Interferon-γ Induces p11 Gene and Protein Expression in Human Epithelial Cells through Interferon-γ-activated Sequences in the p11 Promoter

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The effect of interferon (IFN)-γ on p11 expression was studied in two human epithelial cell lines (BEAS-2B and HeLa). Treatment with IFN-γ resulted in increased steady-state levels of p11 mRNA and protein expression, with a time-dependent and dose-dependent effect. Transient transfection experiments of a reporter gene containing −1498 bp of the 5′-flanking region of the p11 promoter demonstrated that IFN-γ induced p11 gene expression at the transcriptional level. These effects were inhibited at the promoter and protein levels by a specific JAK-2 kinase inhibitor, AG-409. Functional analysis of the p11 promoter indicates that two γ-activated sequence elements (GAS) located at positions −1219 and −1090 are important for the induction of the p11 promoter by IFN-γ. Transfection of mutated reporter constructs demonstrated that the mutation at the GAS-2 site (−1090) inhibited the p11 promoter activity, with a reduction of about −73% and mutation at the GAS-3 site (−1219) eliminated about 26% of the p11 promoter activity. A STAT1 dominant negative mutant vector at Tyr-701 (JAK kinase phosphorylation site) blocked the effect of IFN-γ on the p11 promoter activity. IFN-γ induced a rapid tyrosine phosphorylation and nuclear translocation of STAT1 protein, which is involved in the binding to the GAS-2 site in the p11 promoter by EMSA analysis. These data suggest that IFN-γ-induced p11 expression is mediated through the binding of STAT1 to GAS sites in the p11 promoter. Inhibition of p11 expression by inhibitory antisense RNAs (iRNA) treatment resulted in enhanced IFN-γ and calcium ionophor-stimulated arachidonic acid release suggesting that at least in part IFN-γ-stimulated p11 expression may serve a counterregulatory role.

The S-100 protein family is a multigenic family of low molecular weight (9–11 kDa) calcium-binding proteins (1). S-100A10, known as p11 or calpactin I light chain, is a distinct member of S100 family because its two EF-hands carry mutations that limit its ability to bind calcium (2). p11 is a natural ligand of annexin II, forming an annexin II2-p112 heterotrimer (AIIt)1 (3–5). This complex is implicated in the regulation of exocytosis and endocytosis by reorganization of F-actin (6, 7), the formation of the cornified envelope in epithelial keratinocytes (8), and the stimulation of t-PA-dependent plasminogen activation and the activation of procathepsin B (9, 10). p11 also interacts with the C terminus of cytosolic phospholipase A2 and inhibits cPLA2 activity resulting in reduced arachidonic acid release (11). Antisense inhibition of p11 results in enhanced cPLA2 activity and increased AA release, whereas p11 overexpression reduces cPLA2 activity and AA release (12). Dexamethasone is known to reduce cPLA2 activity, and recent studies suggest that this effect may be mediated by up-regulation of p11 (12). Recent data also suggest that retinoic acid-increased arachidonic acid release may be in part mediated by the reduction of p11 protein expression (13). Seung-Wook Kim et al. (14) have reported that annexin I and annexin II-p11p but not annexin II inhibited cPLA2 activity. Taken together, these data suggest that p11 acts as a regulatory protein of cPLA2 and may play a role in the regulation of cPLA2 activity and AA release.

IFN-γ is a pro-inflammatory cytokine that is secreted by activated T-lymphocytes and natural killer cells and regulates cellular antiviral, antitumor, and immunological responses (15). IFN-γ has been reported to regulate cyclooxygenase-2 (COX-2) expression and induce prostaglandin formation, which may play a major role in the induction of inflammatory processes (16). IFN-γ also induces intercellular adhesion molecule-1 (ICAM-1), which plays an important role in the adherence and migration of leukocytes at sites of inflammation in several cell types (17, 18). The binding of IFN-γ to its surface receptor activates the receptor-associated tyrosine kinases, JAK1 and JAK2. JAKs tyrosine phosphorylate and activate the latent cytosolic transcription factor STAT1, which then dimerizes, translocates to the nucleus, and binds to the γ-activated sequence (GAS) elements of IFN-γ response genes, resulting in gene activation (19, 20). IFN-γ stimulates an increase in PLA2 activity in a variety of cell lines (21, 22). IFN-γ also induces AA release, as well as cPLA2 gene transcription (23). It was of interest to study the effect of IFN-γ on p11 expression. In this

1 The abbreviations used are: AIIt, annexin II-p11 heterotrimer; cPLA2, cytosolic phospholipase A2; IFN, interferon; GAS, IFN-γ activated sequence; γ-IRE, IFN-γ response element; STAT, signal transducer and activator of transcription; JAKs, Janus family tyrosine kinases; CAT, chloramphenicol acetyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; AA, arachidonic acid; ELISA, enzyme-linked immunosorbent assay; CMV, cytomegalovirus; ANOVA, analysis of variance; RPA, ribonuclease protection assay; iRNA, inhibitory antisense RNAs.

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study we investigate the effect of IFN-γ on p11 transcription, steady-state levels of p11 mRNA and protein expression and the signal transduction pathway involved in human epithelial cells.

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa cells (American Type Culture Collection, Manassas, VA) were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. A human bronchial epithelial (BEAS-2B) cell line (American Type Culture Collection) was grown in LHC-8 medium (Biofluids, Rockville, MD) without serum. All experiments were performed when cells were 80–90% confluent.

