Source of heterogeneity in secreted interferon- γ

A study on products of translation *in vitro*

Neil J. BULLEID,* Elisabeth CURLING, Robert B. FREEDMAN and Nigel JENKINS

The Biological Laboratory, University of Kent, Canterbury, Kent, CT2 7NJ, U.K.

A cDNA clone coding for human interferon- γ (IFN- γ) was subcloned into a transcription–translation vector. When the mRNA transcribed *in vitro* was added to a rabbit reticulocyte-lysate system, two polypeptides were synthesized: one corresponding in M_r to pre-IFN- γ (18000) and one with a lower M_r (12000) which corresponds to a polypeptide arising from incorrect initiation of translation. When microsomal vesicles isolated from dog pancreas or Chinese-hamster ovary (CHO) cells were added to the translation system, translocation of the pre-IFN- γ occurred, as judged by protection from exogenous proteinases. The resultant changes in the M_r of the translation products were indicative of signal-peptide cleavage and heterogeneous core glycosylation. When translation products were treated with *N*-glycanase, the higher- M_r products were no longer observed, consistent with removal of all oligosaccharide side chains, leaving a single core polypeptide. Glycosylation of the synthesized protein yielded both singly and doubly glycosylated products compatible with the glycosylation variants seen in secreted IFN- γ . Quantitative differences were seen in the relative amounts of singly and doubly glycosylated products synthesized by dog pancreatic compared with CHO-derived microsomes. These data indicate that the relative amounts of IFN- γ glycosylation variants are determined at an early stage in protein synthesis and that product variants may occur when IFN- γ is expressed in cells derived from different tissues.

INTRODUCTION

Human interferon- γ (IFN- γ) is naturally secreted by antigen-sensitized T-lymphocytes that express potent antiviral activity (Langford *et al.*, 1979). The gene coding for human IFN- γ has recently been cloned and expressed both in Chinese-hamster ovary (CHO) cells (Mustaers *et al.*, 1986) and in bacterial systems (Gray *et al.*, 1982; Seelig *et al.*, 1988), yielding a biologically active recombinant protein. The deduced amino acid sequence of IFN- γ predicts a mature polypeptide of 146 amino acids with an M_r of 17 100 (Gray *et al.*, 1982). However, the core polypeptide is modified both co-translationally by the addition of up to two oligosaccharide side chains and post-translationally by modification of these side chains (reviewed by Kornfeld & Kornfeld, 1985). Glycosylation of both the natural and recombinant IFN- γ occurs either at Asn-28 alone, or at both Asn-28 and Asn-100 to yield products of apparent M_r 20 000 and 25 000 respectively. Differential modification of these side chains with sialic acid can occur, causing extensive peripheral charge differences (Kelker *et al.*, 1983). More recently, core-polypeptide processing was discovered in the recombinant and natural IFN- γ protein, resulting from proteolytic cleavage of the C-terminal residues (Rinderknecht *et al.*, 1984; Arakawa *et al.*, 1986). The significance of these modifications is unclear, but it appears that the C-terminal region of recombinant IFN- γ is particularly critical for maintaining the correct protein conformation for inducing antiviral activity (De la Maza *et al.*, 1987). The ‘correct’ glycosylation of recombinant proteins with potential therapeutic use is usually important for the stability of the protein, but is not always necessary for maintenance of full biological activity *in vivo* (reviewed by Berman & Lasky, 1985). In addition, aberrant glycosylation may lead to increased immunogenicity *in vivo*, with the generation of antibodies which neutralize the activity of IFN- γ (Steis *et al.*, 1988).

In order to study the initial stages in the glycosylation and

processing of IFN- γ , we have subcloned a IFN- γ cDNA clone into a transcription–translation vector. The transcribed RNA has been used to direct the synthesis of IFN- γ in a rabbit reticulocyte-lysate system. When microsomal vesicles derived from dog pancreas or from CHO cells were included in this system, translocation and processing (i.e. signal-peptide cleavage) of the synthesized protein occurred. Core glycosylation of the translocated and processed product followed, yielding both singly and doubly glycosylated products. Some heterogeneity was observed in the glycosylation products, consistent with the removal of mannose residues, but no heterogeneity was observed in the size of the core polypeptide.

