Human interferon $\gamma$ potently induces the synthesis of a 55-kDa protein ($\gamma 2$) highly homologous to rabbit peptide chain release factor and bovine tryptophanyl-tRNA synthetase

Jan Fleckner*, Hanne H. Rasmussen†, and Just Justesen‡

Departments of *Molecular Biology and †Medical Biochemistry, University of Aarhus, DK-8000 Aarhus C, Denmark

Communicated by Joan A. Steitz, September 11, 1991 (received for review June 25, 1991)

ABSTRACT An interferon $\gamma$ (IFN-$\gamma$)-inducible protein, $\gamma 2$, was identified by two-dimensional gel electrophoresis of transformed human amnion (AMA) cell proteins. cDNA clones coding for this protein have been isolated and characterized as encoding a polypeptide with a predicted molecular weight of 53,165 and a pl of 6.16. Both values are in good agreement with those observed in two-dimensional gel electrophoresis. The $\gamma 2$ protein is found to be highly induced by IFN-$\gamma$, whereas no induction was seen after addition of IFN-$\alpha$ to AMA cells. A $\gamma 2$-specific 2.7-kilobase mRNA was likewise seen to accumulate selectively in response to IFN-$\gamma$ in these cells. Comparison of the predicted amino acid sequence of $\gamma 2$ to proteins in GenBank data bases revealed that $\gamma 2$ is highly homologous to rabbit peptide chain release factor [Lee, C. C., Craigten, W. J., Muzny, D. M., Harlow, E. & Caskey, C. T. (1990) Proc. Natl. Acad. Sci. USA 87, 3508-3512] and bovine tryptophanyl-tRNA synthetase [Garret, V., Trezequet, B., Pajot, J. C. Gandar, M. Merle, M. Guegiev, J. P. Benedetto, C. Sarger, J. Alteriot, J. La Bouessec, J. Labouessec, and J. Bonnet (1990), GenBank accession no. X52113]. Amino acid sequence similarities of 94% and 97%, respectively, are found, which in general would indicate that $\gamma 2$ represents the human equivalent to either of these two mammalian genes. Based on these sequence similarities, the current data raise the possibility that tryptophanyl-tRNA charging and peptide chain release are carried out by the same enzyme. The $\gamma 2$ protein is shown to possess tryptophan-dependent aminooxyacid-tRNA synthetase activity and thus constitutes an enzymatic activity involved in the biological activity of IFN-$\gamma$.

Interferons (IFNs) are inducible glycoproteins eliciting an antiviral state in target cells (I). In addition they are potent immunomodulators exerting a large number of other effects. IFN-$\gamma$ (type II IFN) is distinct from IFN-$\alpha$ and IFN-$\beta$ (type I IFNs) molecules on the basis of antiangiogenic, inducer, primary structure, cell receptor, and producer cells. IFN-$\gamma$ is more effective than type I IFNs in inhibiting proliferation of various malignant cells and cultured cells and in modulating the activity of cells in the immune system (for reviews, see refs. 2 and 3).

Specific sets of proteins are induced in various cell lines after IFN treatment (4-6). Addition of IFN-$\gamma$ to various cell lines results in the preferential induction of a set of genes including major histocompatibility complex II genes (7, 8), the Fc receptor gene for IgG (9), $\gamma$IP-10 and m119 (both belonging to the platelet factor 4 family (10, 11)), phagocyte cycthrome b heavy chain gene (12), the gene encoding indoleamine 2,3-dioxygenase (13), and two genes IP-30 and $\gamma$1 of unknown functions (14, 15).

We have previously used two-dimensional gel electrophoresis [isoelectric focusing (IEF) and SDS] to characterize sets of human polypeptides specifically induced by IFN-$\alpha$/IFN-$\beta$ or by IFN-$\gamma$ (4). The cloning of genes of such proteins is an essential step in the analysis of pathways underlying major or specific effects of IFN action. Here we report on cDNA cloning and expression studies of the $\gamma 2$ protein, which is preferentially and very strongly induced by IFN-$\gamma$ in AMA cells (epithelial) and MRC-5 cells (fibroblast) (4). The deduced M, 53,165 amino acid sequence is highly homologous to that of rabbit peptide chain release factor (16) and bovine tryptophanyl-tRNA synthetase [EC 6.1.1.2, M. Garret, V. Trezequet, B. Pajot, J. C. Gandar, M. Merle, M. Guegiev, J. P. Benedetto, C. Sarger, J. Alteriot, J. La Bouessec, J. Labouessec, and J. Bonnet (1990), GenBank accession no. X52113].