To test the transcriptional activity of p11, for all experiments, after replacing culture medium at the same time, the cells were treated with or without 300 units/ml of IFN-γ at 4, 12, 24, or 48 h prior to harvest at the 48-h time point. For the dose response experiments, cells were treated with or without IFN-γ at the indicated dose for 24 h. For the inhibition experiments, cells were preincubated at 4°C for 2 h before treatment with or without IFN-γ. Inhibitors were maintained for the incubation period.

Immunoblot of p11 Protein—BEAS-2B and HeLa cells were grown on 175-cm² collagen-coated tissue culture flasks (BD PharMingen Labware, Franklin Lakes, NJ) and treated with IFN-γ (Roche Molecular Biochemicals, Indianapolis, IN). Cells were harvested with trypsin (E-P Basic Medium, BDH, Poole, Dorset, UK) after washing three times with cold phosphate-buffered saline, cells were resuspended in 50 mM HEPES buffer including Complete protease inhibitor (Roche Molecular Biochemicals) and then sonicated three times for 15 s and centrifuged at 14,000 rpm for 15 min. Total protein was assayed by BCA reagent (Pierce). Ten micrograms of crude cell lysates were separated on 10% Tris-glycine gels (Invitrogen) and electrophoretically transferred onto a nitrocellulose membrane (Invitrogen). p11 protein expression was detected by using 1:2000 dilution of mouse anti-p11 monoclonal antibody (Transduction Laboratories, Lexington, KY) and 1:5000 dilution horseradish peroxidase-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratory, Inc., West Grove, PA). The blot was developed using the ECL Western blotting detection system (Amersham Biosciences) and exposed to radiographic film.

Ribonuclease Protection Assay (RPA)—BEAS-2B and HeLa cells were treated with and without IFN-γ. Total cellular RNA was extracted using Tri-reagent (Molecular Research Inc. Cincinnati, OH) and redissolved in diethyl pyrocarbonate (DEPC) water. To construct the probe for p11 mRNA, a 319-bp product of p11 cDNA was amplified by PCR using the following set of sense and antisense primers: 5’- primer: 5’-ACCCACCAAAAAATGGCATCT-3’ (corresponding to bases 61–80 of the human p11 cDNA sequence, GenBank accession number M81457); 3’- primer: 5’-CTGCTCATTCTGCTACCTT-3’ (corresponding to bases 361–380 of the p11 cDNA sequence). The PCR product was cloned into the pGEM-T Easy vector (Promega, Madison, WI). Orientation of the insert was determined by DNA sequencing. The p11 cRNA probe and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Ambion, Austin, TX) were radiolabeled using an in vitro transcription kit (Ambion) with SP6 polymerase and α-32P-UTP (800 Ci (29.6TBq)/mmol) (PerkinElmer Life Sciences). An RPA assay kit (RPAII, Ambion, Austin, TX) was used to quantitate target mRNA. Ten micrograms of total RNA (for GAPDH) or 50 μg (for p11) were mixed with 10,000 cpm (for GAPDH) or 20,000 cpm (for p11) of α-32P-labeled riboprobe, the mixture was hybridized at 45 °C overnight, and the unprotonated RNA was digested by the addition of 1:100 dilution RNase A/T1 at 37 °C for 60 min. Digestion was terminated by the addition of RNase inactivation and protein precipitation mixture. The remaining samples, pCAT II deletion and mutation constructs were tritium-labeled using the human p11 promoter region (from −188 to −89) to generate deletion plasmids pCAT-1048 to pCAT-188 and pCAT-88 to pCAT-188. pCAT-88 plasmid was cut with BglII and ligated into pCAT basic vector, which also cut with BgIII to create the deletion construct pCAT-T-1434 to +89. The site-directed mutagenesis constructs were generated by ligation of two pieces of PCR product produced from backbone pCA-T-1498 to +89. The reporter construct mutated at the GAS-2 site (TTCACAGA mutated to CTGAGAGA) was created using the following two primer sets: 5’-GGGGTACCGAGGTTCT-3’ and 5’-AAAGTATTCTGAGATATAATTGAGA-3’. To produce the first PCR product from −1498 to −1073, and 5’-CTCTAATTATCGAGAGATTCT-3’ and 5’-TAAAGGCTCAGTGGCCGA-3’ to produce the second PCR product with both PCR products of full-length p11 cDNA were cut with BglII and ligated with T4 ligase. The ligation reaction was used as a template to create a 2 μg-mRNA 2 μg-cDNA construct from −1498 to +89 with the primers 5’-GGGGTACCGAGGATTCTTCTT-3’ and 5’-TAAAGGCTCAGTGGCCGA-3’ to create a PCR product from −1231 to +89. Both PCR products were cut with XhoI and religated. The ligation mixture was used as a template to create the GAS-3 mutation construct from −1498 to +89 using the primers 5’-GGGGTACCGAGGATTCTTCTT-3’ and 5’-TAAAGGCTCAGTGGCCGA-3’.