EXPERIMENTAL

Materials

The human IFN- γ cDNA clone was generously provided by Dr. A. Morris, University of Warwick, Coventry, U.K. Restriction enzymes, phage- T_4 DNA ligase, phage- T_7 RNA polymerase, RNAase inhibitor, nucleotides and 7-methylguanosylguanosine triphosphate (7-mGpppG) were purchased from Boehringer-Mannheim (Lewes, East Sussex, U.K.). L-[35 S]methionine, Amplify and ‘Rainbow’ 14 C-labelled M_r markers were purchased from Amersham International (Amersham, Bucks., U.K.). Bluescript was from Stratagene (Northumbria Biologicals, Cramlington, Northd., U.K.). *N*-Glycanase was purchased from Genzyme Biochemicals Ltd. (Maidstone, Kent, U.K.). Antibody 20D7 was obtained from Celltech (Slough, Berks., U.K.). Protein A–Sepharose was purchased from Sigma (Poole, Dorset, U.K.).

Cells

A CHO-cell line transfected with the human IFN- γ gene was provided by Wellcome Biotechnology (Beckenham, Kent, U.K.).

Abbreviations used: IFN, interferon; CHO, Chinese-hamster ovary; 7mGpppG, 7-methylguanosylguanosine triphosphate; DTT, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride.

* Present address and address for correspondence and reprint requests: Department of Biochemistry and Molecular Biology, School of Biological Sciences, University of Manchester, Manchester M13 9PT, U.K.

This line was used as a source of CHO microsomes and for the production of secreted human IFN- γ in tissue culture (Curling *et al.*, 1989).

Construction of recombinant plasmid

The entire coding sequence for the human IFN- γ , including some 3' and 5' flanking sequences, was supplied as a 1.1 kb fragment cloned into the restriction-endonuclease-*Bam*HI site of plasmid pBR322. After digestion of this plasmid with *Msp*I and then *Sau*3A, a 0.846 kb fragment was purified, by electroelution after electrophoresis through agarose (Maniatis *et al.*, 1982), that contains the 5' sequence up to the *Sau*3A site and the 3' sequence to the second *Sau*3A site, as described by Devos *et al.* (1982). Bluescript digested with *Bam*HI was mixed with the IFN- γ fragment and ligated. Competent cells were prepared by CaCl_2 treatment and were transformed with the recombinant plasmids. The recombinants containing the IFN- γ gene were identified after digestion with *Bgl*II and designated 'pUKC1013'. The orientation of the gene in Bluescript was such that transcription from the phage-T₇ promoter yielded the 'sense' strand. The resulting transcript contains vector-derived sequence (up to the *Bam*HI site in multiple cloning region of Bluescript) and cDNA-derived sequence (83 nucleotides from the *Sau*3A site) 5' to the initiation codon. Plasmid DNA was purified by the method of Birnboim & Doly (1979), as described by Maniatis *et al.* (1982).

Cell-free transcription

Transcription was carried out essentially as described by Krieg & Melton (1984), with the modifications as described by Bulleid & Freedman (1988). Plasmid DNA (pUKC1013) was linearized with *Sac*I, and transcription was initiated at the phage-T₇ promoter with phage-T₇ RNA polymerase.

Cell-free translation

Transcribed RNA was translated in a micrococcal-nuclease-treated rabbit reticulocyte-lysate system prepared by the method of Pelham & Jackson (1976), as described by Bulleid & Freedman (1988).

Post-translational proteolysis

Translation mixtures were assayed for translocation by treating post-translationally with proteinase K as described previously (Bulleid & Freedman, 1988).

