required for dimerization of the subunits, since recombinant IFN-γ produced in *Escherichia coli* forms dimers which are biologically active (Alton et al., 1983). Glycosylation may, however, protect IFN-γ against cellular proteases and prolong its survival time in the circulation (Cantell et al., 1992; Sareneva et al., 1993). The structure of IFN-γ is very different from other types of interferon (type I IFNs), which are monomers and in most cases lack *N*-linked glycan residues (Weissman and Weber 1986; Joklik et al., 1990; Ealick et al., 1991; Senda et al., 1992).

Owing to the unique features of IFN-γ we have studied the role of *N*-linked glycans in the synthesis, dimerization, secretion and biochemical properties of human recombinant IFN-γ. Four different forms of recombinant IFN-γ, i.e. wild type and three mutants lacking either one or both of the *N*-linked glycosylation sites, were constructed and the corresponding proteins were produced in insect cells.

EXPERIMENTAL

Baculovirus expression vector

The coding sequence of IFN-γ (Devos et al., 1982) was obtained from the plasmid pGm8 (kindly provided by Dr. Charles Weissmann, University of Zürich) by PCR using Taq DNA polymerase (Promega) and oligonucleotide containing *Bam*HI sites in the 5′ chain (CTCTCGGATCCAACGATGAAATATAC) and in the 3′ chain (CAGGCAGGATCCAA-

Abbreviations used: IFN-γ, interferon-γ; GST, glutathione S-transferase; TCA, trichloroacetic acid; Wt, wild type; ER, endoplasmic reticulum; RP, reversed phase.

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CCATTACTGG). The amplified fragment of IFN-γ containing the authentic leader sequence was digested with *Bam*HI and ligated into the *Bam*HI site of the vector pGEM^R-3Zf(+) (Promega). The sequence of the cloned PCR product was verified by DNA sequencing (USB sequenase kit). The IFN-γ cDNA was subcloned into baculovirus expression vector pAcYM1 (Matsuura et al., 1987). Transfer of the IFN-γ gene into the baculovirus genome was accomplished by cotransfection of pAcYM1–IFN-γ and wild-type virus (E2 strain) DNA into Sf9 insect cells using Lipofectin (Gibco/BRL) reagent. Medium from transfected cells was subjected to plaque purification (Summers and Smith, 1986), and occlusion-negative recombinant plaques were obtained by visual screening under a dissecting microscope.

IFN-γ antigen production for immunization

The IFN-γ cDNA was modified by PCR as above, except using oligonucleotide containing *Bam*HI site in the 5′ chain (GGCTGTGGATCCCAGGACCCATATGTAAAA) just prior to the signal peptidase cleavage site. The PCR product was ligated into the *Bam*HI site of the pGEM-3Zf(+) vector, subcloned into the pGEX-2T expression vector (Pharmacia) and transformed into the *E. coli B* strain BL21(DE3) (Studier et al., 1990). Glutathione S-transferase (GST) IFN-γ fusion protein was produced and purified by Glutathione Sepharose 4B (Pharmacia) (Smith and Johnson, 1988). The purified GST–IFN-γ fusion protein was used to immunize rabbits with three injections (20 μg/injection per animal) at 0, 2 and 6 weeks and the animals were bled one week after the last injection.

Oligonucleotide directed mutagenesis

The modified IFN-γ cDNA from pGEM-3Zf(+) vector was subcloned into the *Bam*HI site of M13mp9. Two 5′-chain oligonucleotides (GATGTAGCGGATCAGGGAACTCTTTTC and GAAAAGCTGACTCAGTATTCGGTAAC) were designed to replace the asparagine codons (AAT) in the two putative *N*-glycosylation sites by the CAG codon of glutamine at positions 25 and 97 in the mature protein (Figure 1). The oligonucleotide directed *in vitro* M13 mutagenesis kit (Amersham) was used to obtain the mutant constructs which lack either one or both of the Asn sites for *N*-glycosylation. All mutations were verified by sequencing and the mutated IFN-γ cDNAs were subcloned into pAcYM1 vector. The recombinant baculoviruses carrying mutated IFN-γ genes were generated as described above.

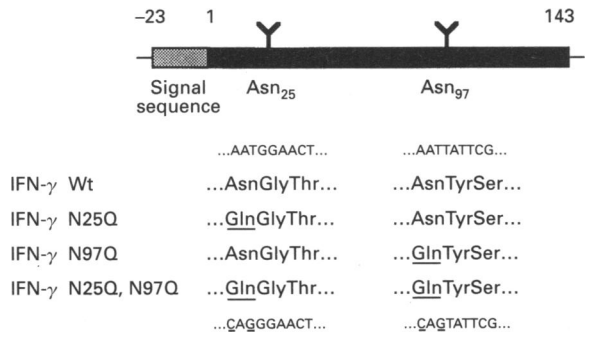


Figure 1 Schematic presentation of human IFN-γ cDNA and glycosylation site mutant constructs

IFN-γ cDNA was modified by site-directed mutagenesis to substitute either one or both of the glycosylation site Asn residues with Gln. Signal sequence (stipled), mature secretory portion of IFN-γ (solid) and glycosylation sites (Y) are indicated. Mutated nucleotides, amino acid sequences (underlined) and corresponding names for the constructs are shown.

RNA isolation and analysis

To analyse the accumulation of IFN-γ specific mRNAs, total cytoplasmic RNA was isolated (Sambrook et al., 1989) from 5×10^6 baculovirus-infected Sf9 cells at different times post-infection. The quantity of total RNA was determined spectrophotometrically and the integrity of RNA was analysed by ethidium-bromide-stained, formaldehyde–agarose gel. Equal cell aliquots (approximately 20 μg) of the total cell RNA were size-fractionated on formaldehyde–agarose gel (Sambrook et al., 1989). After transfer to nylon membranes (Magna Graph, MSI) and cross-linking with u.v.-light (Stratalinker UV crosslinker, Stratagene) the RNAs were hybridized with an IFN-γ cDNA probe labelled with ^{32}P-dATP (Amersham) by using a Random Primed DNA Labeling Kit (Boehringer Mannheim, Germany). The filters were washed and autoradiographed according to standard procedures (Sambrook et al., 1989).

