BIOCHEMICAL CHARACTERIZATION OF A
γ INTERFERON–INDUCIBLE CYTOKINE (IP-10)

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The cellular immune response is characterized by the complex interaction of
many different cells responding to multiple extracellular signals. Activation,
proliferation, and directed migration of both local and blood-borne cells is partly
regulated by soluble mediators released by cells, referred to collectively as
cytokines. IFN-γ, a glycoprotein secreted from activated T cells, has potent
immunomodulatory activities and is an important activator of the cellular im-
une response (reviewed in reference 1). In fact, local intradermal injections of
IFN-γ have been shown to elicit certain features of the delayed-type hypersen-
sitivity reaction, such as induration, T cell and monocyte infiltration, keratinocyte
proliferation, and dermal and epidermal HLA-DR antigen expression (2).

To begin to address the molecular mechanisms that underly the biological
activity of IFN-γ in the immune response, we have isolated and characterized
several genes that are induced in human cells by IFN-γ (3). The deduced amino
acid sequence of one such gene, referred to as IP-10, is predicted to specify a
secreted polypeptide of 10 kD (4). The expression of the IP-10 gene is an early,
primary, transient response to IFN-γ stimulation of responsive cells. Transcrip-
tion is induced as early as 30 min after IFN-γ treatment of cells and mRNA
accumulation peaks by 5 h. IP-10 mRNA is induced >30-fold in a variety of cells,
including mononuclear cells and fibroblasts.

The primary amino acid sequence of the predicted IP-10 protein demonstrates
significant homology to a newly emerging family of chemotactic and mitogenic
proteins associated with inflammation and cell proliferation. This family of
proteins includes: platelet factor 4 (PF4)1 (5); the platelet basic proteins (β-
thromboglobulin [6] and connective tissue–activating peptide III [7]); 9E3, a
protein induced by Rous sarcoma virus transformation and associated with
proliferating fibroblast monolayers (8); and 310c, a protein induced in leukocytes
by the mitogen staphylococcal enterotoxin A (9). The genomic organization of
the IP-10 gene, when compared with the organization of the rat PF4 gene,
indicates that these genes evolved from a common ancestor (10). In fact, the

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1 Abbreviations used in this paper: αMEM, α-modified Eagle’s medium; βTG, β-thromboglobulin;
HUVE, human umbilical cord endothelial cells; KLH, keyhole limpet hemocyanin; PF4, platelet
factor 4; TBS, Tris-buffered saline.

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entire protein family most likely evolved from a common ancestral gene by gene duplication, establishing a novel gene family of chemotactic and mitogenic cytokines, which are released from a variety of cells in response to diverse biological stimuli.

The association of the IP-10 protein with this family of cytokines suggests possible biological functions for this protein in an inflammatory response, which may account for some of the diverse activities seen during the progression of a cellular immune response. In this and an accompanying paper (11) we have begun to address the function, localization, and processing of the IP-10 protein. Through the production of polyclonal, monospecific antisera directed against a recombinant IP-10 protein expressed in Escherichia coli and a synthetic peptide synthesized to the COOH-terminus of the protein, the biosynthetic steps resulting in the secretion of the IP-10 protein have been elucidated. Kaplan et al. (11) describe the results of in vivo studies in which the expression of the IP-10 protein induced during the course of a delayed-type hypersensitivity reaction in the skin and after intradermal injection of IFN-γ is followed. These studies suggest an association between this protein and the development of a cellular immune response.

### Materials and Methods

**Cell Culture.** Human endothelial cells were isolated from umbilical cord veins (12) and grown in M199 medium supplemented with 20% heat-inactivated human serum, penicillin (100 U/ml), and streptomycin (100 µg/ml). All the experiments presented in this study were performed on second-passage human umbilical cord endothelial cells (HUVE). A primary keratinocyte cell line isolated from human foreskin was obtained from Clonetics Corporation (Boulder, CO) and maintained in a defined keratinocyte growth medium (13). PBMC were isolated from venous blood fractionated on a Ficoll-Hypaque gradient. Monocytes were isolated from these PBMC by Percoll gradient fractionation (14) and maintained in α-modified Eagle’s medium (αMEM) supplemented with 10% heat-inactivated autologous human serum or FCS, penicillin, and streptomycin. FS4 cells were grown in αMEM supplemented with 10% FCS, penicillin, and streptomycin.