Immunoprecipitation

Labelled human IFN- γ was immunoprecipitated using monoclonal anti-(human IFN- γ) (20D7) and Protein A-Sepharose. Samples were diluted to 400 μ l in reaction buffer (0.05 M-Tris/HCl/5 mM-EDTA/5 mM-EGTA/0.5% deoxycholate, pH 8.2) and preincubated with 40 μ l of Protein A-Sepharose [10% (v/w) suspension], to 'pre-clear' the samples of Protein A-binding components. Pre-cleared samples were each incubated with 10 μ g of partially purified monoclonal 20D7 for 3 h at 4 °C, then 50 μ l of Protein A-Sepharose (10% suspension) was added. After 30 min, the Sepharose was pelleted and washed three times in wash buffer I [50 mM-Tris/HCl/5 mM-EDTA/0.5% Nonidet P40/0.5 M-NaCl/BSA (1 mg/ml), pH 8.0], then with wash buffer II (as above, but without BSA and NaCl).

SDS/PAGE

Samples were prepared for electrophoresis by mixing with 5 vol. of SDS/PAGE sample buffer [0.02 M-Tris/HCl (pH 8.0)/0.002 M-EDTA/6% (w/v) SDS/20% (w/v) sucrose/0.003% (w/v) Bromophenol Blue/50 mM-dithiothreitol (DTT)] and

boiling for 4 min. SDS/15%-PAGE was performed by the method of Laemmli (1970).

Post-translational deglycosylation

The products of translation were deglycosylated by first immunoprecipitating the IFN- γ as described above. The resulting immunoprecipitate was boiled for 3 min in the presence of 0.5% SDS and 0.1 M-DTT. The sample was diluted with 0.55 M-sodium phosphate buffer, pH 8.6 (the final sodium phosphate concn. was 0.2 M). Nonidet P40 was then added to a final concentration of 1.25%, and the sample was treated with *N*-glycanase (10 units/ml final concn.) for 16 h at 37 °C.

Preparation of microsomal membranes

Microsomal membranes were prepared from dog pancreas as described previously (Austen *et al.*, 1984). Microsomal membranes were prepared from CHO cells by an adaption of the procedure outlined by Walter & Blobel (1983). Approx. 1.2×10^8 CHO cells (transfected with the human IFN- γ gene) were pelleted by centrifugation and washed three times in Buffer A, which contained 50 mM-triethanolamine, pH 7.5, 250 mM-sucrose, 50 mM-potassium acetate, 6 mM-magnesium acetate, 1 mM-EDTA, 1 mM-DTT and 0.5 mM-phenylmethanesulphonyl fluoride (PMSF). The resulting pellet was homogenized in 5 ml of buffer A using a tight-fitting Dounce homogenizer (ten strokes). The efficiency of homogenization was checked by Trypan Blue staining and was found to be approx. 70%. The homogenate was centrifuged at 12000 *g* for 10 min, and the resulting supernatant was loaded on to a Sepharose CL-2B column (2.5 cm $\times$ 50 cm) equilibrated in buffer C [50 mM-triethanolamine (pH 7.5)/1.5 mM-magnesium acetate/1 mM-EDTA/0.5 mM-PMSF/1 mM-DTT]. The turbid void-volume fractions were collected and spun through a 1.3 M-sucrose cushion in buffer A at 140000 *g* for 2 h. The resulting pellet was resuspended to an A_{280} of 50 units/ml in buffer B [50 mM-triethanolamine (pH 7.5)/250 mM-sucrose/1 mM-DTT]. The A_{260}/A_{280} ratio was typically 1.6. Microsomal vesicles were made 1 mM in CaCl_2 and treated with 0.01 mg of micrococcal nuclease/ml at 20 °C for 15 min. EGTA was added to a final concentration of 4 mM, and the microsomal vesicles were snap-frozen in liquid N₂. Nuclease-treated microsomes were added to translation mixtures at a final concentration of 5 A_{280} units/ml.