Metabolic labelling, immunoprecipitation and trichloroacetic acid-precipitation

Recombinant baculovirus-infected Sf9 cells were grown as monolayers, preincubated 15 min in methionine-free Grace's insect cell medium at 27 °C and labelled with ^{35}S-methionine (500 μCi/ml, 1000 Ci/mmol, Amersham). After labelling, the cells and media were collected and the cells were washed with ice-cold PBS (or with fresh medium for pulse-chase experiments). The cells were suspended in a small volume of 10 mM Tris, pH 7.4, containing 1 mM EDTA and lysed in immunoprecipitation binding buffer (150 mM NaCl/5 mM EDTA/0.5% Triton X-100/50 mM Tris, pH 7.4). Lysates and media were adjusted to the same volume with binding buffer before immunoprecipitation. A 5 μl volume of the pool of three rabbit anti-IFN-γ antisera were allowed to bind to 25 μl of Protein A Sepharose CL-4B (Pharmacia) in binding buffer. After washing with the binding buffer the beads were used to precipitate IFN-γ protein from cell lysates or media in a total volume of 500 μl. The precipitated proteins were analysed in SDS/PAGE followed by autoradiography. In short pulse-labelling experiments the protein synthesis and the pulse was stopped by adding anisomycin (Sigma) and L-Met in final concentrations of 0.1 mM and 5 mM, respectively, and the samples were prepared for immunoprecipitation as above. To determine the amount of radiolabelled methionine incorporated into the protein (Braakman et al., 1991; Horwitz et al., 1969), 10 μl of the cell lysate was precipitated with 10% trichloroacetic acid (TCA) containing 1 mg/ml of BSA and 5 mM methionine (15 min, 22 °C), and boiled in 5% TCA for 10 min to release the labelled tRNA (Braakman et al., 1991) from proteins. After washing with 5% TCA, the radioactivity of the pellet was measured.

Tunicamycin treatment

Infected Sf9 cells were treated with tunicamycin (10 μg/ml, Sigma) for 4 h before labelling at 40 h post-infection. Medium was replaced with methionine-free Grace's medium containing ^{35}S-methionine (50 μCi/ml) with or without tunicamycin. Cells were labelled for 1 h and chased for 2 h in regular fresh medium and both the media and the cells were collected for immunoprecipitation.

Chemical cross-linking of cellular IFN-γ proteins

To study the kinetics of dimerization, recombinant IFN-γ baculovirus-infected Sf9 cells were pulse labelled (^{35}S-methionine, 250 μCi/ml) for 3 min. The protein synthesis was stopped by adding fresh medium containing anisomycin (0.1 mM) and unlabelled methionine (5 mM) and the cells were then chased for different time periods. At each time-point a sample of cells was taken and divided into two equal batches. Glutaraldehyde (0.6 %, final concentration) was added to the other tube and proteins were allowed to cross-link for 10 min on ice in the presence of 0.5 % Triton X-100. The cell extracts were then diluted into immunoprecipitation buffer containing BSA (0.2 mg/ml), cell debris was removed by centrifugation and the supernatants were subjected to immunoprecipitation with IFN-γ specific antibodies. The precipitated proteins were separated in 14 % SDS/PAGE followed by autoradiography. The glutaraldehyde concentration for optimal cross-linking was determined prior to the experiment and 0.6 % (v/v) concentration was shown to be sufficient to fully cross-link IFN-γ dimers in cell extracts.

Gel filtration

For analysis of the molecular form of IFN-γ supernatants of Sf9 cell cultures infected with IFN-γ Wt baculovirus (70000 i.u) or N25Q,N97Q mutant (100000 i.u. after concentration by CM Sephadex; see next section) were applied onto a Superdex 75 HR 10/30 gel-filtration column (Pharmacia) and eluted with buffer containing 25 mM Tris/HCl, pH 7.4/500 mM NaCl/ 5 mM EDTA/10 mM β-mercaptoethanol. Fractions of 0.25 ml were collected and IFN-γ in fractions was determined by biological assay and immunoblotting.

Purification of IFN-γ and determination of specific antiviral activity

Suspension cultures (1–3 l) of Sf9 cells were infected with various recombinant baculoviruses and IFN-γ proteins (collected 68 h after infection) were purified by the methods developed for purification of natural leukocyte IFN-γ (Cantell et al., 1986; Kauppinen et al., 1985). Cell culture supernatants were dialysed against 20 mM phosphate buffer, pH 6.5, containing 50 mM NaCl and allowed to bind to CM-Sephadex C-50 (Pharmacia). After washing with the same buffer the bound material was eluted with 50 mM glycine/HCl buffer, pH 9.5 containing 1 M NaCl. Further purification of IFN-γ was achieved by monoclonal anti-IFN-γ antibodies coupled onto Eupergit C-matrix (courtesy of Hannele Tölö, Finnish Red Cross Blood Transfusion Service, Helsinki, Finland). The bound IFN-γ was eluted from the immunoaffinity matrix with 2 M NaCl in 150 mM NH_4OH, pH 10.7, and neutralized immediately with 1.0 M phosphate buffer, pH 5.0.

To analyse the purity and quantity of the recombinant IFN-γ proteins, a sample of each preparation was applied to reversed-phase (RP)-h.p.l.c. About 0.1–0.5 μg of immunoaffinity purified IFN-γ was chromatographed on a 0.21 × 4 cm TSK TMS 250 (C1) (TOSOH Corporation, Japan) reversed phase column using a linear gradient of acetonitrile (3–100 % in 60 min) in 0.1 % trifluoroacetic acid at a flow rate of 250 μl/min. An average of the peak integrals (at 214 nm) from β-lactoglobulin, lysozyme, cytochrome *c* and bovine serum albumin at 214 nm were used as standards. For comparison, the respective IFN-γ bands were also quantitated by densitometric scanning after separation in SDS/PAGE (Figure 5f) and Coomassie Blue staining.

The antiviral activity of various preparations of IFN-γ was assayed by vesicular stomatitis virus (VSV) plaque reduction assay in HEp2 cells as described earlier (Cantell et al., 1991). A laboratory reference IFN-γ preparation was used and all titres are given in international units (i.u.) per milliliter.

SDS/PAGE, autoradiography, quantification and Western blot

The proteins and immunoprecipitates were analysed in 14 % SDS/PAGE (Laemmli, 1970). The gels were either stained with Coomassie Brilliant Blue or processed for autoradiography by fixing, drying and exposing to Amersham Hypermax films. The absorbances of the bands in the autoradiograms and Coomassie stained gels were quantified by Millipore BioImage scanner. For Western blotting, the proteins separated in SDS/PAGE were electrophoretically transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore) and incubated with pooled rabbit anti-IFN-γ sera (1 : 1000 dilution in PBS/5 % nonfat milk, 2 h at +37 °C). After washing (PBS containing 0.05 % Tween-20) the membranes were incubated with peroxidase-conjugated goat anti-rabbit immunoglobulins (1:2000; 1 h at +37 °C) (Bio Rad), washed and the proteins on the membranes were detected by ECL reagent (Amersham).