MATERIALS AND METHODS

Cell Culture, Protein Labeling, and Gel Electrophoresis. Human amnion cells (AMA) were grown in monolayers in Dulbecco's modified Eagle's medium (DMEM) (Biocrom, Berlin) supplemented with 10% (vol/vol) newborn calf serum and antibiotics [penicillin at 100 international units (IU)/ml and streptomycin at 50 $\mu$g/ml]. Recombinant human IFN-$\gamma$ and [35S]methionine were purchased from Amersham. Recombinant human IFN-$\alpha$ 2B (IFN-$\alpha$) was purchased from Schering. Cells were labeled in DMEM lacking methionine and containing 10% (vol/vol) dialyzed fetal calf serum and [35S]methionine at 100 $\mu$Ci/ml (1 Ci = 37 GBq). After labeling cells were lysed in buffer E [20 mM Tris Cl, pH 7.5, 5 mM Mg(OAc)$_2$, 10 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 10% (wt/vol) glycerol] containing 0.5% Nonidet P-40 and analyzed by SDS/PAGE (17) or two-dimensional gel electrophoresis (18). The $\gamma 2$ protein purified to ~80% purity (SDS/PAGE estimate) was subjected to two-dimensional gel electrophoresis. The $\gamma 2$ spots were cut from the gels, concentrated by SDS/PAGE, electroblotted onto a ProBlot membrane (Applied Biosystems), and tryptic peptides were subjected to amino acid sequencing on a gas-phase sequencer (Applied Biosystems model 470A) (18).

Oligonucleotide Primers and PCR. Based on the amino acid sequence of peptide 4 (see Results), two degenerate oligonucleotides were synthesized using an Applied Biosystems model 381A DNA synthesizer. The 17-base pair (bp) oligonucleotide 4a represents amino acids 3-8, whereas the 17-bp oligonucleotide 4b is the reverse complement of the DNA encoding amino acids 15-19. All possible codons were represented for each amino acid, except for serine where the oligonucleotide sequence choice was based on the mammalian codon usage. PCRs contained (in 25 $\mu$l) 25 pmol of each primer, 1 $\mu$l of first-strand cDNA primed by oligo(dT) (19), 10

Abbreviations: IFN, interferon; IEF, isoelectric focusing; IU, international unit(s).

†To whom reprint requests should be addressed.

‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. X59892).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.
mM Tris Cl (pH 8.3), 50 mM KCl, 2 mM MgCl₂, 0.1% gelatin, and 0.2 unit of Taq polymerase (Cetus). For the amplification, 40 cycles of denaturation (94°C, 1 min), annealing (45°C, 1 min), and extension (72°C, 3 min) were used.

Construction and Screening of cDNA Library. Twice poly(A)⁺-selected RNA (5 µg) was prepared from AMA cells that had been exposed to IFN-γ (100 IU/ml) for 4 h or 8 h (post-tau). cDNA was synthesized (19) and a library was constructed in Agt10 (20). Screening was performed by plaque hybridization using a 32P-labeled PCR-derived partial γ2 cDNA (see Results).

Sequencing of cDNA Clones. BAL-31 exonuclease (Amer- sham) deletions of the cDNA clones were inserted into bacteriophage M13 (21). DNA sequencing of overlapping deletions was done by the dideoxy-nucleotide chain- termination method using reagents from United States Biochemical.

RNA Preparation and Northern Blot Analysis of RNAs. All RNA samples were prepared by the acid guanidinium thiocyanate/phenol/chloroform extraction method (22). Northern blot analysis was done by electrophoresis of total cellular RNA through 1% formaldehyde/agarose denaturing gels and transfer to Zeta-Probe membranes (Bio-Rad) in 10× SSC (1.5 M NaCl/0.15 M sodium citrate, pH 7.0). Radioactive DNA probes were prepared by random oligonucleotide priming (23).