Preparation of secreted IFN- γ

The CHO cells were plated at 10^5 cells/well in six wells of a 96-well plate and incubated overnight (at 37 °C; CO₂/air, 1:19). The following day the cells were washed three times with methionine-free MEM medium before 13 μ Ci of [³⁵S]methionine in 100 μ l of the above medium was added and the cells were then incubated for 3 h at 37 °C (130 μ Ci/ml final concn.). The conditioned medium was then harvested, centrifuged to remove floating cells, and IFN- γ was immunoprecipitated as described above.

RESULTS

A cDNA clone containing the uninterrupted coding sequence for human IFN- γ was subcloned into the transcription-translation vector Bluescript. The resulting plasmid, pUKC1013, was linearized with *Sac*I and the cloned gene transcribed with phage-T₇ RNA polymerase. The resulting transcript was translated in a cell-free translation/translocation system consisting of rabbit reticulocyte lysate supplemented with dog pancreatic microsomal vesicles (Fig. 1). In the absence of added microsomal vesicles (lane 3), the major polypeptide synthesized had an apparent M_r of 18000, consistent with the M_r for pre-IFN- γ calculated from

Fig. 1. Cell-free translation of IFN- γ mRNA in the presence or absence of dog pancreatic microsomal vesicles

Translation was carried out in a rabbit reticulocyte lysate system, and translation products were resolved by SDS/11%-PAGE and detected by fluorography. Lane 1, Amersham ^{14}C -labelled 'Rainbow' M_r markers; lane 2, products of translation in the presence of microsomes, but without added mRNA; lane 3, products of translation in the presence of mRNA, but without added microsomes; lane 4, products of translation in the presence of mRNA with added microsomes.

Fig. 2. Cell-free translation of IFN- γ mRNA: protection from proteolysis in the presence of microsomal vesicles

Translation of IFN- γ and SDS/PAGE was carried out as described in Fig. 1. Lane 1, products of translation in the absence of microsomes after proteinase K treatment; lane 2, products of translation in the presence of microsomes after proteinase K treatment; lane 3, products of translation in the presence of microsomes, treated with Triton X-100 (1% v/v) and proteinase K.

the deduced amino acid sequence (Devos *et al.*, 1982). In the presence of microsomal vesicles, a polypeptide was synthesized with an apparent M_r of 24 000 (lane 4), which corresponds to the M_r of the mature, fully-glycosylated, IFN- γ (Yip *et al.*, 1982).

The susceptibility of translation products to proteolysis was investigated to ascertain whether the synthesized protein had been translocated to the interior of the microsomal vesicle. Translation was carried out in either the presence or absence of microsomal vesicles. As Fig. 2 (lane 1) shows, the translation products synthesized in the absence of microsomal vesicles were completely degraded, whereas significant protection from proteolysis was observed for translation products synthesized in the presence of vesicles (lane 2). When samples were pre-treated with detergent to solubilize microsomal vesicles before proteinase K treatment, translation products were degraded (lane 3). Thus the products of translation in the presence of microsomal vesicles appear to be translocated across the membrane and segregated in the vesicle interior.

In order to verify that the protein synthesized *in vitro* was

Fig. 3. Immunoprecipitation of IFN- γ from products of translation in the presence of dog pancreatic and CHO microsomal vesicles

Translations were carried out in the presence or absence of dog pancreatic or CHO microsomes. Products of translation were immunoprecipitated with a specific antibody for IFN- γ (20D7) and resolved by SDS/15%-PAGE. Lanes 1 and 7, Amersham ^{14}C -labelled 'Rainbow' M_r markers; lane 2, presence of IFN- γ mRNA, absence of microsomes; lane 3, presence of IFN- γ mRNA, presence of dog pancreatic microsomes; lane 4, presence of IFN- γ mRNA, presence of dog pancreatic microsomes, treated post-translationally to remove oligosaccharide side chains; lane 5, absence of IFN- γ mRNA and presence of CHO microsomes; lane 6, presence of IFN- γ mRNA and presence of CHO microsomes. The inferred products of translation are labelled as follows: x, product arising from incorrect initiation of translation; 0, IFN- γ core polypeptide, signal-peptide cleaved; pIF, pre-IFN- γ ; I, singly glycosylated IFN- γ ; II, doubly glycosylated IFN- γ .