RESULTS

Synthesis of IFN-γ by insect cells

Wt and glycosylation-site mutant cDNAs (Figure 1) were used to construct recombinant baculoviruses. These viruses were used to infect Sf9 cells and the synthesis of IFN-γ proteins was visualized by radiolabelling and immunoprecipitation. Pulse-chase experiments show that Wt IFN-γ has a typical (three major bands)

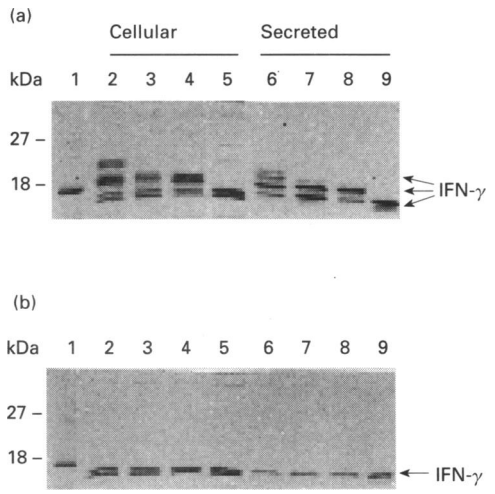
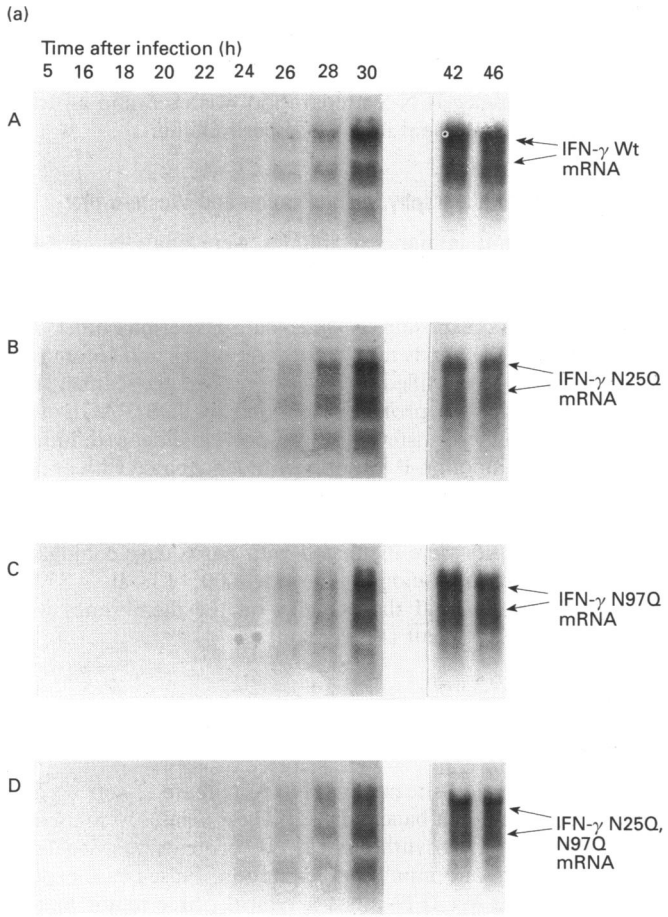


Figure 2 Synthesis of recombinant IFN-γ by baculovirus-infected Sf9 cells

Monolayers of Sf9 cells were infected with IFN-γ Wt and glycosylation-site mutant baculoviruses at a multiplicity of 5–10 plaque forming units per cell. After 36 h of infection the cells were treated with tunicamycin (10 μg/ml) for 4 h prior to metabolic labelling. The cells were labelled (^{35}S-methionine, 50 μCi/ml) for 1 h in the absence (**a**) or presence (**b**) of tunicamycin. After 2 h chase, cells and media were collected and labelled IFN-γ molecules were immunoprecipitated with polyclonal rabbit anti-IFN-γ antibodies and the proteins were visualized by autoradiography after separation in 14% SDS/PAGE. (**a**) Lane 1, reticulocyte lysate translated *in vitro*, 166 amino acid containing full length IFN-γ; lanes 2–5, immunoprecipitable material from cells (lane 2, Wt; lane 3, N25Q; lane 4, N97Q; lane 5, N25Q,N97Q); lanes 6–9 corresponding material immunoprecipitated from media. (**b**) Tunicamycin-treated cells. Lanes as in (**a**).



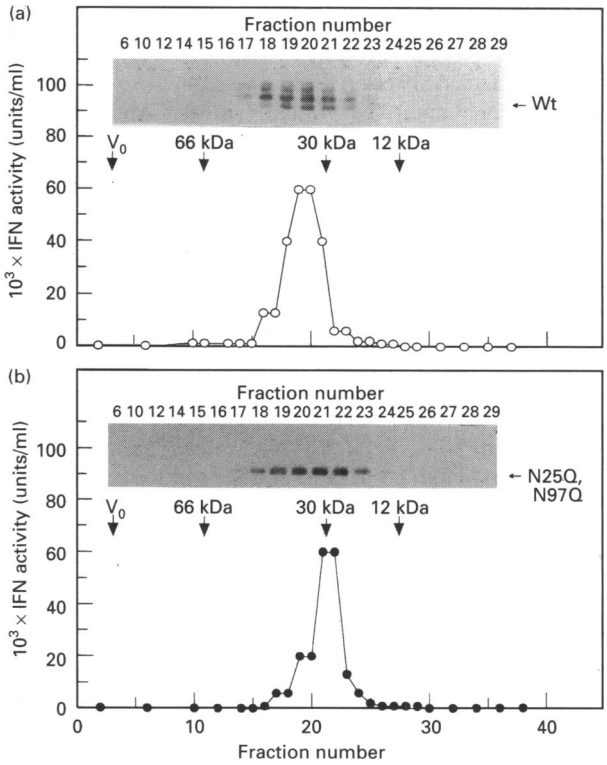
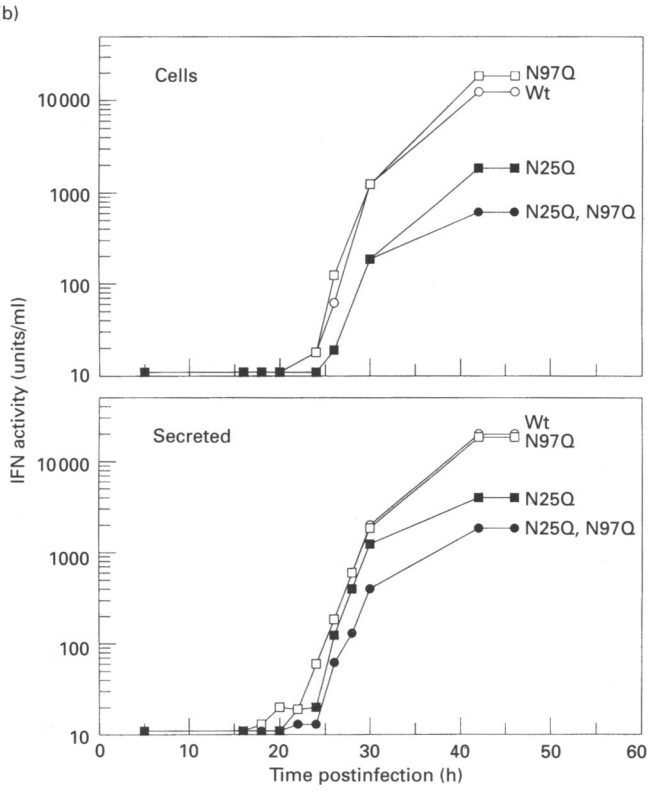