Assay for Tryptophan Aminocyl Synthetase Activity. To test for enzymatic activity of γ2 protein the trytophan-dependent ATP–pyrophosphate exchange assay (24) was used in a modified form using TLC to identify ATP and pyrophosphate (25). The 20-µl reaction mixture of 10 mM Tris Cl (pH 7.5), 10 mM MgCl₂, 4 mM sodium pyrophosphate, 2 mM ATP, 0.02% gelatin, and where indicated 0.1 mM t-tryptophan or 0.1 mM t-leucine, contained 0.25 µCi of [γ-32P]-ATP and 1–4 µl of purified γ2 protein. After incubation at 30°C for 10–50 min, 3 µl was spotted onto a polyeth- ylenimine-cellulose (PEI) thin layer plate. The chromatograms were developed in 0.75 M KH₂PO₄, and the labeled spots of ATP and pyrophosphate were localized by autoradiography. The spots were cut from the chromatograms and the radioactivity was measured.

RESULTS

Induction of the γ2 Polypeptide in AMA Cells. As described (4), IFN-γ induces a specific set of peptides in AMA cells. The location of γ2 in two-dimensional gels of AMA cell proteins in untreated (Fig. 1A) and IFN-γ-treated (Fig. 1B) cells is shown. The strongly induced γ2 polypeptide has an apparent molecular mass of 55.3 kDa (Figs. 1 and 2) and a pI of 5.99 (Fig. 1). To study the induction kinetics, AMA cells were pulse-labeled for 1 h at various times after IFN-γ addition. At 4–6 h after induction, a γ2 band became clearly visible, increased dramatically in intensity, and reached a maximum level after 10 h (Fig. 2). This high level was maintained for at least 24 h in the presence of IFN-γ.

Isolation of γ2 cDNA. Amino acid sequences of internal tryptic peptides were obtained by microsequencing of the γ2 protein spots excised from two-dimensional gels as described (18). Amino acid sequences were obtained from the following four peptides ranging in length from 12 to 19 amino acids: 1, MSASPNSSSTLTDTA; 2, GIGFTDSDXIG; 3, XX- TDIXLIXPXDIDQDPX; 4, ISPAFQAAAPSFSNSPQI.

Based on the sequence of peptide 4, two degenerate oligonucleotides were designed (4a, TTYCCNCGNATH- CARGC; 4b, ATYGANGGRAANGARRT, where Y is a pyrimidine, N is any nucleotide, R is a purine, H is A, T, or C) and used as primers for the amplification of a 50-bp PCR fragment. Since several attempts failed to amplify a γ2-specific cDNA fragment using oligonucleotide 4a and oli-
clones were identified by plaque hybridization. Insert sizes were assessed by PCR identifying two inserts of 2.6 kb (clones y2A1 and y2A71), which were subcloned into bacteriophage M13. Both clones had sequences corresponding to a polypeptide of 471 amino acids. All four tryptic peptide sequences obtained by microsequencing of the y2 polypeptide were identified in the predicted polypeptide. The deduced polypeptide has a calculated molecular weight of 53,165, consistent with the observed apparent molecular mass of 55.3 kDa. The calculated PI for the predicted polypeptide is 6.16, compared to the observed PI of 5.99. This difference might implicate the presence of secondary modifications of the y2 protein. The predicted sequence was confirmed by sequencing the first 15 N-terminal amino acids of purified native y2 (data not shown). In the native y2, the initiator methionine was found to be absent. The stop codon at position 1527 was followed by an ~1-kb untranslated sequence including a conventional polyadenylation signal AATAAA at nucleotide position 2588.

Expression of y2 mRNA in Response to IFNs. Northern blot analysis of RNA from IFN-γ-treated AMA cells using the y2 cDNA insert as a [32P]-labeled probe detected a major transcript of 2.7 kb (Fig. 4A) and, at high doses of IFN-γ, two minor transcripts of 2.0 and 1.5 kb. A response to IFN-γ was observed at doses as low as 10 units/ml and y2 mRNA was fully induced at IFN-γ levels of 100 units/ml (dot blot analysis, data not shown). The major band of 2.7 kb indicates that the y2 cDNA represents at least 95% of the y2 mRNA and likely the entire exon sequences, depending on the length of the poly(A) tail. The identity of the minor bands remains uncertain and might represent differential splice products or specific degradation products.

