**Gamma Interferon Potently Induces Tryptophanyl-tRNA Synthetase Expression in Human Keratinocytes**

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Incubation of human keratinocytes with gamma interferon (γ-IFN) induces the synthesis of a 53-kDa protein of unknown nature and function. We report the identification of this protein through amino acid microsequencing. The NH2-terminal amino acid sequence of the 53-kDa antigen demonstrated that this protein was tryptophanyl-tRNA synthetase (Frolova et al, Gene 109:291–296, 1991, Genbank accession number 61715). This result was validated by the sequencing of tryptophanyl peptides. Identification of the 53-kDa γ-IFN–induced protein was confirmed by immunoblotting with an antiserum directed against beef pancreas tryptophanyl-tRNA synthetase. Northern blot analysis using a synthetic oligonucleotidic 32P-labeled probe evidenced a 3.1-kb transcript in γ-IFN–treated cells indicating that the gene was regulated at the pre-translational level.

These data show that γ-IFN potently induces in keratinocytes the expression of an enzyme directly involved in protein biosynthesis. Elevated levels of tryptophanyl-tRNA synthetase in treated cultured keratinocytes might be involved in the cell-growth–inhibitory activity of gamma interferon. *J Invest Dermatol* 100:775–779, 1993

**G**amma interferon (γ-IFN), which is synthesized and secreted by activated T lymphocytes, exerts a broad spectrum of biologic activities in target cells with properties similar to those of alpha and beta IFNs but with a greater efficiency (for review see [1]). In the skin, γ-IFN induces the synthesis and expression of HLA-DR antigens on keratinocytes [2,3] as well as with other molecules such as the intercellular adhesion molecule-1 (ICAM-1) and the IP-10 protein [4–6]. In inflammatory dermatoses characterized by an activated T-cell infiltrate, these molecules contribute to the direct activation of keratinocytes by production of pro-inflammatory cytokines and can profoundly influence the course of the local immune response [7–9]. γ-IFN interacts with keratinocytes in a biologically relevant fashion, because specific high-affinity receptors have been characterized on the keratinocyte cell membrane [10,11].

In *vitro*, γ-IFN interferes with the biosynthesis of numerous products of the keratinocyte metabolism with consequent effects on cell behavior. Modulations of keratinocyte antigens involved either in the differentiation process such as cell-surface glycoproteins [12,13] or in cell adhesion such as fibronectin [14], thrombospondin [15], and bullous pemphigoid antigens [16] are evidence of drastic modifications of the normal keratinocyte metabolism upon stimulation with γ-IFN. Moreover, specific sets of proteins are induced by γ-IFN in various cells including human keratinocytes [17–20], including in particular, the biosynthesis of a 53-kDa protein [20,21].

The model of expression of this component, which is localized in the cell cytoplasm, has been previously determined [21]. However, the nature and the biologic significance of this γ-IFN–induced protein are still unknown [20,21]. In the experiments reported here we show that the 53-kDa protein is tryptophanyl-tRNA synthetase and that induction of tryptophanyl-tRNA synthetase protein by γ-IFN reflects induction of specific mRNA.

**MATERIALS AND METHODS**

**Cell Culture** Human epidermal cell cultures were prepared from breast skin removed during plastic surgery. Primary cultures of keratinocytes were grown on 3T3 feeder layers [22]. Subcultures were obtained by seeding culture dishes at 2 × 10⁶ cells/cm² on irradiated 3T3 cells. Culture medium was as previously described [12], a 3:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium, supplemented with 10% fetal calf serum, hydrocortisone 0.4 μg/ml, insulin 5 μg/ml, triiodothyronine 2 × 10⁻⁹ M, choleraen 10⁻¹⁰ M, epidermal growth factor 10 ng/ml, and antibiotics. Cultures were maintained at 37°C in 5% CO₂. Under these conditions, a confluent layer of epidermal cells was obtained within 1 week. At this time, some cultures were maintained in the same medium; others were exposed to γ-IFN (Amersham International Pic, Buckinghamshire, England, specific activity 1.7 × 10⁷ IU/mg), 100 IU/ml culture medium. Medium was changed daily. All experiments were carried out on first-passage cells.