Figure 4 Gel filtration of IFN-γ proteins

Supernatants from Sf9 cell cultures expressing (**a**) Wt IFN-γ (0.7×10^5 i.u.) or (**b**) N25Q,N97Q mutant (1.0×10^5 i.u.; CM Sephadex concentrated) were applied to a Superdex 75 column. The arrows indicate the fractions where the molecular mass marker proteins were eluted. V_0, void volume. The antiviral activities of the fractions and the corresponding immunoblots (insets) are shown.



protein pattern in immunoprecipitates of secreted material (Figure 2a, lane 6). This heterogeneity is due to the efficiency of *N*-glycosylation and is also found in IFN-γ purified from leukocytes (Yip et al., 1982; Cantell et al., 1992). The slowest migrating band represents molecules that have both Asn-X-Ser/Thr signals of IFN-γ glycosylated. The next band represents proteins with only one glycosylation site occupied. The IFN-γ proteins that have no *N*-glycosylation at all show the fastest mobility in SDS/PAGE. Accordingly, the single-site mutants N25Q and N97Q have two major bands in immunoprecipitates of cell media (Figure 2a, lanes 7 and 8, respectively), while the IFN-γ subunit from which both *N*-glycosylation signals were removed exhibit only the lowest molecular mass band (Figure 2a, lane 9). As shown in Figure 2, all differentially glycosylated as well as the non-glycosylated IFN-γ is secreted into the culture medium. The observed heterogeneity in molecular masses was due to

Figure 3 Kinetics of IFN-γ mRNA accumulation and IFN-γ protein production

Sf9 cells were infected with various IFN-γ recombinant baculoviruses and grown in a spin culture at a density of 2×10^6 cells/ml. Samples were collected at various time-points for mRNA isolation and for the assays of IFN-γ antiviral activity. (**a**) Steady-state IFN-γ mRNA levels of different IFN-γ recombinant baculovirus infected cells A, IFN-γ; B, N25Q; C, N97Q; D, N25Q,N97Q. 20 μg of total cell RNA were size-fractionated in formaldehyde–agarose gels, transferred to nylon membranes and hybridized with ^{32}P-labelled random primed IFN-γ cDNA probe. (**b**) Time-course of the appearance of cellular and secreted IFN-γ. Equal cell aliquots were used in the titration of antiviral activity of IFN-γ from the cells and media.

asparagine-linked glycosylation, since labelling of the cells in the presence of tunicamycin resulted into IFN-γ molecules with an apparent molecular mass of the non-glycosylated form (Fig. 2b). In addition, the result shows that elimination of *N*-glycosylation did not block the secretion of any one of the different IFN-γ proteins (Figure 2b, lanes 6–9).

A band with slightly faster mobility than the major IFN-γ was seen in immunoprecipitates from cell lysates and to a lesser extent in precipitates of secreted material (Figure 2). This is most likely due to C-terminal proteolytic processing also described for natural IFN-γ produced by leukocytes (Rinderknecht et al., 1984) and for recombinant IFN-γ proteins expressed by *E. coli* or CHO cells (Arakawa et al., 1986; Curling et al., 1990), and appears to account for the absence of 6–8 amino acids from the C-terminus of baculovirus-produced IFN-γ.

N-Glycosylation of Asn-25 is necessary for efficient production of IFN-γ

The influence of N-glycosylation on IFN-γ production was examined in Sf9 cells infected with different recombinant viruses. The amounts of IFN-γ from the cells and secreted into the cell culture medium were monitored by measuring the antiviral activity (Figure 3b). The results indicated that elimination of N-glycosylation sites decreased the overall levels of IFN-γ production and secretion. Interestingly, glycosylation at Asn-25 was more critical for high-level production of IFN-γ than glycosylation at Asn-97. Measurements of antiviral activity from several batch cultures showed that elimination of the *N*-glycosylation site at Asn-97 (N97Q) resulted in secreted protein yields of 70–90% as compared to Wt production. However, the yields were only 10–25% (N25Q) and 1–10% (N25Q,N97Q) when the first or both of the glycosylation sites were mutated, respectively. The result was confirmed by Western blotting, which indicated that the amount of different recombinant IFN-γ proteins correlated well with the observed antiviral activities (results not shown). Analysis of the steady-state IFN-γ mRNA levels (Figure 3a) indicated that the accumulation of IFN-γ specific mRNAs was similar between all four constructs. This suggests that the mutations created in the IFN-γ gene did not affect the transcription efficiency or the stability of IFN-γ mRNAs.

Recombinant IFN-γ molecules produced by insect cells are biologically active dimers

Biologically-active IFN-γ molecule is a dimer, consisting of two identical non-covalently-linked subunits (Ealick et al., 1991). To study whether the *N*-glycans affected the production and secretion of IFN-γ dimers, supernatants of Sf9 cell cultures expressing wild type or N25Q,N97Q IFN-γ were subjected to gel filtration and the fractions were tested for the presence of IFN-γ by immunoblotting and biological assay. Figure 4 shows that all secreted IFN-γ was in a dimeric form, since no monomers were detected.