The time course of y2 induction in AMA cells was followed by RNA dot blot analysis. After a 4-h incubation with IFN-γ, an increase in y2-mRNA level was observed that continued for 10–12 h, where a maximum level of 60-fold induction was reached. y2 mRNA stayed at this maximum level for 24 h in the continued presence of IFN-γ. This pattern of induction was reproducibly obtained with other preparations of human IFN-γ (recombinant IFN-γ (Boehringer Mannheim) and native IFN-γ (kindly provided by Kurt Berg, University of Copenhagen)). In contrast virtually no response was observed when IFN-α was added to the AMA cells (Fig. 4B).

y2 mRNA was measured relatively to GAPDH mRNA, which is unaffected by IFN treatment.

In various human cell lines, variation in the induction of y2 mRNA by IFN-γ was observed. In MRC-5 and HT1080 fibroblasts, y2 mRNA was strongly induced by IFN-γ and unaffected by IFN-α. In HeLa (epithelioide) cells, y2 mRNA was highly inducible by IFN-γ (~35-fold) weakly inducible by IFN-α (~5-fold). In the lymphoblastoid cell lines MOLT-4 and NALM-6, y2 mRNA was not induced by either IFN-γ or IFN-α (data not shown).

Comparison of the Nucleotide and Predicted Polypeptide Sequence of y2 with Other Sequences. The nucleotide sequence of the y2 CDNA was compared to sequences in the GenBank data bases using the FASTA program from the Genetics Computer Group of the University of Wisconsin, based on the Lipman and Pearson (27) search for similarity. Homology was found to mammalian (rabbit) peptide chain release factor (16) and bovine tryptophanyl-tRNA synthetase (GenBank accession no. X52113). At the nucleotide level extensive homology was found throughout the coding region to both sequences, 85% to tryptophanyl-tRNA synthetase and 84% to peptide chain release factor. In the noncoding regions homology to these sequences dropped to 59% and 54%, respectively. At the polypeptide level the homology was even greater as shown in Fig. 5, where y2, peptide chain release factor and tryptophanyl-tRNA synthetase amino acid sequences show extensive homology.

**Fig. 3.** Complete nucleotide sequence and predicted amino acid sequence of y2 (clone y2A1). The numbers indicate the nucleotide and amino acid positions. The predicted initiator methionine codon begins at nucleotide 112. Peptide sequences obtained by microsequence analysis of y2 are shaded. The polyadenylation signal AATAAA is underlined and the poly(A) sequence is indicated by A•.
The GAPDH was exposure originally for y2 in interfered the region with GAPDH mRNA. The dot IFN-a total RNA in were extreme similarity for membranes amino acid of aids amino acid motif (28). (28). (B) Induction of y2 transcript by IFN-γ. AMA cells were treated with IFN-γ at 100 IU/ml and IFN-α at 1000 IU/ml for the indicated times. For each spot on the dot blot, ~4 μg of total RNA was applied to Zeta-Probe membranes by using a minifold. Hybridization conditions were as described in A. After autoradiography filters were stripped and rehybridized for measurement of GAPDH mRNA levels. Quantitation were done by scanning the films, and the densitometric values for y2 mRNA were normalized relative to values obtained for GAPDH mRNA. Each point is the mean of double determinations.

sequences are aligned. y2 displays identity with 94% of the amino acids in tryptophanyl-tRNA synthetase and with 87% of the amino acids of peptide chain release factor. When conservative amino acid substitutions were considered, similarity rose to 97% and 94% for these sequences, respectively. The region with the highest degree of divergence was the extreme N-terminal region, in which the three proteins differed in length.