**Protein Extraction and Polyacrylamide Gel Electrophoresis** Epidermal cell cultures were rinsed with phosphate-buffered saline on ice, and the cells were scraped into extraction buffer: 0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.5% Nonidet P-40 (NP-40), 10 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 5 mM N-ethylmaleimide, and 1 μg/ml aprotinin. Cultures were ground at 4°C by 30 strokes in a Teflon/glass homogenizer and the homogenate was briefly sonicated. After centrifuga-

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Abbreviations: CAPS, 3-cyclohexylamino-1-propanesulfonic acid; ICAM-1, intercellular adhesion molecule-1; γ-IFN, human recombinant gamma interferon; IU, international unit; PVDF, polyvinylidene difluoride.
tion (13,000 × g, 4°C, 15 min), the supernatant was collected and stored at −70°C. Sodium dodecylsulfate–polyacrylamide gel electrophoresis was performed on slab gels using the system of Laemmli [23] modified by the use of 5–15 or 8–12% gradients of polyacrylamide [12].

N-Terminal Amino-Acid Sequencing NP-40 soluble proteins from 8-d γ-IFN–treated cultures were run on 8–12%, 1-mm–thick gradient gels, then electrophotoretherically transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon, Millipore Corp., Bedford, MA) in 0.1 M CAPS pH 10, 10% methanol buffer. Proteins were visualized by Coomassie Brilliant blue R250 staining (Serva, Heidelberg, Germany), and the 53-kDa band was carefully excised using a sharp blade. After further washings in H2O, the 53-kDa protein containing PVDF bands were stored at −70°C until N-terminal amino acid analysis.

The N-terminal sequence determination was performed by Edman degradation using a 470-A Applied Biosystems gas phase sequencer. Phenylthiohydantoin (PTH) amino acids were identified on-line with a 120-A Applied Biosystems PTH-Analyzer. An IBM PC-AT microcomputer was connected to the PTH-Analyzer and was used to quantify the PTH amino acids by means of the System Gold software (Beckman). All the products and reagents used for sequencing were from Applied Biosystems (Foster City, CA).

Before sequencing, the Immobilon pieces were cut into 1 × 1 mm pieces and placed into the cartridge of the sequencer; no polybrene-treated glass fiber filters were used. The sequencing was performed using the 03RPTH program provided by Applied Biosystems, with slight modifications. Chemical delivery rates were carefully monitored during the sequence determination.

The quantity of PTH-proline at the first cycle was 12–14 pmoles; nevertheless, as the exact amount of material loaded on the sequencer was not known, the initial yield could not be calculated. The repetitive yield was determined through semi-logarithmic regression analysis of all identified PTH amino acids, except PTH-Ser, which were recovered in low yield. The repetitive yield was between 88 and 93%.

Internal Amino Acid Sequence Determination The 53-kDa protein contained on PVDF membranes was in situ digested by trypsin according to Bauw et al [24]. Briefly, pieces of PVDF bands were treated for 30 min with 400 μl of a 0.2% polyvinylpyrrolidone solution in methanol; then an equal volume of water was added and the supernatant discarded after 10 min. PVDF pieces were washed four times with water and once with 0.1 M Tris HCl pH 8.5 buffer. They were replaced in 100 μl of the same buffer; 1 μl of a trypsin solution (1 mg/ml) was added and the digestion proceeded for 6 h at 37°C. Then the supernatant was collected and the PVDF pieces were washed with 100 μl of 80% formic acid and four times with 100 μl of water; all the washing solutions were added to the digestion mixture.

The peptides contained in the combined washing solutions were separated by reversed-phase high-performance liquid chromatography, using a C8 Aquapore RP300 column (22 × 0.4 cm; Brownlee) and a linear gradient of acetonitrile in 0.1% trifluoroacetic acid. Eluting peptides were detected by UV absorbance at 214 nm, collected in Eppendorf tubes, and stored at −20°C prior to sequence analysis.

Peptides were loaded on a polybrene-treated glass fiber filter and amino acid determinations were performed with the equipment described above.