Recombinant proteins were purified from cell-culture supernatant by ion-exchange and immunoaffinity chromatography. The purified material was analysed in RP-h.p.l.c. to reveal the purity and quantity of the different IFN-γ proteins (Figure 5). All preparations exhibited at least 80% purity based on h.p.l.c. The actual amount of each protein was determined from the peak integrals and densitometric scanning of Coomassie Blue-stained SDS/PAGE gels run from the peak fractions, the values

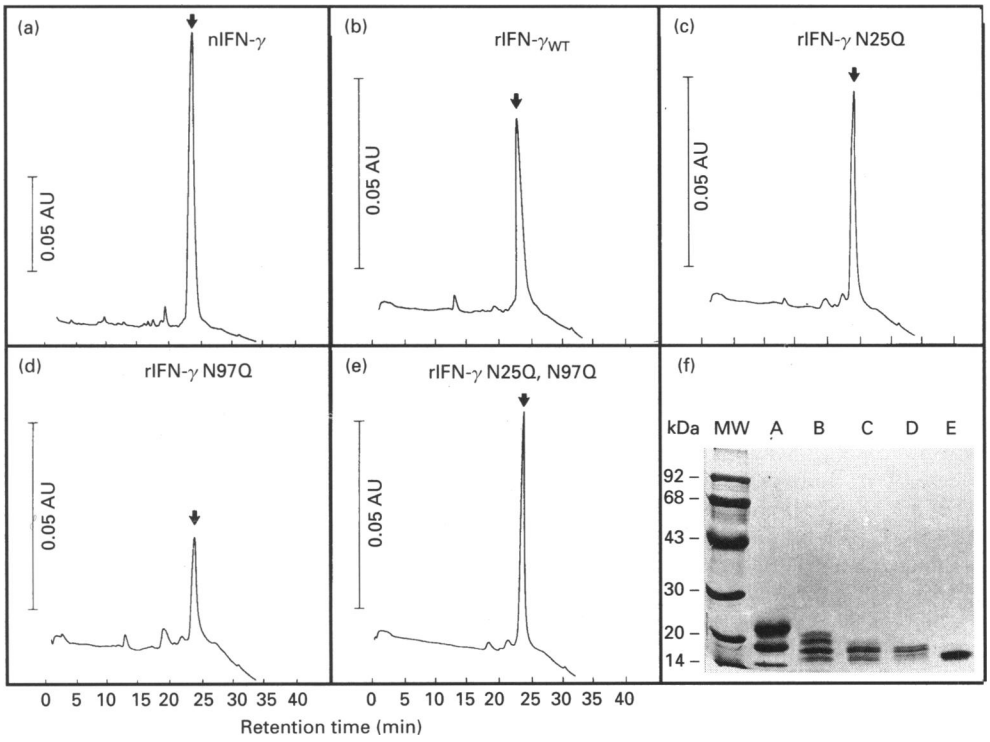


Figure 5 Reversed-phase chromatography of purified IFN-γ

Leukocyte-produced IFN-γ [nIFN-γ, (**a**)] and baculovirus-produced recombinant [rIFN-γ (**b–e**)] IFN-γ molecules were purified as described in Experimental. Purified preparations were subjected to RP-h.p.l.c. The proteins were eluted with a linear gradient of acetonitrile (3–100% in 60 min) in 0.1% trifluoroacetic acid using a flow rate of 250 μl/min. The major peaks (marked by arrows) represent different IFN-γ forms as indicated in the figure. The peak fractions were collected and the proteins were separated in 12% SDS/PAGE and stained with Coomassie Blue (**f**). AU, absorbance units; MW, molecular-mass standards (kDa).

Table 1 Specific antiviral activity of purified IFN-γ from leukocytes and differentially glycosylated recombinant proteins

The antiviral activity was determined by plaque reduction assay (means of three titrations) and correlated to the actual amount of IFN-γ protein in the preparation based on h.p.l.c. analysis and quantification from Coomassie Blue-stained gel (Figure 5).

Interferon	Specific antiviral activity (i.u./mg of protein)
Leukocyte IFN-γ	1.1×10^7
Baculo IFN-γ Wt	1.1×10^7
Baculo IFN-γ N25Q	0.83×10^7
Baculo IFN-γ N97Q	0.93×10^7
Baculo IFN-γ N25Q, N97Q	0.47×10^7

of which correlated well with each other. The specific antiviral activity of IFN-γ recombinant proteins was calculated on the basis of their antiviral titre and the actual amount of IFN-γ protein. The specific activities for glycosylated natural (leukocyte IFN-γ) or recombinant IFN-γ molecules were approximately 1×10^7 i.u./mg of protein, whereas the activity for unglycosylated N25Q,N97Q was slightly lower (Table 1).

Glycosylation at Asn-25 is necessary for efficient dimerization of IFN-γ

The specific activities of differentially glycosylated IFN-γ molecules were relatively similar and could not explain the differences in the production kinetics. Therefore we wanted to study the early events in the biosynthesis of IFN-γ. Firstly we determined the translation rate of IFN-γ molecules in insect cells. As revealed from Figure 6, the time period between the intercepts of the two lines is 35 s for both Wt and N25Q,N97Q proteins. This corresponds to half the time required for the synthesis of one molecule as previously described by Braakman et al. (1991) and Horwitz et al. (1969). This gives the average synthesis time of one IFN-γ molecule to be 70 s (2.8 amino acids/s). As expected, lack of glycosylation did not have any effect in the translation rate of IFN-γ.

Efficient recombinant IFN-γ protein synthesis allows us to use short pulses for studying the kinetics of dimerization. Since mature IFN-γ molecules lack cysteine residues and thus intermolecular disulphide bridges, we had to use chemical cross-linking with glutaraldehyde to follow the kinetics of dimer formation. After a 3 min pulse (equal to the synthesis time of 2.5 molecules) the cells were permeabilized, cross-linked and the solubilized material was immunoprecipitated with IFN-γ specific antibodies. Dimers started to accumulate approximately 2 min after the pulse in the case of Wt and N97Q molecules, whereas the extent of dimerization of N25Q and N25Q,N97Q were considerably lower (Figure 7). Based on quantitative analysis (Figure 8) Wt IFN-γ dimerizes 10-fold more efficiently than its unglycosylated counterpart. The $t_{\frac{1}{2}}$ for dimerization of IFN-γ Wt was approximately 5 min. This suggests that although the synthesis time of different IFN-γ recombinant molecules was the same, the extent of dimerization depends on glycosylation. *N*-linked oligosaccharides at position Asn-25 seem to play an important role in this dimerization process.