At position 170, y2 possesses a HIGH amino acid motif as identified in prokaryotic class I tRNA synthetases (Fig. 5) (28). Sequencing of the region around amino acid positions 164–170 initially resulted in the translated amino acid sequence PLLKQCN. As this sequence can be transformed into that of tryptophanyl-tRNA synthetase by a simple +1 frameshifting over eight codons, the sequence analysis of area was repeated using ITP instead of GTP to resolve any compressions. This analysis indeed revealed a compression around nucleotide 602 adding an extra nucleotide to the sequence, which again was compensated by another sequencing error 21 nucleotides further downstream. The new nucleotide sequence translated into the amino acid sequence PSSEAMNH forming part of the HIGH motif (Fig. 5). Prokaryotic class I amino acid tRNA synthetases are also characterized by the KMSKS amino acid motif, which in combination with the HIGH motif is indicative of the presence of a Rossman fold, thought to be involved in nucleotide binding (29, 30). In y2 and WRS (and eRF), a C-terminal motif KMSAS homologous to KMSKS was also found (Fig. 5).

**Tryptophan-Dependent Aminoacyl-tRNA Synthetase Activity of Purified y2 Protein.** The y2 protein was purified to ~90% purity by a series of column chromatography steps including DEAE-cellulose, heparin-Sepharose, and Procion red-Sepharose. The purified fraction was tested for aminoacyl-tRNA synthetase activity using the pyrophosphate exchange assay (24). Fig. 6A shows a polyethyleneimine-cellulose chromatogram giving evidence for a tryptophan-dependent pyrophosphate exchange reaction. Further purification of the y2 protein through a Superose 12 (Pharmacia) column yielded a protein fraction eluting at ~110 kDa. Fig. 6B shows the time course of the tryptophan-dependent pyrophosphate exchange reaction using this fraction. These data suggest that native y2 protein exists as a dimer in agreement with studies of bovine tryptophanyl-tRNA synthetase (31).

**DISCUSSION**

We have isolated complete 2.6-kb cDNA clones encoding the IFN-γ-inducible y2 polypeptide. The clones were isolated from a λgt10 cDNA library constructed from poly(A)⁺-selected RNA isolated from IFN-γ-induced human amniotic epithelial cells by screening with a partial y2 cDNA fragment. This fragment was obtained by PCR amplification using oligonucleotides synthesized on the basis of information obtained by microsequencing tryptic peptides from the y2 polypeptide. The 2.6-kb cDNA contains an open reading frame of 1413 nucleotides (Fig. 3). In AMA cells the amount of y2 polypeptide was found to increase dramatically upon IFN-γ treatment (Figs. 1 and 2) as did the level of mRNA (Fig. 4A), where a 60-fold increase was observed (Fig. 4B). The y2 mRNA was not induced by IFN-α in AMA cells, indicating that the gene belongs to the group of genes that preferentially respond to type II IFN. In other cell lines (five tested), y2 mRNA induction can also be observed preferentially in response to IFN-γ or not at all.

Comparison of the predicted amino acid sequence to the available sequence information in GenBank data bases (release 26.0) demonstrated high sequence similarities to rabbit polypeptide chain release factor (16) and bovine tryptophanyl-tRNA synthetase (WRS, GenBank accession no. X52113) amino acid sequences. Only amino acids differing from the y2 sequence are indicated (one-letter code). Dashes, positions where the amino acids of eRF or WRS are identical to that of y2; star, inserted gap.

**FIG. 5.** Alignment of y2 amino acid sequence to rabbit eukaryotic polypeptide chain release factor (eRF, ref. 16) and bovine tryptophanyl-tRNA synthetase (WRS, GenBank accession no. X52113) amino acid sequences. Only amino acids differing from the y2 sequence are indicated (one-letter code). Dashes, positions where the amino acids of eRF or WRS are identical to that of y2; star, inserted gap.
We thank O. Sønderskov for photography. We also thank B. J. Christensen for technical assistance. Special thanks go to N. Pallisgaard for help with construction of the library, L. K. Rasmussen for the N-terminal sequencing, Prof. N. O. Kjeldgaard for critical reading of the manuscript and valuable discussions, and Prof. J. E. Celsis for help running the 2D gels. This research was supported by grants from Danish Biotechnology program, the Danish Natural Science Research Council, the Danish Cancer Foundation (H.H.R.), and the Velux Foundation.