Computer Search for Identity The amino acid sequence homology searches were carried out using the Biscane computer program of Citi 2 for screening protein databases [25]. The protein database was Genbank (release 73, September 1992).

Immunoblotting The proteins from unstained gels were transferred electrophoretically to nitrocellulose membranes (Schleicher & Schuell BA 86) essentially according to the immunoblot technique described by Towbin et al [26]. In these assays, the polyclonal antibody to beef pancreas tryptophanyl-tRNA synthetase was diluted to 1/50,000. Non-fat powdered milk 5% in Tris-buffered saline (10 mM Tris HCl, pH 7.6, 140 mM NaCl (TBS)) was used to saturate the free-binding capacity of the nitrocellulose membrane.

For immunodetection of the antigens, the sheets were incubated at room temperature for 2 h in diluted antibody, washed for 1 h in six changes of washing buffer (0.5% NP-40 in TBS), and incubated for 2 h with peroxidase-labeled Fab goat anti-rabbit IgG (Bioys, Compiegne, France). After further washings, the specific staining reaction was visualized with 3-3’ diaminobenzidine (0.05 mg/ml) and 0.03% H2O2 in 100 mM Tris HCl, pH 7.6. Controls were obtained by using the preimmune serum or by omitting the first layer antibody.

RESULTS

Identification of the γ-IFN–induced 53-kDa Protein by Microsequencing Sodium dodecylsulfate–polyacrylamide gel electrophoresis analysis of NP-40 keratinocyte-stratified culture extracts revealed the presence of numerous protein bands distributed over a wide range of M, (Fig la–d). Comparison of protein profiles from cultures grown with (Fig 1c,d) or without (Fig 1a,b) γ-IFN (100 IU/ml) showed subtle differences in the high and low M proteins. However, the most prominent change was the large amount of a 53-kDa protein, which was selectively altered in extracts from treated cultures. From NH2-terminal sequence analysis of the following sequence was identified: PNPESAPLELXNISAXQ. Comparison of this sequence with those available in databank revealed complete homology to human tryptophanyl-tRNA synthetase, EC number “6.1.1.2” (Frolova et al, Gene 109:291–296, 1991, Genbank accession number 61715).

The identity of the 53-kDa protein was further investigated by internal sequences determinations. Five peptides produced during in

RNA Isolation and Northern Blotting Analysis A single-strand oligonucleotide probe (30 mer) synthesized from the nucleotide sequence of the cDNA encoding for human tryptophanyl-tRNA synthetase (nucleotide +1577 to +1606) [27] was used to characterize mRNAs specific for the protein by Northern blot analysis. A specific probe for human β-actin was purchased from Oncogene Science (Manhasset, NY). These probes were end labeled with γ32P ATP (3000 Ci/mmol, Amersham) using T4 polynucleotide kinase (Appligene, Strasbourg, France). Oligolabeling was done according to Miyada’s method [28] and labeled probes were purified on Sephadex G25 column (Pharmacia LKB Biotechnology, Uppsala, Sweden). Labeled oligoprobes were eluted using TNE buffer (10 mM Tris-HCl, pH 7.5 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid). Specific activity was typically in the range 2.5 to 5 x 108 cpm/μg.