To create the GAS-2 mutation construct from −1498 to −1204 and −5’-GGGGGCTTCTGCGAGACGTC-3’ and 5’-CAGCTGTTTCTGCGAGAAGCGGCAATTT-3’ to create a PCR product from −1498 to −1204 and −5’-ATTCGGCTTCTGCGAGACGTC-3’ and 5’-TAAAGGCTCAGTGGCCGA-3’ to create a PCR product from −1231 to +89. Both PCR products were cut with XhoI and religated. The ligation mixture was used as a template to create the GAS-3 mutation construct from −1498 to +89 using the primers 5’-GGGGTACCGAGGATTCTTCTT-3’ and 5’-TAAAGGCTCAGTGGCCGA-3’. The designated names for the mutation constructs are mGAS2 and mGAS3. The sequences of the deletion and mutation constructs were confirmed by bi-directional DNA sequencing.

Transient Transfection Assay—HeLa cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. The day before transfaction, 5 × 10⁶ cells were seeded into 6-well plates. 1.8 μg of p11 promoter constructs were cotransfected with 0.2 μg of the PCMV/β-gal construct (Clontech) into serum-free cells using 6 μl of the LipofectAMINE reagent (Invitrogen) in each well. After 5 h of transfection, the cells were allowed to recover overnight in fresh Dulbecco’s modified Eagles medium with 10% fetal bovine serum. At the indicated time point, SEAP activity was measured in 25-μl aliquots of culture medium with a SEAP reporter assay kit according to the manufacturer (Tropix, Bedford, MA) in a luminometer (Packard Bioscience, Meriden, CT). At the final time point, the transfected cells were lysed and were centrifuged for 10 min. β-galactosidase activity was measured with a β-galactosidase ELISA kit (Roche Molecular Biochemicals). Data are expressed as a ratio of SEAP activity to β-galactosidase activity. For dose response experiments, SEAP-1498 to +89 transfected cells were treated with different concentrations of IFN-γ for 24 h. SEAP and β-galactosidase activity were measured as described above. For deletion and mutation constructs, the promoter reporter constructs were cotransfected with 0.2 μg of the pCAT II plasmid. The transfected cells were treated with 300 units/ml of IFN-γ for 24 h and then harvested. PCAT and β-galactosidase activity were assayed separately using pCAT and β-galactosidase ELISA kits according to the manufacturer’s instructions (Roche Molecular Biochemicals). The PCAT activity was normalized to β-galactosidase activity to represent relative pCAT activity. Deletion and mutation constructs, the pCAT II deletion and mutation constructs were expressed as a percentage of the full-length pCAT-1498 to +89 promoter construct that was set at 100%.

Transient Transfection with Wild-type STAT1 Expression Plasmid and Mutant STAT1 Dominant Negative Expression Plasmid—Wild-type STAT1 and mutant STAT1 dominant negative expression plasmids (Stratagene, La Jolla, CA) were used to transiently transfect the human p11 cDNA (encoding the 750 amino acid STAT1 protein), the STAT1 dominant negative plasmid is mutated at amino acid 701 (the JAK1/2 phosphorylation site), pcDNA3.1 with no insert was used as a control vector. All three plasmids have a potent CMV promoter. HeLa cells
were transfected with 1 µg of the p11 promoter construct pcAT-1498-89 alone or cotransfected with 1 µg of wild-type STAT1, mutant STAT1, or control vector pcDNA3.1 individually. Controls for transfection efficiency were performed using cotransfection with 0.2 µg of PCMVβ-gal. Transient transfection was performed as described above. After transfection, some cells were exposed to IFN-γ (300 units/ml) for 12 h. Cells were harvested, lysed, and pCAT and β-galactosidase activity were assayed by ELISA.

Effect of AG-490 on IFN-γ-induced p11 Promoter Activity and p11 Protein Levels—HeLa cells were seeded into 6-well plates and cotransfected with the SEAP-1498-89 plasmid and PCMVβ-gal control plasmid as described above. Cells were pretreated with the JAK-2 kinase inhibitor AG-490 (Calbiochem, San Diego, CA) (50, 100, or 1000 µM) for 2 h, and then treated with or without IFN-γ (300 units/ml) for 24 h. SEAP activity and β-galactosidase activities were determined as described above. For p11 Western blot studies, HeLa cells were grown on T-75-cm² flasks until 80% confluent. Cells were pretreated with AG-490 (100 µM) for 2 h and then incubated with IFN-γ (300 units/ml) for 12 h and lysed. Western blotting for p11 protein was performed as described above.

Electrophoretic Mobility Shift Assay and Immunoblot of STAT1 Protein—HeLa cells were incubated with and without IFN-γ (300 units/ml) at 10 min, 30 min, 2, 4, 6, or 24 h prior to harvest at the 24 h time point, and nuclear extracts were prepared using a nuclear extraction kit according to the manufacturer’s directions (Sigma). DNA binding was performed by incubating 2 µg of nuclear protein in a total volume of 10 µl of binding buffer (50 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.5 mM dithiothreitol, 0.5 mM EDTA, 1 mM MgCl₂, 0.05 µg/ml poly(dI-dC), 15% glycerol) and 10,000 cpm of 32P-labeled double-stranded GAS-2 oligonucleotide 5′-TTATTCCAGAAAATTCTT-3′ and non-specific double-stranded oligonucleotide SP-1 consensus sequence were preincubated with nuclear extracts for 20 min at room temperature followed by an additional incubation for 15 min at room temperature with the labeled probe. To identify bands containing specific STAT1 protein, the sample was incubated with anti-STAT1 antibody (provided by Dr. Jahan Haque) antibody for 1 h on ice before the DNA binding reaction was performed. For the immunoblot of STAT1 protein, 10 µg of crude cell lysate or nuclear extracts from HeLa cells were separated using 8% Tris-glycine gel, and Western blotting was performed as described with 1:1000 dilution of anti-phospho-STAT1 or anti-STAT1 polyclonal antibody (Cell Signaling Technology, Beverly, MA) and 1:5000 dilution horseradish peroxidase-conjugated goat anti-rabbit IgG as secondary antibody.