All induction experiments were performed in the regular cell growth medium, using cells just before reaching confluence. Monocytes were induced at 10^6 cells/ml in Teflon dishes.

**IFN-γ.** The IFN used in this study was highly purified recombinant protein synthesized in E. coli generously provided by Genentech, Inc., San Francisco, CA. The endotoxin levels were determined in a limulus amoebocyte lysate assay before shipping. The human rIFN-γ had a specific activity of 2–4 × 10^7 U/mg as determined in a human lung carcinoma A549 inhibition assay using the encephalomyocarditis virus.

**Peptide Synthesis.** The peptide was synthesized by the solid-phase method (15) using chloromethylated, 1% crosslinked, styrene-divinylbenzene copolymer (Merrifield Resin). Deprotection was followed by a coupling program that used the symmetric anhydrides of the appropriate Boc amino acids (Bachem Inc., Torrance, CA; or Peninsula Laboratories, Inc., Belmont, CA) (16). The side-chain functionalities were protected by benzyl-type protecting groups. The peptide was cleaved from the resin and deprotected by treatment with liquid HF-anisole at −0°C (17). The peptide was purified by gel-permeation chromatography, ion-exchange chromatography, and reverse-phase HPLC. The final product was homogeneous by analytical reverse-phase HPLC.

The peptide was glutaraldehyde coupled to keyhole limpet hemocyanin (KLH) (18). 2 µg of peptide was dissolved 10 µl of H2O and added to 15 µg KLH in 2 ml 0.1 M PBS. Glutaraldehyde (21 mM) was added over 1 h at room temperature. The mixture was allowed to stand overnight at room temperature and then was dialyzed against PBS.
γ INTERFERON-INDUCIBLE CYTOKINE IP-10

~100 µg of protein in CFA was used to immunize two 8-wk-old female New Zealand white rabbits. The rabbits were boosted twice at 1-mo intervals with the same amount of protein in IFA. 10 d after the second boost, the rabbit was bled and serum was isolated and used for Western blotting and immunoprecipitation.

Polyacrylamide Gel Electrophoresis (PAGE). SDS-PAGE was performed according to the method of Laemmli (19) using slab gels with 10, 12.5, and 15% polyacrylamide. A modification of the Laemmli gel system was used for gels that had to be dried down because of gel cracking problems. This modified SDS-PAGE system uses a lower ratio of bisacrylamide to acrylamide (20). For samples that contained ³⁵S, gels were processed for fluorography using Enhance (DuPont Co., Wilmington, DE) and exposed at -70°C on Kodak x-ray film.

E. coli Expression and Production of Antiserum. A 576-bp Xba I–Eco RI derived from the IP-10 cDNA plasmid pIFNγ-31.7 (4) was cloned into the fusion expression vector pB4+ (21). The pB4+ vector contains the gene for the influenza viral protein NSI. The plasmid was transformed into the E. coli strain AR58, which is a λ lysogen containing a temperature-sensitive mutation in the CI gene (CI857). The resulting strain, harboring the recombinant expression plasmid, produced a fusion protein at the nonpermissive temperature that consisted of 81 amino acids of NSI and 72 amino acids of IP-10.

A 325-bp Fnu4H fragment derived from the IP-10 cDNA plasmid pIFNγ-31.7 was cloned into the nonfusion expression vector pT17. This recombinant plasmid was transformed into the E. coli strain AR58, which upon temperature induction synthesized 88 amino acids of the IP-10 protein.

Bacterial cells were grown in L-broth at 30°C to an OD₆₅₀ of 0.3, then shifted to 42°C for 1 h of growth. The bacterial cells were pelleted by centrifugation and resuspended in 1/20 vol of PBS. The cells were lysed by sonication and subjected to centrifugation at 10,000 rpm for 5 min. The pellet, which included the recombinant proteins and cellular envelopes, was suspended in sample buffer and subjected to preparative SDS-PAGE. The gel was stained with Coomassie Brilliant Blue R for 1 min and immediately washed five times in deionized water. The faintly stained band was excised from the gel with a razor blade and minced. The gel pieces were soaked for 24 h in 50 mM Tris, pH 7.5, containing 0.15 M NaCl and 0.1% SDS. The amount of eluted protein recovered was estimated by comparison to protein standards (Bio-Rad Laboratories, Richmond, CA) after SDS-PAGE. This treatment of the sample resulted in a preparation highly enriched for recombinant proteins.