indeed IFN- γ , the translated products were immunoprecipitated with a monoclonal antibody raised against human IFN- γ (Fig. 3). In the absence of microsomes, two translation products were observed, one with an M_r of 18 000 and one with an M_r of 12 000 (lane 2). The polypeptide at 12 000 M_r was not observed in the non-immunoprecipitated material, as it co-migrated with endogenously labelled globin. This translation product probably arises from the incorrect initiation of translation at an internal site, as is the case with thyroid-hormone-binding protein (Cheng *et al.*, 1987), and β -hexosaminidase B (Sonderfeld-Fresko & Proia, 1988). Such an internal site does exist, and it would code for a polypeptide with a calculated M_r of 10 600. The 12 000- M_r polypeptide is unlikely to arise from premature termination, as such a truncated protein would still contain a signal sequence. The 12 000- M_r polypeptide was not translocated, as judged by post-translational proteolysis (Fig. 2, lane 2) and lack of glycosylation. The polypeptide could arise from proteolysis within the lysate; however, the protein runs as a single band, and there is no evidence of smaller degradation products. In the presence of added dog pancreas microsomes, an additional major translation product was seen with an M_r of 24 000 (lane 3) and several minor

products with M_r values of 20 000–21 000 were also observed. An additional translation product was also seen, with M_r 16 500, which is probably processed IFN- γ with the signal peptide cleaved. When the products of translation in the presence of dog pancreas microsomes were deglycosylated, all the higher- M_r bands were lost (lane 4), with the major band corresponding to processed IFN- γ (16 500). Translation products in the presence of microsomes were quantified by densitometry. The results of three experiments revealed that $21 \pm 3\%$ of the IFN- γ immunoprecipitated was not processed, $7.6 \pm 1\%$ was processed, but not glycosylated, and $49.6 \pm 5\%$ was glycosylated, with $41.3 \pm 5\%$ being doubly glycosylated and $8.3 \pm 0.6\%$ singly glycosylated. The mistranslated 12 000- M_r IFN- γ accounted for $19.3 \pm 2.3\%$ of the total.

Microsomal vesicles were also isolated from CHO cells in order to develop a cell-free system to study the initial stages in the glycosylation of recombinant IFN- γ . A CHO-cell line that was active in the secretion of IFN- γ was used; endogenous mRNA was removed by treatment with micrococcal nuclease. When these microsomes were added to a rabbit reticulocyte-lysate system in the absence of added mRNA, no IFN- γ was synthesized (Fig. 3, lane 5).

When mRNA coding for IFN- γ was added, several translation products were immunoprecipitated (lane 6). The M_r values for the major proteins synthesized were the same as for those synthesized in the presence of dog pancreas microsomes (cf. lanes 3 and 6). However, there were some differences in the minor glycosylation products having M_r values in the range of 20 000–21 000. Also the efficiency of translocation and processing of IFN- γ with these microsomes was much reduced. Thus the results from three experiments revealed that, in the presence of CHO microsomes, $51 \pm 1.7\%$ of the immunoprecipitated IFN- γ was not processed, $1 \pm 1\%$ was processed, but not glycosylated, and only $15.3 \pm 5.8\%$ was glycosylated, $9.3 \pm 3.2\%$ being doubly glycosylated and $6.0 \pm 2.6\%$ singly glycosylated. It appears that there was proportionally less doubly glycosylated product in

the presence of CHO microsomes than in the presence of dog pancreas microsomes. Specifically, $82.6 \pm 1.5\%$ of the glycosylation products synthesized in the presence of dog pancreas microsomes were doubly glycosylated, whereas in the presence of CHO microsomes only $61 \pm 4.3\%$ were doubly glycosylated.