Quantification of Autoradiographs—A Molecular Dynamics 301 computing densitometer (Molecular Dynamics, Sunnyvale, CA) was used to digitalize images. The optical density of bands was analyzed with background subtraction using Image Quant software (Molecular Dynamics).

Transfection of HeLa Cells with Small Inhibitory Antisense RNAs (iRNAs)—iRNAs were prepared by IDT (Coralville, IA) and targeted to the coding sequence. Untemplated TT were added to each single strand in annealing buffer (100 mM potassium acetate, 30 mM HEPES, pH 7.4, 2 mM magnesium acetate) for 2 h at 90 °C. Cells grown in 6-well plates were transfected with 100 nm iRNA duplexes using LipofectAMINE reagent (Invitrogen) (5 µl in 1 ml of culture media) for 5 h. After transfection, media was changed, and cells were labeled with [3H]AA (1 µC/ml of culture media, Amersham Biosciences) for 16 h. Cells were then treated with or without IFN (300 units/ml) for 24 h. The effect of treatment of cells with iRNA duplexes on p11 protein levels was determined by immunoblotting of cell lysates. The effect of iRNA treatment on arachidonate release was determined by scintillation counting of media from labeled cells after treatment with the calcium ionophore A23187 or vehicle (Me₃SO) for 30 min. Media was harvested and after centrifugation at 750 × g for 10 min, [3H]AA release was measured in a scintillation counter (Beckman, Fullerton, CA).

Statistical Analysis—The dose-related effects were analyzed with one-way ANOVA. For other experiments, comparisons with control levels were performed using a two-tailed unpaired Student’s t test. Values of p < 0.05 were considered statistically significant.

RESULTS

IFN-γ Increases the Level of the p11 Protein in BEAS-2B Cells—Western blot studies demonstrated that p11 is constitutively expressed in BEAS-2B cells. IFN-γ (300 units/ml) increased p11 protein expression in BEAS-2B cells over a 4–48 h period, with a maximum effect observed at 12–24 h (Fig. 1, A and B). Treatment of cells with 10, 100, 300, 1000 units/ml of IFN-γ for 24 h resulted in a dose-dependent increase in p11 protein levels (Fig. 1, C and D).

The Effect of IFN-γ on p11 Protein Levels in HeLa Cells—As IFN-γ increased p11 protein levels in a human airway epithelial cell line, BEAS-2B cells, we further investigated the effect of IFN-γ on p11 expression in another epithelial cell line, HeLa cells. HeLa cells were treated with or without 300 units/ml of IFN-γ for 4–48 h or with IFN-γ (10, 100, 300, 1000 units/ml) for 24 h and p11 protein expression was determined by Western blot analysis. Fig. 2, A–D show that IFN-γ also induced a time and dose-dependent increase in p11 protein in HeLa cells.

IFN-γ Increases mRNA Levels in BEAS-2B Cells—To determine if the observed increases in p11 protein levels correlate with changes in p11 mRNA expression, BEAS-2B cells were incubated with or without IFN-γ (300 units/ml) for 4–48 h or IFN-γ (10, 300, 1000 units/ml) for 24 h. Total RNA was isolated and steady-state p11 mRNA levels were studied by RPA. As shown in Fig. 3, A and B, IFN-γ treatment stimulated increased p11 mRNA levels over 4–48 h, compared with the untreated control cells. BEAS-2B cells treated with 10, 300, 1000 units/ml of IFN-γ for 24 h demonstrated a significant dose-related increase in steady-state p11 mRNA levels as shown in Fig. 3, C and D. GAPDH mRNA levels are presented as controls for equivalent RNA loading and to normalize for p11 mRNA expression.

IFN-γ Induces p11 mRNA Expression in HeLa Cells—To further investigate if IFN-γ-induced p11 mRNA expression was increased in HeLa cells, cells were incubated with or without IFN-γ (10, 300, 1000 units/ml) for 24 h, and the steady-state levels of p11 mRNA were measured by RPA. Untreated HeLa cells constitutively produced p11 mRNA, and IFN-γ treatment induced the steady-state p11 mRNA expression in a dose-dependent manner (Fig. 4, A and B).

IFN-γ Induces p11 Expression at the Transcriptional Level in HeLa Cells—To further investigate if the observed increase in steady-state p11 mRNA levels reflected an IFN-γ-induced increase in p11 gene transcription, a reporter gene construct containing a 1498-bp sequence of the 5′-flanking region of the p11 promoter in the SEAP vector was created (SEAP-1498-89) and transient transfection of this p11 promoter construct into HeLa cells was accomplished as described under “Experimental Procedures.” The results are shown in Fig. 5A. IFN-γ (300 units/ml) treatment of HeLa cells resulted in a significant increase in p11 promoter activity (3–4-fold) over a 3–24 h time course. HeLa cells treated with IFN-γ (0, 30, 300 units/ml) for 24 h demonstrated a significant dose-related effect in relative p11 promoter activity as shown in Fig. 5B. β-galactosidase activity was used to evaluate the transfection efficiency. These results demonstrate that IFN-γ induces p11 expression at the transcriptional level.