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IgG was isolated from serum by protein A-Sepharose affinity chromatography (Pharmacia Fine Chemicals, Piscataway, NJ). 1 mg of the gel-purified recombinant protein was coupled to cyanogen bromide–activated Sepharose 4B (Pharmacia Fine Chemicals). 100–200 µg IgG was affinity purified on the recombinant protein column. The bound IgG was eluted with 1 M glycine-HCl, pH 2.8, quickly neutralized with 2 M Tris, and dialyzed against PBS. The affinity-purified antiserum was used for immunoprecipitation, immunofluorescence, and immunohistochemistry.

Pulse-chase Experiments. HUVE cell monolayers (~10⁶ cells) were washed twice with PBS and maintained for 30 min in αMEM lacking methionine but containing 200 mM glutamine. Cells were then pulsed for 30 min in this cell-starvation medium supplemented with [³⁵S]methionine (500 µCi/ml). After removal of the pulse medium, the cells were washed twice with PBS and then incubated with cell growth medium supplemented with unlabeled methionine (5 mM) for the chase periods indicated. At the completion of the chase, the monolayers were washed twice with PBS and scraped into an SDS solution (0.5% SDS, 50 mM Tris, pH 7.4, 100 mM NaCl, 2 mM EDTA). After being heated for 2 min at 100°C, the samples were frozen at ~20°C. For immunoprecipitation, the samples were again heated for 2 min at 100°C, sonicated for 2 min, and adjusted to contain 0.2 U/ml aprotinin (Sigma Chemical Co., St. Louis, MO), and 1 mM PMSF (Sigma Chemical...
Co.). Affinity-purified antibodies were added to a final concentration of 5 μg/ml and the immunoprecipitation was continued as described below.

**Immunoblotting.** E. coli cells or human cell lysates (prepared as described) were dissolved in sample buffer (2% SDS/0.0625 M Tris, pH 7.4/10% glycerol/0.01% bromophenol blue/and 5% 2-ME), boiled for 5 min, and fractionated by SDS-PAGE using slab gels of 12.5 and 15% acrylamide. Prestained protein molecular weight standards (Bethesda Research Laboratories, Gaithersburg, MD) were used to calculate apparent molecular weights. Protein was transferred electrophoretically to nitrocellulose (Schleicher & Schuell, Inc., Keene, NH). All of the following steps were performed in Tris-buffered saline (TBS; 50 mM Tris, pH 7.5, 2 mM EDTA, 0.15 M NaCl), 0.5% NP-40, and 0% FCS. The nitrocellulose filter was first treated overnight in 5% nonfat dry milk, followed by incubation for 2 h with a 1:1,000 dilution of antiserum. The nitrocellulose was washed and reacted for 1 h with 10⁶ cpm/ml of ¹²⁵I Staphylococcus protein A (Amersham Corp., Amersham, Arlington Heights, IL). After extensive washing in TBS + 0.5% NP-40, the filter was exposed to x-ray film at -70°C in the presence of an intensifying screen.

**Immunoprecipitation.** ~10⁶ cells were lysed in PBS containing 1% NP-40, 0.2 U/ml aprotinin, 1 mM PMSF, and 0.1% diisopropylfluorophosphate (Sigma Chemical Co.). Nuclei and debris were removed by centrifugation at 14,000 g for 5 min. The lysate was adjusted to 0.2% SDS and boiled for 5 min at 100°C. The lysate was further clarified by centrifugation at 45,000 g, 15 min at 4°C. The supernatant was passed through a 0.45-μm millipore filter and further clarified by centrifugation at 45,000 g, 15 min at 4°C. Affinity-purified antibodies were added to a final concentration of 5 μg/ml to both lysate and supernatant. The solution was incubated at room temperature for 4–16 h. Antigen-antibody complexes were precipitated by incubation with protein A–Sepharose for 2 h at room temperature.