Glycan residues to position Asn-97 are added mainly post-translationally

The extent of core glycosylation appeared to continue after the synthesis and dimerization of the Wt and N25Q molecules which

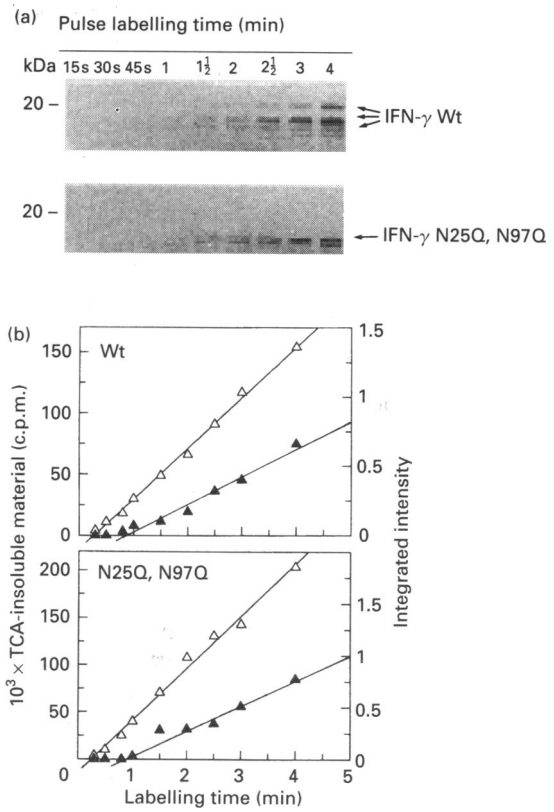


Figure 6 Synthesis time of IFN-γ molecules

Insect cells were infected with Wt and N25Q,N97Q recombinant baculoviruses and labelled with ^{35}S-methionine (250 μCi/ml) at 38 h post-infection with short pulses from 15 s to 4 min. The protein synthesis was stopped by adding 100 μM anisomysin and 5 mM L-methionine. Cells were lysed and immunoprecipitated with polyclonal rabbit anti-IFN-γ antiserum. Precipitated proteins were separated in 14% SDS/PAGE (**a**), autoradiographed and quantified (**b**) by scanning (▲). Total incorporation of the radioactivity was measured from TCA-precipitates (△). The difference between the intercepts of these two lines with the *x*-axis (15 s and 50 s) corresponds to half (35 s) of the synthesis time of IFN-γ and is equal to 70 s for the completion of one molecule (Braakman et al., 1991; Horwitz et al., 1969).

have an intact asparagine residue at position 97 (Figure 7, right panel). Quantitative analysis (Figure 9) reveals that the extent of glycosylation of these molecules is increasing (up to 10–15 min) during the chase. In the case of Wt and N25Q IFN-γ the amounts of fully glycosylated (two sites occupied, Wt) and partially glycosylated (one site occupied, N25Q) molecules increase with a corresponding decrease in the amount of unglycosylated molecules. N97Q molecules, which have intact glycosylation sites at position 25 are more extensively glycosylated already after the pulse and no further glycosylation appears to occur during the chase (Figure 9).

Secretion of glycosylated and unglycosylated IFN-γ dimers

To study the influence of glycosylation on secretion kinetics of different IFN-γ proteins, the infected cells were pulse-labelled for 10 min and chased for up to 5 h (Figure 10). Recombinant IFN-γ proteins were all secreted with a $t_{\frac{1}{2}}$ (50% of maximal amount secreted) of about 45–60 min, although the yields of secreted, immunoprecipitable IFN-γ proteins varied greatly between different constructs (Figure 10b). The amount of secreted, differentially glycosylated IFN-γ correlates well with the differences observed in the extent of dimerization (c.f. Figure 8).