AG-490 Inhibits IFN-γ-induced p11 Promoter Activity—To further understand the mechanism of IFN-γ induction of p11 expression, HeLa cells transfected with pSEAP-1498-89 promoter construct were pretreated with the JAK-2 tyrosine kinase inhibitor, AG-490 (50 or 100 µM) for 2 h and then treated with or without IFN-γ (300 units/ml) for 24 h. The results are presented in Fig. 6 and demonstrate that AG-490 significantly inhibited IFN-γ-induced p11 promoter activity in a dose-related...
manner (p < 0.001, n = 6). AG-490 alone had no effect on p11 promoter activity, indicating that it is not cytotoxic at the concentration used in this experiment.

**Fig. 1.** Western blots demonstrate that IFN-γ treatment increases p11 protein levels in BEAS-2B cells. After treatment with IFN-γ, cells were lysed and 10 μg of total protein lysate was examined by Western blot analysis using anti-annexin II light chain monoclonal antibody. A, time course (4–48 h) effect of IFN-γ (300 units/ml)-treated and untreated BEAS-2B cells showing an increased p11 protein level at 4 h, with peak levels at 12–24 h. The result shown is representative of four separate experiments. B, densitometry measurements from four time course experiments of IFN-γ (300 units/ml) treated and control BEAS-2B cells demonstrating increased p11 protein levels at 4–48 h (data presented as mean ± S.E., *, p < 0.05 compared with control). C, IFN-γ (10, 100, 300, 1000 units/ml) treatment for 24 h induces a dose-dependent increase in p11 protein level. The result shown is representative of three separate experiments. D, densitometry measurements from three dose response (0–1000 units/ml) experiments demonstrating a dose-related increased p11 protein levels (data presented as mean ± S.E., p < 0.001 for dose effect by one-way ANOVA).

**Fig. 2.** Western blots demonstrate that IFN-γ treatment increases p11 protein levels in HeLa cells. HeLa cells were grown to near confluence and treated with and without IFN-γ. Ten micrograms of total crude cell lysates were subjected to gel electrophoresis and immunoblotting with anti-annexin II light chain monoclonal antibody. A, time course (4–48 h) of IFN-γ (300 units/ml)-treated and untreated control HeLa cells showing increased p11 protein levels at 4 h, with the greatest increase at 12–24 h. The result shown is representative of four separate experiments. B, mean densitometry measurements from four time course experiments of IFN-γ (300 units/ml)-treated HeLa cells demonstrating increased p11 protein levels at 4–48 h (data presented as mean ± S.E., *, p < 0.05 compared with control values). C, IFN-γ (10, 100, 300, 1000 units/ml) treatment for 24 h induces a significant dose-dependent increase in p11 protein levels. The result shown is representative of three separate experiments. D, mean densitometry measurements from three dose response (0–1000 units/ml) experiments demonstrating a dose-related p11 protein level increase (data presented as mean ± S.E., p < 0.001 for dose effect by one-way ANOVA).
AG-490 Inhibited the Effect of IFN-γ/H9253 on p11 Protein Level in HeLa Cells

To confirm the pathway of IFN-γ/H9253 induced p11 expression, HeLa cells were preincubated with AG-490 (100 μM) for 2 h and then treated with or without IFN-γ/H9253 for 12 h. IFN-γ/H9253 significantly increased p11 expression whereas in the cells pretreated with AG-490, this effect was substantially diminished as shown in Fig. 7. Therefore, IFN-γ/H9253 induced the expression of p11 was blocked by JAK-2 tyrosine kinase inhibitor.

Identification of IFN-γ/H9253 Response Elements in the p11 Promoter

In order to further study the transcriptional regulation of p11 promoter by IFN-γ/H9253, we characterized putative IFN-γ/H9253 response elements in the p11 promoter. The sequence analysis of 1498 bp of the p11 5′-flanking promoter region for putative IFN-γ response elements is shown in Fig. 8A. There are three interferon GAS and one IFN-γ response elements (γ-IRE) located separately at positions -215 (GAS-1), -1090 (GAS-2), for 4–48 h resulted in increased p11 steady-state mRNA expression in BEAS-2B cells. GAPDH mRNA levels are presented as an internal control to evaluate RNA loading. The results are representative of three different experiments. B, densitometry measurements from three dose response experiments of IFN-γ (10, 300, 1000 units/ml) treatment for 24 h demonstrating a significant dose effect on p11 mRNA expression in HeLa cells (data presented as mean ± S.E., p < 0.001 for dose effect by one-way ANOVA).

AG-490 Inhibited the Effect of IFN-γ on p11 Protein Level in HeLa Cells—To confirm the pathway of IFN-γ induced p11 expression, HeLa cells were preincubated with AG-490 (100 μM) for 2 h and then treated with or without IFN-γ for 12 h. IFN-γ significantly increased p11 expression whereas in the cells pretreated with AG-490, this effect was substantially diminished as shown in Fig. 7. Therefore, IFN-γ induced the expression of p11 was blocked by JAK-2 tyrosine kinase inhibitor.