The immunoabsorbed protein A–Sepharose beads were collected by centrifugation, washed twice in buffer that contained 0.6 M NaCl, 0.0125 M KPO₄, pH 7.4, and 0.02% NaN₃ (HSA buffer), twice at room temperature with a mixed detergent solution (0.05% NP40, 0.1% SDS, 0.3 M NaCl, and 10 mM Tris-HCl, pH 8.6), once again with HSA buffer, and finally, once in PBS. The antigen-antibody complexes were released from the beads by incubation at 100°C for 2 min in 2X PAGE sample buffer.

**NH₂-Terminal Sequence Determination.** Human keratinocytes were biosynthetically labeled with [³H]leucine and [⁵⁵S]cysteine for 8 h. Radiolabeled IP-10 was purified from the keratinocyte media by immunoprecipitation and SDS-PAGE. The gels were dried down without fixing or staining. The IP-10 protein was located in the gel by autoradiography, electroeluted from the gel, and concentrated by precipitation (22). Samples were subjected to automated Edman degradation in Gas Phase Sequencer (Model 470A; Applied Biosystems, Inc., Foster City, CA). The amino acid derivative obtained at each cycle was dissolved in 20% acetonitrile and transferred to 10 ml of Aquasolv for scintillation counting.

**ELISA.** The antipeptide antisera were checked for their ability to react with the synthetic peptide by an ELISA assay. Microtiter plates were coated with peptide (100 ng/well) in a sodium carbonate buffer, pH 9.6, and then saturated with 3% BSA, PBS, 0.1% Tween 20. 100 μl of antipeptide antisera dilutions from 10⁻² to 10⁻⁷ were placed in the wells and incubated for 2 h. After extensive washing in PBS/0.05% Tween, the plates were incubated for 2 h with peroxidase-labeled affinity-purified goat anti–rabbit IgG. The wells were then washed and the substrate 0-phenylenediamine (100 μl of 10 mg/25 ml in 0.05 M citrate-phosphate buffer, pH 5) was added. The reaction was allowed to proceed for 10 min at room temperature and was stopped by addition of 50 ml of 2.5 M H₂SO₄. Absorbance was read at 410 nm in an automatic plate reader (Minireader II; Dynatech Laboratories, Inc., Alexandria, VA). Positive ELISA readings were obtained at dilutions of 10⁻⁷ after boosting with peptide conjugate. The purified peptide was attached to CNBr-activated Sepharose 4B. This matrix was used to affinity purify the rabbit antipeptide antiserum. The affinity-purified antibodies were used for Western blotting and immunoprecipitation experiments.

**RNA Isolation and Blotting.** Total cellular RNA was isolated by the guanidinium
isothiocyanate-cesium chloride method (23). RNA was fractionated on a 1% agarose gel containing 2.2 M formaldehyde (24) and transferred to nitrocellulose (25) and hybridized with a random primed (26) IP-10 cDNA probe (pIFNγ-31.7). Hybridization was performed at 40°C for 16 h in a solution containing 50% formamide, 10% dextran sulfate, 5X SSC (1X SSC: 0.15 M sodium chloride, 0.015 M sodium citrate), 1X Denhardt's (0.02% polyvinyl-pyrrolidone, 0.02% Ficoll, and 0.02% BSA), and 200 μg/ml of sonicated herring sperm DNA. The filters were washed at 50°C in 0.1X SSC containing 0.1% SDS and exposed at -70°C to Kodak XAR film in the presence of one intensifying screen (Cronex Lightning Plus).

Results

Peptide Synthesis. A peptide was synthesized to the carboxyl 22 amino acids of the deduced IP-10 protein. The carboxyl 22 amino acids were chosen for two reasons. First, the carboxyl 13 amino acids of the homologous protein PF-4 have been reported to be biologically active in chemotactic chambers (27). Second, Chou-Fasman computer analysis (28) strongly predicts an amphiphilic α-helical COOH-terminus for the IP-10 protein. Since some amphiphilic α-helices are stable in aqueous solution (29, 30), this COOH-terminal 22-amino-acid peptide could exist as an α-helix in an aqueous environment. It is possible then that this peptide could mimic the secondary structure of the COOH-terminus of the native polypeptide. This would theoretically make it an ideal immunogen.