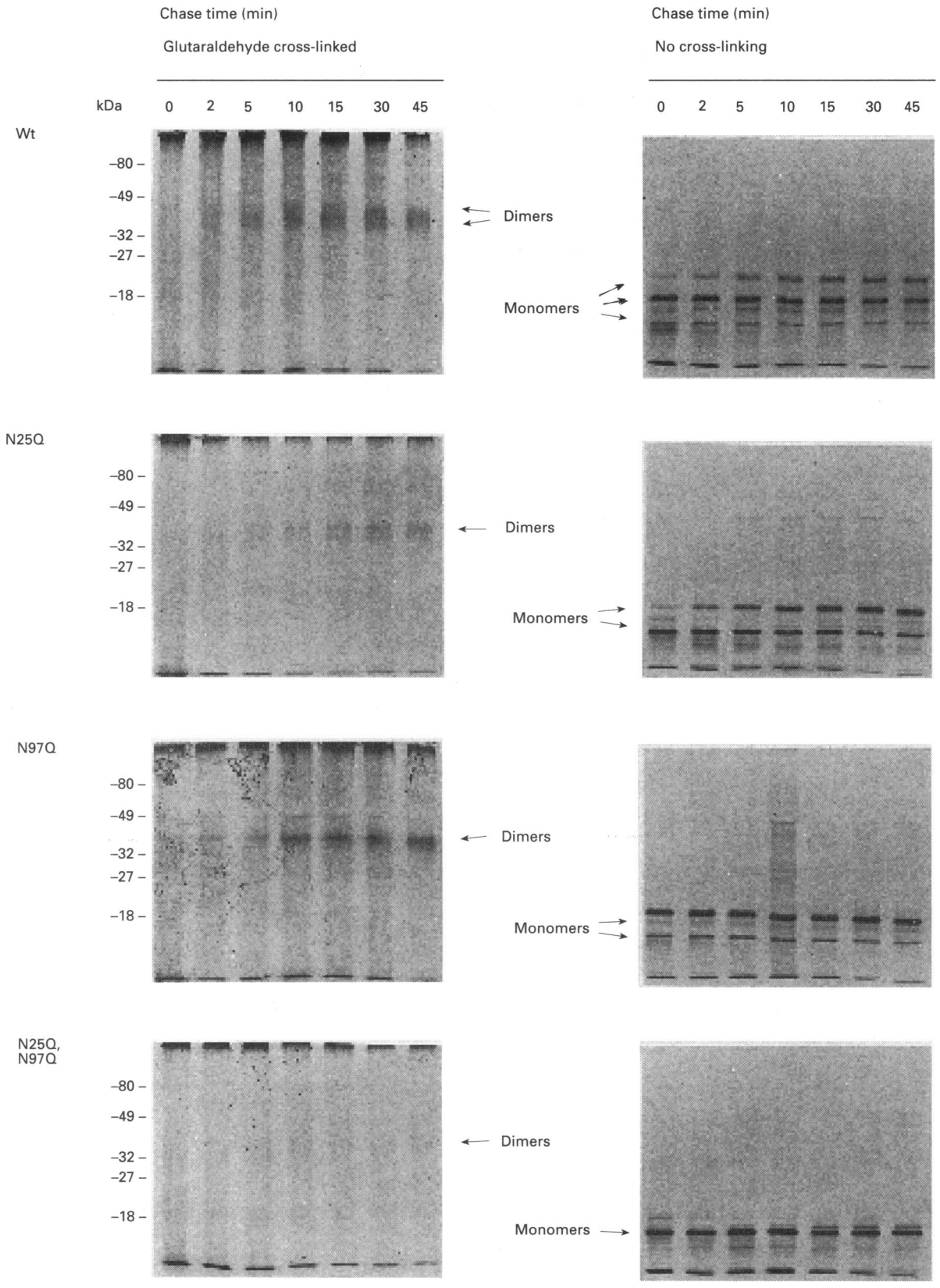


Figure 7 Kinetics of dimerization of differentially glycosylated IFN-γ

IFN-γ recombinant baculovirus-infected Sf9 cells were pulse labelled for 3 min with ^{35}S-methionine (500 μCi/ml), the protein synthesis was stopped by adding anisomycin (100 μM final concentration) and the cells were chased for various periods of time as indicated in the figure. At each time-point cells were collected and disrupted with immunoprecipitation buffer with or without chemical cross-linking with 0.6% glutaraldehyde (final concentration) for 10 min on ice. Cell extracts were further diluted in immunoprecipitation buffer containing BSA (0.2 mg/ml) and cell debris was removed by centrifugation. Supernatants were subjected to immunoprecipitation with anti-IFN-γ specific antibodies followed by analysis in 14% SDS/PAGE and autoradiography. IFN-γ specific dimer and monomer bands are shown by arrows.

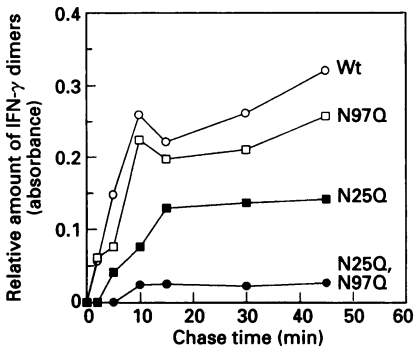


Figure 8 Quantitative analysis of IFN-γ dimerization

The band intensities from Figure 7 were quantified by densitometry and the values were used to demonstrate graphically the kinetics of dimerization of different IFN-γ molecules. Relative band intensities of chemically cross-linked IFN-γ dimers are shown. IFN-γ forms are as indicated in the figure.

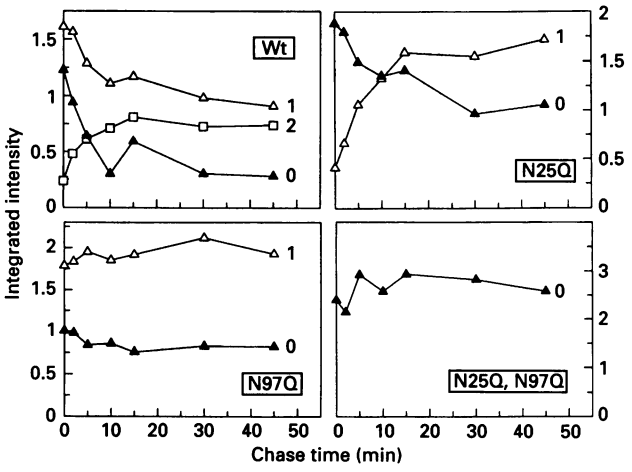


Figure 9 Glycosylation of IFN-γ molecules

The integrated intensities of the monomeric protein bands from Figure 7 (no cross-linking) were quantified. The number after the line indicates how many *N*-glycosylation sites (0, 1 or 2) are occupied in the given protein molecule.

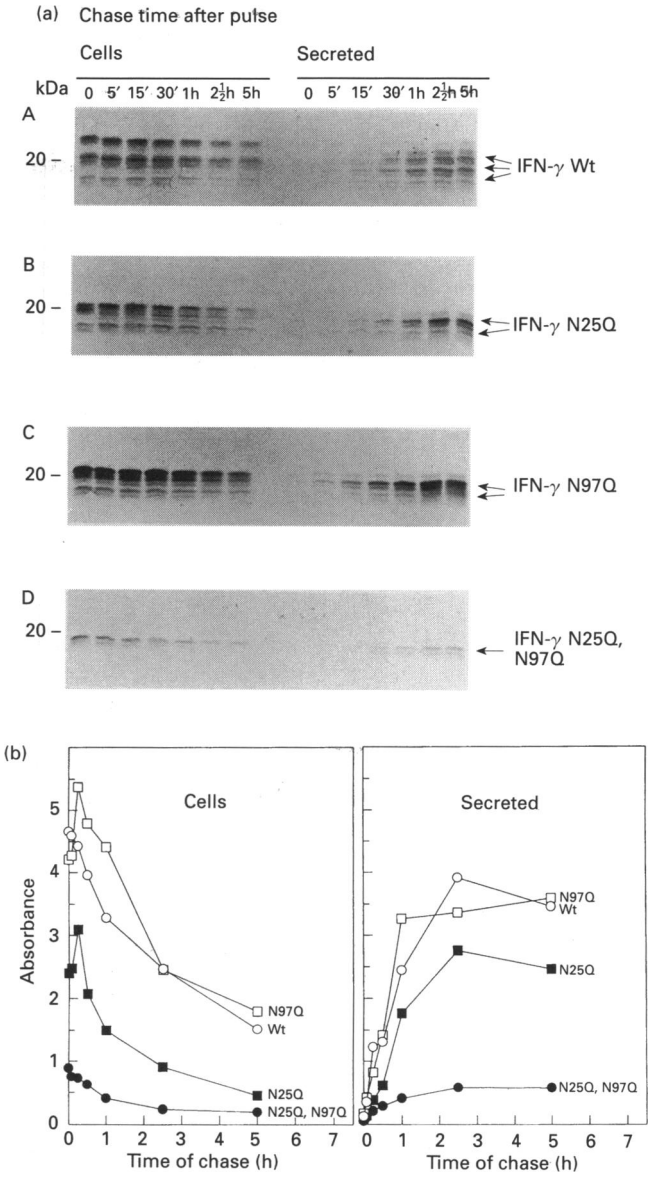


Figure 10 Secretion of glycosylated and unglycosylated IFN-γ molecules

IFN-γ recombinant baculoviruses were used to infect monolayers of Sf9 cells with 5 plaque-forming units/cell and the cells were pulse-labelled with ^{35}S-methionine (100 μCi/ml) for 10 min 36 h postinfection. After thorough washing the cells were chased for various periods of times and the cells and media were collected and IFN-γ protein was immunoprecipitated. The proteins were separated in SDS/PAGE, dried and autoradiographed. (**a**) Autoradiography of one representative pulse-chase experiment of various IFN-γ constructs. A, Wt IFN-γ; B, N25Q; C, N97Q; D, N25Q,N97Q. (**b**) The band intensities of various IFN-γ constructs were quantified from (**a**).