Identification of IFN-γ Response Elements in the p11 Promoter—In order to further study the transcriptional regulation of p11 promoter by IFN-γ, we characterized putative IFN-γ response elements in the p11 promoter. The sequence analysis of 1498 bp of the p11 5′-flanking promoter region for putative IFN-γ response elements is shown in Fig. 8A. There are three interferon GAS and one IFN-γ response elements (γ-IRE) located separately at positions -215 (GAS-1), -1090 (GAS-2),
reporter gene construct containing 1498 bp of the p11 promoter region and with the pCMV/β-gal plasmid as a transfection control. After overnight incubation in fresh Dulbecco's modified Eagle's medium with 10% fetal bovine serum, the cells were treated with or without IFN-γ in modified Eagle's medium with 10% fetal bovine serum. The mean demonstrates a dose-dependent effect on p11 promoter activity in HeLa cells (p < 0.001 for dose effect by one-way ANOVA). Values represent the mean ± S.E. of three different experiments.

To ascertain the role of the GAS-2 and GAS-3 elements in IFN-γ-induced p11 promoter activity, two full-length mutation constructs (mGAS-2, mGAS-3) were created (Fig. 9A). HeLa cells were transfected with the wild-type pCAT-1498 + 89, mGAS-2 or mGAS-3 promoter construct. As shown in Fig. 9B, mutation of the GAS-2 site significantly diminished IFN-γ-induced p11 promoter activity, with a reduction about 73% of p11 promoter activity compared with that of the full-length wild-type pCAT-1498 + 89 promoter construct. Mutation of the GAS-3 site inhibited to a lesser degree (about 26% reduction) the IFN-γ-induced p11 promoter activity of the wild-type pCAT-1498 + 89 promoter construct. These data suggest that the GAS-2 site has an important role in IFN-γ-induced p11 expression.

GAS-2 and GAS-3 Elements Are Involved in IFN-γ Induction of the p11 Promoter Activity—To ascertain the role of the GAS-2 and GAS-3 elements in IFN-γ-induced p11 promoter activity in the context of the full-length reporter construct, two full-length mutation constructs (mGAS-2, mGAS-3) were created (Fig. 9A). HeLa cells were transfected with the wild-type pCAT-1498 + 89, mGAS-2 or mGAS-3 promoter construct. As shown in Fig. 9B, mutation of the GAS-2 site significantly diminished IFN-γ-induced p11 promoter activity, with a reduction about 73% of p11 promoter activity compared with that of the full-length wild-type pCAT-1498 + 89 promoter construct. Mutation of the GAS-3 site inhibited to a lesser degree (about 26% reduction) the IFN-γ-induced p11 promoter activity of the wild-type pCAT-1498 + 89 promoter construct. These data suggest that the GAS-2 site has an important role in IFN-γ-induced p11 expression.
expression whereas the GAS-3 site also makes a contribution to this response.

**STAT1 Dominant Negative Mutant Vector Blocked IFN-γ-induced p11 Promoter Activity**—To further investigate the pathway of IFN-γ-induced p11 expression, the expression plasmid encoding wild-type STAT1 or mutant STAT1 (Tyr-701) were cotransfected with the p11 promoter construct pCAT-1498 + 89 (Fig. 10). IFN-γ treatment significantly increased pCAT-1498 + 89 activity in cells cotransfected with wild-type STAT1 expression vector or empty vector, pcDNA3.1. However, in cells cotransfected with the dominant negative vector, STAT1 (Tyr-701), IFN-γ treatment had substantially less effect on the activity of the p11 reporter gene.

**IFN-γ Induces a Rapid Tyrosine Phosphorylation and Nuclear Translocation of STAT1 Protein**—To determine the time course of IFN-γ induction of tyrosine phosphorylation of STAT1 protein in these cells, HeLa cells were treated with IFN-γ (300 units/ml) for 10 min, 30 min, 2 h or 4 h and crude cell lysates were prepared. Western blot analysis with anti-phospho-STAT1 and anti-STAT1 were performed. As shown in Fig. 11, A and B, IFN-γ induces a rapid tyrosine phosphorylation of STAT1, starting at 10 min whereas no change was observed in total STAT1 protein levels. Next, to determine whether tyrosine phosphorylation of STAT1 in response to IFN-γ was accompanied by translocation into the nucleus, nuclear protein extracted from HeLa cells was studied by Western blot. As shown in Fig. 11C, STAT1 rapidly translocated to the nucleus following treatment with 300 units/ml of IFN-γ for 10 min, 30 min, 2 h, and 4 h. These data suggest that IFN-γ treatment of these cells causes a rapid tyrosine phosphorylation and nuclear translocation of STAT1 protein in response to IFN-γ.

**Electrophoretic Mobility Gel Shift of the GAS-2 Sequence**—To confirm that IFN-γ induces p11 expression through binding to a specific GAS site, an electrophoretic mobility gel shift assay was used. Since the GAS-2 site appears to play a pivotal role in IFN-γ-induced p11 expression, we synthesized an 18-bp double-stranded oligonucleotide GAS-2 probe. Nuclear protein extracted from HeLa cells was used for the electrophoretic mobility gel shift assay. The ratio of pCAT/β-galactosidase ELISA. The ratio of pCAT/β-galactosidase ELISA was studied by Western blot. As shown in Fig. 11, A and B, IFN-γ inducers a rapid tyrosine phosphorylation and nuclear translocation of STAT1 protein in response to IFN-γ.