The COOH-terminal 22 amino acids were synthesized using the solid-phase method (15). After completion of the synthesis and HF cleavage from the resin, the peptide was purified by gel filtration, ion exchange, and finally, reverse-phase HPLC. The peptide was then glutaraldehyde-coupled to KLH and used to elicit a monospecific polyclonal rabbit antiserum. The polyclonal antibodies reacted very strongly with the peptide in solid-phase ELISA assays (data not shown). Nonetheless, they did not Western blot or immunoprecipitate a product from IFN-γ-induced human cells. This result can be explained in one of two ways: Either the antipeptide antibodies are not reactive with the IP-10 protein or the IP-10 protein undergoes COOH-terminal processing, removing from the mature polypeptide the region chosen as the synthetic immunogen.

E. coli Expression. To resolve the issue proposed above for the antipeptide antiserum's inability to recognize the predicted human protein, the IP-10 open reading frame was expressed in E. coli and the recombinant protein purified by SDS-PAGE and used to elicit a polyclonal monospecific antiserum. The open reading frame of the IP-10 cDNA was first expressed as a fusion protein with the influenza nonstructural protein NS1 (Fig. 1, lane 2). This fusion protein was purified by elution from SDS-polyacrylamide gels (lane 3) and then used to immunize rabbits. Subsequently, IP-10 was expressed as a nonfusion protein, purified from SDS-polyacrylamide gels (Fig. 2B), coupled to CNBr-activated Sepharose, and used to affinity purify the high titer NS1-IP-10 antiserum. This resulted in affinity-purified polyclonal antibodies directed against the entire open reading frame of IP-10.

To determine the reactivity of both the antipeptide and the anti--recombinant IP-10 antibodies, the E. coli lysate expressing IP-10 was subjected to Western blot analysis using these antibodies (Fig. 2A). Both the antipeptide and the anti-rIP-10 antibodies recognize the E. coli--synthesized nonfusion IP-10. This indi-
FIGURE 1. *E. coli* expression and purification of the NS1-IP-10 fusion protein. (Top) 12.5% SDS-PAGE analysis of *E. coli* lysates expressing the NS1 protein alone (lane 1), *E. coli* lysates expressing the NS1-IP-10 fusion protein (lane 2), the purified NS1-IP-10 protein (lane 3), and molecular weight markers (×10^6) (lane 4). The gel was stained with Coomassie Brilliant Blue. (Bottom) A schematic of the IP-10 cDNA where the rectangle indicates coding sequence and the hatched rectangle indicates the predicted signal sequence. A schematic of the expression vector used to construct the NS1-IP-10 fusion is also depicted. Numbers refer to the predicted amino acid sequence beginning with the initiating methionine (4).

FIGURE 2. *E. coli* expression, immunoblot, and purification of the nonfusion IP-10 protein. (Top) (A) Immunoblot of paired extracts of *E. coli* lysates, control (lane 1), and those expressing the IP-10 protein (lane 2) were fractionated by 15% SDS-PAGE. Proteins were electrophoretically transferred to nitrocellulose and reacted with a 1:1,000 dilution of the indicated antisera follow by 125I-Protein A (10⁶ cpm/ml) and subjected to autoradiography. Control lysates were isolated from *E. coli* cells harboring an expression plasmid that contained the IP-10 cDNA in the reverse orientation inserted into the expression site. Lysates from the control and expressing *E. coli* were prepared identically from ~8 × 10⁷ bacteria. M, ×10⁶ markers are indicated on the right. (B) Coomassie Brilliant Blue stained 15% SDS-PAGE of the purified *E. coli*-expressed nonfusion IP-10 protein. M, markers (×10⁶) are indicated on the left. (Bottom) A schematic of the cDNA is presented indicating the region of the IP-10 protein to which the synthetic peptide was synthesized. The rectangle indicates coding region and the hatched rectangle indicates the predicted signal sequence. Below this schematic, another schematic illustrates the nonfusion expression vector and the particular construct used in the experiment shown in A. Numbers refer to the predicted amino acid sequence beginning with the initiating methionine (4).
cates that the antipeptide antibodies recognize the predicted open reading frame encoded by the IP-10 cDNA.