DISCUSSION

IFN-γ is a potent immunomodulatory molecule which is produced by activated subpopulations of T-lymphocytes in minute quantities. This makes it very difficult to study the early events of the synthesis and secretion of natural IFN-γ. We chose another approach and produced IFN-γ by recombinant methods using a baculovirus expression system. We specifically addressed the question of whether the *N*-linked glycan residues, which exist in the natural IFN-γ molecules, have a role in the synthesis and biochemical properties of human IFN-γ. To do that, we substituted the asparagine residues at positions 25 and/or 97 for glutamine residues and obtained four different IFN-γ molecules with the expected glycosylation patterns (Figure 2). These amino acid substitutions are conservative and are unlikely to affect the three-dimensional structure or the surface charge of IFN-γ dimers. The production of the differentially glycosylated molecules was, however, dramatically different. Fully glycosylated Wt and partially glycosylated N97Q proteins were produced in very high yields up to 2×10^4 i.u/ml, whereas N25Q gave approximately 4–10-fold lower and N25Q,N97Q 10–100-fold lower yields in insect cells. This suggested that the position (25 *versus* 97) of glycosylation is of importance for IFN-γ production. The changes introduced into the IFN-γ gene did not affect the polyhedrin promoter driven transcription or mRNA stability since the steady-state levels of IFN-γ mRNAs were the same at every time-point of baculovirus infection (Figure 3).

Efficient synthesis of the IFN-γ protein in insect cells allowed us to look at the early posttranslational events including dimer-

ization. We were unable to study the folding of IFN-γ molecules, since they are lacking intra-chain disulphide bridges. Present knowledge of folding *in vivo* is mainly based on studies of molecules with disulphide bridges (Gething et al., 1992; Helenius et al., 1992; Doms et al., 1993). Alternatively, conformational specific monoclonal antibodies could be used, but to our knowledge none of them are available against IFN-γ. We used chemical cross-linking in pulse-chase experiments to follow the kinetics of formation of IFN-γ dimers. The extent of dimerization was clearly higher for the wild-type and N97Q polypeptides, which both have an intact glycosylation site at Asn 25, than for N25Q and N25Q,N97Q proteins (Figures 7 and 8). A large portion of cross-linked, immunoprecipitated IFN-γ was found as insoluble aggregates on top of the gel. This most likely represents unfolded or misfolded material that is tightly bound to cellular chaperonins, which regulate the early events during protein folding (Gething et al., 1992). There was a clear difference in the observed amounts of IFN-γ dimers between the glycosylated and unglycosylated molecules. Taken together, the differences in the extent of dimerization between different IFN-γ molecules most likely explain the differences in the production levels. The sugar residues at asparagine 25 are essential for efficient (folding and) dimerization and subsequent high-level secretion of functional IFN-γ dimers into the cell culture supernatant. The unglycosylated IFN-γ polypeptides may be misfolded so that they are incapable of forming proper dimers and remain as insoluble aggregates in the endoplasmic reticulum. In many cases glycan residues are known to facilitate folding (Helenius et al., 1992) and different glycosylation sites may play a differential role in the folding process (Gallagher et al., 1992). The degradation of the misfolded polypeptides may take a relatively long time, since in pulse-chase experiments we did not see any degradation of the proteins.

The two glycosylation sites displayed another interesting difference. In the Wt and N25Q molecules the extent of glycosylation increases during the first 15 min of chase (Figure 9). In N97Q proteins there appears to be no further increase in the extent of glycosylation after the 3 min pulse. The glycosylation is almost complete already after the pulse. This indicates that Asn-25 is glycosylated cotranslationally. On the other hand, the carboxyterminal glycosylation site may be hidden during translation, leading to inefficient glycosylation of this site (Asn-97). An interesting possibility is that both the folding and dimerization of IFN-γ molecules may start almost simultaneously during translation so that the carboxyterminal domain of one monomer binds to the aminoterminal domain of the nascent chain of the other molecule. By this mechanism the observed tight structure of IFN-γ dimers would be established. In favour of this possibility is the finding that the kinetics of IFN-γ dimerization appears to occur relatively fast ($t_{\frac{1}{2}}$ 4–5 min). The oligomerization of more complex structures such as viral membrane proteins may vary considerably ($t_{\frac{1}{2}}$ from 5 to 80 min; Doms et al., 1993) and only a few of those proteins show fast oligomerization kinetics ($t_{\frac{1}{2}}$ 5–10 min).

It is known that the lack of glycosylation may impair the secretion of some molecules such as plasminogen activator (Jarvis et al., 1990), whereas IFN-β is efficiently secreted in the presence of tunicamycin in insect cells (Jarvis and Summers, 1989). We did not observe any major differences in the secretion kinetics of the differentially glycosylated IFN-γ molecules, although the total production of unglycosylated molecules was clearly lower than that of the glycosylated forms. In the case of IFN-γ, if unglycosylated molecules are folded and dimerized in a correct fashion they become competent for secretion and are apparently secreted with similar kinetics to their glycosylated counterparts (Figure 10).

The apparent molecular masses of baculovirus-produced and natural IFN-γ molecules varied (Figure 5). These differences may, in addition to carboxy-terminal proteolytic-trimming (Rinderknecht et al., 1984), be due to differences in the composition of sugar chains. The insect cells lack the ability to trim *N*-glycans efficiently, and the glycoproteins synthesized by these cells contain mainly high mannose-type oligosaccharides with occasional fucose and galactose residues (Kuroda et al., 1990). The *N*-glycans of IFN-γ derived from human lymphocytes are more complex containing fucose and *N*-acetylneuraminic acid residues (Yamamoto et al., 1989).

Production of differentially glycosylated forms of IFN-γ in the same system allowed us to compare reliably the biological activity of these proteins. After the purification, all glycosylated IFN-γ molecules including the natural human leukocyte IFN-γ, exhibited similar specific antiviral activities ranging between 0.8 and 1.1×10^7 i.u/mg of IFN-γ protein. All the different forms were purified in a similar fashion. The completely unglycosylated IFN-γ showed a 2-fold lower specific activity (0.47×10^7 i.u/mg) as compared to the glycosylated forms. Whether this difference is due to the presence of inactive forms of unglycosylated IFN-γ or whether it is a reflection of a different receptor affinity is presently not known. However, the two-fold lower specific activity does not sufficiently explain the reduced production of unglycosylated N25Q,N97Q protein.

In this paper we have demonstrated that the *N*-linked glycan residues are important for the dimerization and efficient production of human IFN-γ. The differentially glycosylated IFN-γ molecules prepared in insect cells will also give us powerful tools to analyse whether glycosylation affects the receptor-mediated gene-activation and its biological effects at cellular level. Recent data indicate that glycan residues protect IFN-γ from proteolysis and enhance the half-life of the protein in the circulation (Cantell et al., 1992; Sareneva et al., 1993). These data may also have implications in the clinical use of IFN-γ.

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