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bility gel shift assays. To confirm the binding specificity, excess of unlabeled GAS-2 or an irrelevant consensus oligonucleotide, SP-1, was used as a competitor. Fig. 12A shows that there is complex formation with the GAS-2 probe and this complex can be partially inhibited by 50-fold excess of the cold GAS-2 probe and completely inhibited by 200-fold excess of the cold probe; whereas incubation with cold SP-1 oligonucleotide did not affect the complex formation. These data suggest that this complex is specific for the GAS-2 probe. Preincubation with anti-STAT1 antibody completely abolished complex formation suggesting the presence of STAT1 protein in this complex (Fig. 12B and C).
The image contains a page from a scientific document discussing the effects of interferon (IFN-γ) on gene expression and protein levels in human epithelial cells. The text is focused on the study of p11, a subunit of annexin II, and its role in calcium ionophore-stimulated arachidonic acid release. The text also mentions studies on the effects of IFN-γ on cPLA2 activity and phospholipase A2 (PLA2) expression.

### DISCUSSION

p11 is a unique member of the S-100 family of calcium-binding proteins. p11 is present in a variety of cells, including as a heterotetramer with annexin II (p36). The heterotetramer is composed of two copies of 36-kDa heavy chain, annexin II subunits, and two copies of 11-kDa light chain, p11 subunits. (p36)_2 (p11)_2 (4). Recently, Waisman and colleagues demonstrated that IFN-γ induced arachidonic acid release from iRNA-treated cells was significantly greater with IFN-γ than from control cells. Further, calcium ionophor-stimulated arachidonic acid release from iRNA-treated cells was increased compared with control cells. This increase was observed in control cells treated with or without IFN-γ. Calcium ionophor-stimulated arachidonic acid release from iRNA-treated cells was significantly greater with IFN-γ-treated cells than from control cells treated with IFN-γ alone. Therefore, IFN-γ induced changes in cellular p11 protein levels subsequently may serve to down-regulate IFN-γ-induced changes in PLA2 activity.

### Effect of Inhibition of p11 Protein Expression

IFN-γ treatment of cells may result in a variety of effects including an increase in gene expression of cPLA2 as well as an increase in cPLA2 activity (23, 25). In order to determine if IFN-γ-induced changes in cellular p11 protein levels have an effect on cellular function, cells were transfected with iRNA directed against the p11 coding sequence. IRNA treatment of HeLa cells was associated with a reduction in cellular p11 protein levels (Fig. 1A). 3H[Ara-arachidonic acid release from HeLa cells was studied with and without pretreatment with the calcium ionophor, A23187. A23187 stimulated release of arachidonic acid was similar in control cells treated with or without IFN-γ. Calcium ionophor-stimulated arachidonic acid release from iRNA treated cells was increased compared with control cells. Further, calcium ionophor-stimulated arachidonic acid release from iRNA treated cells was significantly greater with IFN-γ-treated cells than iRNA-treated cells not treated with IFN-γ or control cells treated with IFN-γ (Fig. 1B). Therefore, IFN-γ-induced changes in cellular p11 protein levels subsequently may serve to down-regulate IFN-γ-induced changes in PLA2 activity.

### Induction of p11 Gene Expression in Human Epithelial Cells

IFN-γ induced changes in PLA2 activity. HeLa cells were transfected with double-stranded iRNA directed against codons 2–7 of the p11 coding sequence with Lipofectamine for 5 h as described under “Experimental Procedures.” Control cells were transfected with Lipofectamine alone. A Western blot for p11 protein. After transfection for 5 or 16 h of incubation in media, and treatment with or without IFN (300 units/ml) for 24 h, cells were harvested and cell lysates prepared. The immunoblotting was performed as described under “Experimental Procedures.” In control cells, IFN-γ treatment resulted in an increase in cellular p11 protein levels compared with control cells treated with vehicle alone. IRNA treatment resulted in a substantial decrease in cellular p11 protein levels compared with control cells. The blot presented is representative of three separate experiments each with similar results. B, cellular 3H[Ara-arachidonic acid release from HeLa cells transfected with iRNA against p11 or treated with Lipofectamine alone (control cells). After transfection, cells were labeled with 3H[Aaa] for 16 h. Media were changed, and cells were treated with IFN-γ (300 units/ml) for 24 h. Media was changed, and cells were treated with the calcium ionophor, A23187 (10^6 M) or vehicle for 30 min. The media were collected and centrifuged and aliquots taken for scintillation counting. IRNA treatment resulted in an increase in calcium ionophor-induced arachidonate release compared with control cells. IFN-γ treatment resulted in an increase in calcium ionophor-induced arachidonate release from iRNA-treated cells compared with interferon-treated control cells suggesting that IFN-γ induced changes in phospholipase A2 are not inhibited by p11 in the iRNA-treated cells. Data presented are mean ± S.E., (n = 3). * indicates p < 0.01 by Student’s t test.

### Inhibition of p11 Protein Expression

IFN-γ treatment for 10 min, 30 min, and 2, 4, 6, and 24 h significantly increased GAS-2 binding with maximum observed at 30 min and 2 h as shown in Fig. 13. These data suggest that STAT1 protein mediates IFN-γ induction of p11 expression through binding to the GAS-2 element in the p11 promoter.