Immunoprecipitation Analysis. However, when these antibodies are used to immunoprecipitate (Fig. 3) or Western blot (data not shown) IFN-γ-induced human cells, only the anti-rIP-10 antibodies recognize a human polypeptide. HUVE cells were either induced with 100 U/ml IFN-γ for 4 h in their regular growth medium containing 20% human serum, or left untreated and then biosynthetically labeled for 10 h with [35S]methionine (Fig. 3). During this labeling, 94% of the immunoprecipitable protein is found in the cell supernatant, confirming the prediction based on the cDNA sequence that IP-10 is a secreted polypeptide. In addition, it is evident that the synthesis and secretion of this protein is profoundly induced in endothelial cells by IFN-γ. However, the form immunoprecipitated from endothelial cell lysates and supernatants migrates as a 6-7-kD doublet on reducing SDS-PAGE and not as the predicted 10-kD polypeptide, suggesting that posttranslational processing may be occurring.

Since the cDNA sequence of IP-10 predicts a 10-kD secreted polypeptide containing four cysteines, the 6-7-kD doublet immunoprecipitated from IFN-γ-induced endothelial cell lysates and supernatants might exist natively as a 12-kD disulfide-crosslinked polypeptide migrating in SDS-polyacrylamide gels. To address this possibility, the endothelial cell immunoprecipitates were analyzed by nonreducing SDS-PAGE (Fig. 4). Under nonreducing conditions, three bands that migrate very tightly around 6 kD are evident in the cell supernatant. The two fastest migrating bands seen under nonreducing conditions comigrate with the two bands seen under reducing conditions. This suggests that there is a small disulfide-linked peptide, attached to one or both of the 6-7-kD forms, that accounts for the slowest migrating band seen only under nonreducing conditions.

NH2-Terminal Sequence Analysis of Secreted IP-10. NH2-terminal sequence analysis was performed on the IP-10 protein secreted from human keratinocytes.
Figure 4. Immunoprecipitable IP-10 analyzed by nonreducing (−2-ME) and reducing (+2-ME) SDS-PAGE. rIFN-γ HUVE cell lysate (L) and supernatant (S) was immunoprecipitated as described in Materials and Methods. The antigen-antibody complexes were released from the protein A-Sepharose beads by incubation in 2X PAGE sample buffer without (−) 2-ME, and with (+) 2-ME. Samples were fractionated by 15% low-bis SDS-PAGE, treated with Enhance, and exposed for 14 d.

Figure 5. Forms of IP-10 synthesized in vivo in a pulse-chase experiment. ~10^6 HUVE cells were pulsed with [35S]methionine, and the label was chased with unlabeled methionine for the time periods indicated in hours. Immunoprecipitated proteins were subjected to 15% low SDS-PAGE, treated with Enhance, and exposed for 21 d. (Lane 1) In vivo-labeled immunoreactive products isolated immediately after a 30-min pulse; (lanes 2–5) As in lane 1 except that the 30-min pulse was followed by a 2-h chase for lane 2, a 4-h chase for lane 3, a 8-h chase for lane 4, and a 16.5-h chase for lane 5, ^14C-labeled molecular weight markers were obtained from Bio-Rad Laboratories.

Keratinocyte monolayers were simultaneously biosynthetically labeled with [3H]-leucine and [35S]methionine. The IP-10 protein was purified by immunoprecipitation and subsequent SDS-PAGE and then subjected to 20 consecutive cycles of automated Edman degradation. The secreted mature form of IP-10 shows a Leu at position 3 and Cys at positions 9 and 11. These positions coincide with the amino acid sequence predicted by the cDNA sequence (4). This confirms the prediction for the signal peptidase cleavage site based on the cDNA sequence analysis. In addition, it definitively establishes that the anti-rIP-10 recognizes IP-10 synthesized and secreted from human cells.

Posttranslational Processing. A pulse-chase experiment was performed using HUVE cells to address the biosynthetic relationship between these various forms of immunoprecipitable IP-10 (Fig. 5). Essentially identical results are obtained.
when the pulse-chase experiments are analyzed by reducing and by nonreducing SDS-PAGE, so only an autoradiogram of the nonreducing gel is presented (Fig. 5). Immunoprecipitation following a 30-min in vivo labeling with \[^{35}S\]methionine reveals only the 6–7-kD forms in the cell lysate. During this 30-min labeling, ~50% of the immunoprecipitable material is found in the cell supernatant. This indicates that IP-10 is synthesized and secreted in <30 min. After 2 h of chase, very little of the IP-10 protein can be immunoprecipitated from the cell lysate; it is either secreted or degraded; it is not stored intracellularly. The extracellular form of IP-10 seems to be processed over time in the culture medium. After 16.5 h of chase, ~50% of the immunoprecipitable material seen after 30 min of labeling still remains immunoprecipitable. In addition, IP-10 appears to undergo further processing once secreted, as is evident from its faster mobility at later times during the chase.