The IFN- γ synthesized in the cell-free system was also compared with IFN- γ secreted by the same CHO-cell line. Radiolabelled IFN- γ was isolated by incubating CHO cells for 3 h with [35 S]methionine and immunoprecipitating the secreted IFN- γ from the culture medium with a specific monoclonal antibody. The mobilities of the various forms of IFN- γ secreted from CHO cells were compared with those of the products of translation *in vitro* (Fig. 4). Several IFN- γ glycosylation variants were secreted by the CHO cells, some of which co-migrated with the glycosylated forms synthesized *in vitro*, in the presence of either CHO or dog pancreas microsomes (cf. lanes 3, 4 and 5). The increase in the complexity of bands seen in the secreted form is probably due to both modification of the oligosaccharide side chain (Kelker *et al.*, 1983) and proteolytic trimming of the core polypeptide chain (Rinderknecht *et al.*, 1984).

DISCUSSION

The production of recombinant proteins in heterologous mammalian cells permits the isolation of proteins that are difficult to purify in high yields from natural sources. This is particularly true for growth factors and lymphokines (Parekh *et al.*, 1989). However, the protein produced often differs significantly from the protein isolated from natural sources due to a variety of post-translational modifications which may affect the biological activity of the protein (Berman & Lasky, 1985). In the case of IFN- γ , these modifications are extensive and involve both glycosylation variants and variations in the size of the core polypeptide.

In order to understand more about the generation of these variants, we have studied the cell-free expression of IFN- γ , using a rabbit reticulocyte-lysate system supplemented with microsomal vesicles isolated either from dog pancreas or from CHO cells. A cDNA clone coding for IFN- γ was subcloned into a transcription-translation vector and transcribed *in vitro*. The resulting transcript was translated *in vitro* and, in the presence of microsomal vesicles, co-translational translocation of the nascent polypeptide chain and signal-peptide cleavage occurred. Glycosylation of the protein also occurred, with both the singly and doubly glycosylated forms being synthesized. Some variation was seen in the size of the singly and doubly glycosylated forms, which is probably due to the removal of mannose residues from the core oligosaccharide side chain (Walter & Blobel, 1983). There were also quantitative differences between the glycosylation products synthesized in the presence of dog pancreas and CHO microsomes. This suggests that variations in the yield of either singly or doubly glycosylated protein may occur when IFN- γ is expressed in cells derived from different tissues, and this heterogeneity is determined at an early stage in protein synthesis.

When the products of translation in the presence of microsomal vesicles are deglycosylated, the only products observed are the completely unprocessed pre-IFN- γ and the mature-length IFN- γ polypeptides generated by signal-peptide cleavage (Fig. 2). There is no variation in the size of the processed IFN- γ , suggesting that the endoplasmic reticulum is not the site of the proteolytic trimming of the core polypeptide, which occurs in the secreted form (Fig. 4).

By isolating microsomal vesicles from tissue-culture cells and including these in a cell-free system synthesizing IFN- γ , we have

Fig. 4. Comparison of products of translation *in vitro* with secreted IFN- γ .

Translation of IFN- γ was carried out in the absence or in the presence of dog pancreatic and CHO microsomes, and translation products were immunoprecipitated as described in the text. Radiolabelled IFN- γ was prepared from the conditioned culture medium. Proteins were resolved by SDS/15%-PAGE and detected by fluorography. Lane 1 and 6, Amersham 14 C-labelled 'rainbow' M_r markers; lane 2, products of translation *in vitro* in the absence of microsomes; lane 3, as lane 2, but translated in the presence of dog pancreatic microsomes; lane 4, as lane 2, but translated in the presence of CHO microsomes; lane 5, IFN- γ secreted from CHO cells. x, 0, pIF, I and II are explained in Fig. 3.

been able to reproduce *in vitro* the initial stages in the synthesis, translocation, processing and glycosylation of this protein and to demonstrate that the relative efficiencies of these processes vary with the source of the membranes.

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