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IFN-γ Induces p11 Gene Expression in Human Epithelial Cells

IFN-γ induces p11 gene expression in human epithelial cells. Transient transfection studies demonstrated that IFN-γ induces steady-state p11 mRNA expression and p11 protein production in human epithelial cells. Transient transfection studies also showed that IFN-γ induces p11 expression at the transcriptional level. p11 has been reported to be regulated at the transcriptional and post-transcriptional level (17, 18, 34). Matsuura et al. (16) reported that IFN-γ induces COX-2 mRNA expression at the transcriptional level and increases PGE₂ synthesis in normal human epithelial keratinocytes. However, IFN-γ may also down-regulate gene expression. IFN-γ-primed macrophages demonstrate decreased COX-2 gene expression in response to IL-1β stimulation (35). IFN-γ inhibits IL-4-induced 15-lipoxygenase mRNA and protein expression in cultured human monocytes (36). The binding of IFN-γ to its receptor leads to the recruitment and activation of STAT1 protein that, after being activated by tyrosine phosphorylation, translocates to the nucleus and mediates the transcription of IFN-γ response genes (19, 20, 37). STAT1 activation involves specific members of JAK family that are associated with receptors. IFN-γ treatment activates JAK1, JAK2, and STAT1 (38). In this study, we demonstrated that IFN-γ up-regulates p11 expression in human epithelial cells. This effect was confirmed using two different cell lines, BEAS-2B cells and HeLa cells. Studies in two different cell lines demonstrate that IFN-γ induces steady-state p11 mRNA expression and p11 protein production in human epithelial cells. Transient transfection studies showed that IFN-γ induces p11 expression at the transcriptional level. p11 has been reported to be regulated in different cell types. Nerve growth factor (NGF) increases p11 mRNA expression in rat pheochromocytoma (PC12) cells (39). Munz et al. (40) reported that wound-derived growth factors (TGF-β1, EGF, and KGF) differentially regulate p11 and annexin II expression in cultured keratinocytes during skin injury and modify the ratio between p11 and annexin II (39). Dexamethasone treatment of BEAS-2B cells or of HeLa cells increases steady-state levels of p11 mRNA and p11 protein levels and results in a reduction of cPLA₂ activity (12). TGF-α or the mixture of TGF-α and IL-1β induces an increase in p11 protein expression in rat gastric epithelial cells (29, 30). However, little is known about the mechanism of regulation of p11 expression. A recent report has shown that retinoic acid reduces p11 protein levels by a post-translational mechanism. In this study, we demonstrated that IFN-γ-induced p11 expression is mediated by STAT1 binding to GAS in the p11 promoter. Several lines of evidence confirmed this effect. First, AG-490, a specific JAK-2 kinase inhibitor abolished the effect of IFN-γ on p11 promoter and protein level, suggesting that JAK-2 tyrosine kinase is required for activation of the p11 promoter. Second, functional analysis of the p11 promoter, which contains four potential IFN-γ cis-acting elements, one γ-IRE, and three GAS elements, indicated that two elements are important for the induction by IFN-γ of p11 expression. Third, cotransfection of a STAT1 dominant negative plasmid and p11 promoter construct abolished the effect of IFN-γ on p11 promoter activity. Fourth, IFN-γ induces a rapid tyrosine phosphorylation and nuclear translocation of STAT1 protein, indicating that STAT1 is involved in the induction of IFN-γ in p11 expression. Fifth, electrophoretic mobility shift assays demonstrate that IFN-γ induces a time-dependent STAT1 binding to the GAS-2 site of the p11 promoter. Taken together, we believe that IFN-γ induces activated STAT1 binding to GAS elements resulting in p11 gene and protein expression.

STAT1-deficient mice show complete unresponsiveness to IFN-γ, but still respond normally to several other cytokines that also activate STAT1, illustrating the critical role of STAT1 in IFN-γ-induced gene expression (41). IFN-γ-induced expression of the Class II transactivator, ICAM, is mediated through STAT1 binding to one or two of the GAS elements in the promoters of these genes (42, 43). IFN-γ has also been reported to induce CD40 expression through STAT1 binding to one of three GAS sites, another GAS element also is important to IFN-γ-induced CD40 gene expression, but maximal induction of CD40 requires additional cis-acting elements (44). In this study we have shown that both GAS-2 and GAS-3 sites are involved in IFN-γ-induced p11 expression. STAT1 has been shown to cooperate with other transcriptional factors to induce gene expression. Therefore, whether other transcriptional factors are involved in IFN-γ-induced p11 expression remains to be explored.

Given the effect of IFN-γ on cPLA₂ gene expression and activity, we studied the effect of IFN-γ-induced changes in p11 expression using small inhibitory RNAs to inhibit p11 protein expression in cells treated with or without IFN-γ. iRNA treatment resulted in a reduction in cellular p11 protein levels and in an increase in calcium ionophor-induced arachidonic acid release. The arachidonic acid release from iRNA-treated cells that also activate STAT1, illustrating the critical role of STAT1 in IFN-γ-induced gene expression (41). IFN-γ-induced expression of the Class II transactivator, ICAM, is mediated through STAT1 binding to one or two of the GAS elements in the promoters of these genes (42, 43). IFN-γ has also been reported to induce CD40 expression through STAT1 binding to one of three GAS sites, another GAS element also is important to IFN-γ-induced CD40 gene expression, but maximal induction of CD40 requires additional cis-acting elements (44). In this study we have shown that both GAS-2 and GAS-3 sites are involved in IFN-γ-induced p11 expression. STAT1 has been shown to cooperate with other transcriptional factors to induce gene expression. Therefore, whether other transcriptional factors are involved in IFN-γ-induced p11 expression remains to be explored.

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Interferon-γ Induces p11 Gene and Protein Expression in Human Epithelial Cells through Interferon-γ-activated Sequences in the p11Promoter

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