The predicted unprocessed forms of IP-10, however, are not evident from these experiments. After a 30-min labeling (Fig. 5), or after a 10-h labeling (Fig. 3), the largest form of immunoprecipitable IP-10 is 7 kD. Hybrid selection (4), cDNA sequence (4) and E. coli expression (Figs. 1 and 2) indicate that the primary translation product of the IP-10 protein is 12 kD. The predicted signal peptidase cleavage would then produce a 10-kD polypeptide. Thus, it appears that the proteolytic processing of IP-10 is too rapid to capture the predicted unprocessed 12-kD and 10-kD forms of the IP-10 protein using this type of immunoprecipitation analysis in primary endothelial cells.

Inducible Expression of the IP-10 Protein and RNA in Human Cells. To determine the generality of the secretory and protein processing patterns seen with HUVE cells, immunoprecipitation analysis was performed on several other primary human cells (Fig. 6). Parallel cultures of human monocytes, fibroblasts, and keratinocytes were either left untreated or induced with 100 U/ml of IFN-γ for 4 h and then biosynthetically labeled with \[^{35}S\]methionine for 10 h. The cell lysates and supernatants were immunoprecipitated using the anti-rIP-10 antibodies and analyzed by SDS-PAGE. The three additional cell types examined reveal a pattern of induction, secretion and processing of the IP-10 protein similar to that seen with HUVE cells. Densitometric scanning of autoradiograms containing immunoprecipitated IP-10 reveals that keratinocytes secrete ~3 times more IP-10 than endothelial cells, 33 times more IP-10 than monocytes, and 165 times more IP-10 than fibroblasts.

Total cellular RNA isolated from control and rIFN-γ-stimulated primary HUVE cells, monocytes, and keratinocytes was analyzed for the expression of the IP-10 mRNA (Fig. 7). The cells examined are stimulated by IFN-γ to accumulate the IP-10 mRNA in a manner consistent with the pattern of IP-10 protein induction (Figs. 3 and 6).

Discussion

In this study we have generated polyclonal monospecific antiserum to an rIP-10 protein synthesized in E. coli and have demonstrated that IFN-γ alone induces the de novo synthesis and subsequent constitutive secretion of the IP-10 protein from a variety of human cells, including endothelial cells, monocytes, fibroblasts, and keratinocytes. The four cell types tested, however, do not synthesize and
FIGURE 6. Inducible secretion of the IP-10 protein from human cells: immunoprecipitation analysis of primary fibroblasts, monocytes, and keratinocytes. The cells were biosynthetically labeled with [35S]methionine and then used for immunoprecipitation analysis as described in Materials and Methods. $\sim 10^6$ cells were used for each immunoprecipitation. The fibroblasts samples were analyzed by 15% SDS-PAGE under reducing conditions and the monocyte and keratinocyte samples were analyzed by 15% SDS-PAGE under nonreducing conditions. The SDS-PAGE gels were treated with Enhance and subjected to autoradiograph, the fibroblast gel was exposed for 21 d, the monocyte gel for 6 d, and the keratinocyte gel for 14 d.

FIGURE 7. IFN-γ inducibility of the IP-10 mRNA in human cells. Total cellular RNA was isolated (22) from control and rIFN-γ treated HUVE, human monocytes (MO) and human keratinocytes (Ker). 2 μg of HUVE and 10 μg of MO and Ker total RNA was fractionated on a 2.2 M formaldehyde/1% agarose gel, transferred to nitrocellulose, and hybridized with a nick-translated IP-10 cDNA probe. The migration of 28s and 18s ribosomal RNA is indicated.
secrete equal amounts of IP-10 protein after IFN-γ treatment. Of the cells tested, keratinocytes induced by IFN-γ secrete the most IP-10, with endothelial cells, monocytes, and fibroblasts secreting lesser amounts of IP-10, in that order. This is consistent with the amount of IP-10 mRNA that accumulates in these cells after IFN-γ treatment. This suggests that IP-10 mRNA accumulation is an accurate reflection and the primary determinant of the amount of IP-10 protein secreted by cells.