Total cellular RNA was prepared from keratinocytes grown with or without γ-IFN (100 IU/ml) for 48 h, according to the method developed by Chomczynski and Sacchi [29] and estimated by absorbance at 260 nm. Isolated total RNA was electrophoresed in a 1.2% agarose gel with 6.5% formaldehyde and 48.5% formamide, then transferred onto a nylon membrane (Hybond-N, Amersham). The Hybond-N sheets were baked at 80°C for 30 min, then pre-hybridized at 65°C for at least 1 h. The pre-hybridization solution contained 10% Dextran sulfate, 1% sodium dodecylsulfate, 100 μg/ml of denatured non-homologous DNA (i.e., salmon sperm DNA) in 1 M NaCl, 50 mM Tris-HCl buffer, pH 7.5. Hybridization was performed overnight at 65°C in the above mixture supplemented with the 32P-labeled oligonucleotide probe (1 x 106 cpm/ml hybridization buffer). The filters were washed 4 times with 2 x sodium citrate/sodium chloride buffer, 0.1% sodium dodecylsulfate at room temperature; the last washing was done without sodium dodecylsulfate. The filters were then exposed to Amersham films in the presence of intensifying screens for 24 h and 3 d.
**Figure 1.** Effect of γ-IFN on keratinocyte protein profile. NP-40 extracts from stratified epidermal cell cultures are separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis on 5–15% gel and visualized by Coomassie Brilliant blue R250 staining. Extracts from control keratinocyte cultures (a,b) and γ-IFN–treated cultures (c,d); asterisk, the 53-kDa protein (see text). The M₅ of molecular mass markers X1000 is shown on the left; myosin, 205 kDa; B-galactosidase, 116 kDa; phosphorylase B, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 44 kDa; and carbonic anhydrase, 29 kDa.

**Table 1.** Partial Amino Acid Sequences of the 53-kDa Protein

<table>
<thead>
<tr>
<th>Amino Acid Sequences*</th>
<th>Identified Protein*</th>
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<tbody>
<tr>
<td>PVDF membrane</td>
<td></td>
</tr>
<tr>
<td>Peptide 1</td>
<td>MSASDPPNSIFLTDTAK</td>
</tr>
<tr>
<td>Peptide 2</td>
<td>DLTLQAYSYAVENAK</td>
</tr>
<tr>
<td>Peptide 3</td>
<td>DMNQVLDAVENKFFLYTGR</td>
</tr>
<tr>
<td>Peptide 4</td>
<td>AL2EVQLPAE4HVAR</td>
</tr>
<tr>
<td>Peptide 5</td>
<td>IPFPAPIQAPFSNSNPSQIFR</td>
</tr>
</tbody>
</table>

*The amino acid residues are given in the one-letter notation. X: no PTH-amino acid could be detected. †, the corresponding residue could not be identified due to a PTH-Analyzer failure.

**Confirmation of the Identification of the γ-IFN–induced Protein as Tryptophanyl-tRNA Synthetase by Immunoblotting** To confirm that tryptophanyl-tRNA synthetase is induced by γ-IFN, we raised an antiserum against beef pancreas tryptophanyl-tRNA synthetase [30] and used it to probe a blot of NP-40 soluble proteins from human keratinocytes treated with control medium or γ-IFN (Fig 2). In an immunoblot of extract from γ-IFN–treated keratinocyte cultures, the antiserum strongly reacted with a protein band of molecular mass 53 kDa (Fig 2b). When blotted on extract from epidermal cell cultures grown in the absence of γ-IFN, the antiserum failed to detect any protein (Fig 2a).

**Northern Blotting** To characterize the γ-IFN–induced 53-kDa protein specific transcript in human keratinocytes, we performed Northern blot analysis. Because it was demonstrated that this protein is tryptophanyl-tRNA synthetase, we used a single-strand oligonucleotide probe synthesized from the sequence of the cDNA encoding for human tryptophanyl-tRNA synthetase [27]. In these experiments a ~3.1-kb transcript was found in γ-IFN–treated cells (Fig 3d,c), whereas it was not detected in keratinocytes grown in control medium (Fig 3a,b,c). However, Northern blot analysis of poly(A)⁺ RNA revealed a faint expression of tryptophanyl-tRNA synthetase transcripts in control keratinocytes (results not shown).

**DISCUSSION**

We demonstrate that the 53-kDa protein induced in human keratinocytes by γ-IFN is tryptophanyl-tRNA synthetase and that induction of the protein is a consequence of induction of tryptophanyl-tRNA synthetase mRNA.

It was recently reported that the γ2 protein induced by γ-IFN in transformed human amnion cells was highly homologous to rabbit peptide chain release factor and bovine tryptophanyl-tRNA synthetase [31]. Tryptophanyl-tRNA synthetase was also demonstrated in
induced expression is regulated should help elucidate some mechanisms of \( \gamma \)-IFN action on target cells that may be important in inflammatory processes.

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