Immunoprecipitation of biosynthetically labeled cells and radiochemical sequence analysis indicate a complex posttranslational processing pattern for the IP-10 protein, including signal peptidase cleavage and COOH-terminal processing. Based on the data presented here and the disulfide structure of the homologous protein β-thromboglobulin (βTG) (6), a model is presented for the disulfide structure and processing of IP-10 (Fig. 8).

βTG is found in the serum as a homotetramer of four noncovalently associated chains, each containing two intrachain disulfide bonds; no free sulfhydryl groups are detected in βTG with 5,5′-dithio-bis-2-nitrobenzoic acid. The disulfide structure of βTG was deduced from amino acid analysis of CNBr and Staphylococcal protease cleavage fragments of βTG. Disulfide bridges link cysteine 16 of the mature βTG polypeptide to cysteine 42 and cysteine 18 to cysteine 58. After signal peptidase cleavage, the processed form of IP-10 is predicted to contain four cysteines that align perfectly with βTG and PF-4. It is not unreasonable to suspect then, that IP-10 will fold into a disulfide structure analogous to βTG. This is illustrated diagramatically in Fig. 8, with cysteine 9 of the mature polypeptide disulfide-linked to cysteine 36 and cysteine 11 disulfide-linked to cysteine 53.

One possible processing pattern consistent with the data presented here is illustrated in Fig. 8. There are three consecutive lysines beginning at residue 46 and a lysine followed by an arginine at residue 51 and 52 that are potential cleavage sites for dibasic endopeptidases. If the lys-lys bonds are less susceptible to digestion than the lys-arg bond, cleavage at these two positions would produce the 6–7-kD doublet that is seen when immunoprecipitates are analyzed under reducing conditions. An additional cleavage carboxyl to cysteine 53 would leave

**Figure 8.** Hypothetical model of the predicted primary structure of the IP-10 protein. The NH₂-terminal valine is predicted from signal peptidase cleavage. The disulfide structure is that determined for βTG superimposed on the IP-10 protein. Arrows indicate sites of potential cleavage that are predicted to occur based on mobility of the IP-10 protein in SDS-PAGE and the inability of the COOH-terminal directed antipeptide antibody to immunoprecipitate a product from human cells.
a small peptide disulfide-linked to both of the carboxyl trimmed mature forms. This is the simplest processing pattern that would explain the additional, slowest migrating form only seen under nonreducing SDS-PAGE. Given the conservation of the four cysteines throughout this family of secreted polypeptides and knowing the disulfide structure of βTGF, it seems likely that IP-10 would contain two internal disulfide crossbridges. This hypothesis awaits the definitive analysis that will come from protein purification and subsequent characterization.

Using the affinity-purified antibodies directed against the rIP-10 protein described in this study, Kaplan et al. in the accompanying study (11), demonstrate that the IP-10 protein is expressed during the development of a cutaneous delayed cellular immune response by keratinocytes, endothelial cells, and infiltrating dermal mononuclear cells. In addition, pronounced IP-10 expression is seen in the epidermis and dermis on the cutaneous lesions of tuberculoid leprosy (11) and psoriasis (Gottlieb, A. and A. D. Luster, manuscript in preparation). Both conditions are associated with keratinocytic proliferation and the influx of mononuclear cells into the dermis. IP-10’s homology to chemotactic and mitogenic proteins suggests that it may play a role in these cellular changes; this hypothesis awaits a direct analysis.

Summary

An IFN-γ-inducible protein, IP-10, has previously (4, 10) been described to belong to a gene family of chemotactic and mitogenic proteins, associated with inflammation and proliferation. Biochemical characterization of this predicted protein has been pursued through the development of polyclonal monospecific antisera to recombinant protein and synthetic peptides. These reagents establish that the IP-10 protein is secreted from a variety of cells (endothelial, monocyte, fibroblast, and keratinocyte) in response to IFN-γ. Posttranslational processing occurs in the biosynthesis of this protein, resulting in a 6–7-kD species, which may reflect COOH-terminal cleavage. Pulse-chase studies indicate that this processing is a rapid event in the primary cell lines studied, completed in the 30-min labeling period. A model is presented for the processing and secondary structure of this protein. In an accompanying study, Kaplan, et al. (11) using these antisera, demonstrate that the IP-10 protein is associated, in vivo, with a delayed-type hypersensitivity response.

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