

## **The Endoplasmic Reticular Heat Shock Protein gp96 Is Transcriptionally Upregulated in Interferon-treated Cells**

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### **Summary**

A cDNA clone complementary to an interferon (IFN)-induced mRNA approximately 3 kb in length was identified and sequenced revealing homology with the endoplasmic reticular heat shock protein/ATPase gp96. Both IFN- $\alpha$  and - $\gamma$  transcriptionally upregulate expression of this gene. gp96 transcripts, protein, and ATPase activity are shown to be enhanced as a result of IFN treatment in two human cell lines and this effect requires de novo protein synthesis. gp96 molecules have recently been implicated in the presentation of endogenous antigens. A number of the key elements in this pathway, the transporter proteins, the major histocompatibility complex (MHC)-linked units of the proteasomes and the MHC class I molecules are known to be IFN inducible. Our results show that yet another molecule suggested to play an accessory role in the endogenous presentation pathway is IFN inducible. Further, our studies represent the first demonstration of modulation of expression of a heat shock protein by a cytokine and identify a new enzymatic activity upregulated in IFN-treated cells.

The IFNs are a family of regulatory molecules which, after binding to cell surface receptors of responsive cells, are capable of mediating an antiviral state, an antiproliferative effect, and a variety of immune responses (1–5). The establishment of an antiviral effect by the IFNs has been shown to require de novo synthesis of cellular mRNAs and proteins (6–8). Much recent research on the mechanism of action of the IFNs has focused on the induction of genes and protein products in response to IFN treatment. To date, IFN treatment has been shown to result in elevated levels of several new mRNAs, proteins and at least four enzyme activities, 2',5'-oligoadenylate synthetase, protein kinase, indoleamine 2',3'-dioxygenase, and tryptophanyl tRNA synthetase (1, 2, 4, 5, 9, 10). We report here that IFN treatment results in transcriptional upregulation of a constitutively expressed heat shock protein (hsp) gp96 (11), which is also an ATPase (12). These studies represent the first example of IFN-induced modulation of a hsp and identify a new IFN-induced enzyme activity. Further, the gp96 hsp has recently been implicated in presentation of endogenous antigens by MHC class I molecules (12, 13). A number of the key elements in this pathway, the transporter proteins, the MHC-linked units of the proteasomes and the MHC class I molecules are known to be IFN inducible. Our results show that yet another molecule suggested to play an accessory role in the endogenous presentation pathway is IFN inducible.

### **Materials and Methods**

**Cell Lines.** The Daudi and HeLa cell lines were obtained from the American Type Culture Collection (Rockville, MD). Daudi cells were maintained in RPMI 1640 (GIBCO BRL, Gaithersburg, MD) and HeLa cells were maintained in minimal essential media (MEM) (GIBCO BRL). All media was supplemented with 10% FCS and 100  $\mu$ g/mL penicillin and streptomycin.

**IFN.** Natural human IFN- $\alpha$  (specific activity,  $>10^8$  IU/mg of protein) was purified using an NK-2 monoclonal antibody column (Celltech, Slough, UK). Purified recombinant IFN- $\gamma$  was purchased from Amgen Biologicals (Thousand Oaks, CA). All IFN values are expressed in international reference units (IU).

**Construction of a cDNA Library.** mRNA was isolated from Daudi cells using the FastTrack mRNA isolation kit (Invitrogen, San Diego, CA) and used to construct a  $\lambda$ gt10 recombinant cDNA library essentially as described (14, 15).

**Synthesis of  $^{32}$ P-labeled Single-stranded cDNA Probes.**  $^{32}$ P-labeled single-stranded cDNA probes were generated from mRNA of untreated Daudi cells and Daudi cells treated with IFN- $\alpha$  for 18 h as described (16).

**Differential Screening of the cDNA Library.** The  $\lambda$ gt10 library was screened with the  $^{32}$ P-labeled single-stranded cDNA probes generated from mRNA isolated from untreated and IFN-treated Daudi cells as described (16).

**Northern Blot Analysis.** Poly (A<sup>+</sup>) RNA isolated as described above was run on denaturing agarose gels, transferred to nylon membranes, and hybridized as described (16). Where indicated, hybridization signals were quantitated with a pdi scanning system (pdi, Huntington Station, NY). For all data presented, the relative amounts of mRNA loaded for each sample were determined by measuring the signal generated with a radiolabeled 770-bp actin

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cDNA probe (Oncor, Inc., Gaithersburg, MD). All densitometric values presented have been standardized against actin mRNA, whose levels have been observed to be unaffected by IFN treatment under the conditions tested.

**Isolation of cDNA Inserts and Preparation of cDNA Probes.** Insert DNA from the gp96  $\lambda$ gt10 clone was isolated as described (16). hsp70 cDNA was a kind gift of Dr. J. Thomas (New York University, NY). cDNA was radiolabeled as described (16, 17).

**Plasmid Construction and DNA Sequencing.** cDNA isolated from  $\lambda$ gt10 as described above was subcloned into the EcoRI site of pGEM-3Z (Promega Corp., Madison, WI), propagated and sequenced as described (9, 18).

**Measurement of Transcription Rate.** Nuclear run-on assays were performed as described (19, 20). Hybridization to the cDNA insert and quantitation of bound radiolabeled RNA were as described (9). As controls, the radiolabeled RNA samples were also hybridized to nitrocellulose strips to which 200 ng of a 770 base pair cDNA recognizing actin (Oncor, Inc.) or a 1.8-kb cDNA insert encoding the IFN-induced tryptophanyl tRNA synthetase (WRS) was bound. The IFN treatment did not affect the amount of actin-specific mRNA produced by these nuclei.

**ATPase Assay.**  $10^7$  Daudi cells incubated for 18 h with or without 300 IU/mL IFN- $\alpha$  were harvested, washed three times with PBS, then dounced in 0.5 mL 20 mM Hepes, pH 7.2, 20 mM NaCl, and 2 mM MgCl<sub>2</sub>. After centrifugation at 1,000 g for 5 min, the supernatant was harvested and centrifuged at 10,000 g for 10 min. 250  $\mu$ g of the resulting supernatant were assayed for ATPase activity by the method of Flynn et al. (21). 5  $\mu$ L were then spotted onto a precoated polyethyleneimine (PEI) cellulose plate (Sigma, Chemical Co., St. Louis, MO) and chromatography was performed for 3 h against a 1:1 ratio of 1 M LiCl and 1 M HCOOH. The plate was dried and autoradiographed.

**Antibody Neutralization Studies.** Before being added to the ATPase assay, 250 ng of cell extract were incubated with or without 1  $\mu$ g of a rat anti-gp96 monoclonal antibody (Stressgen, Victoria, BC, Canada) or 1  $\mu$ g of normal rat IgG (Accurate Chemical and Scientific Corp., Westbury, NY) for 1 h at room temperature.

**Immunoprecipitations.** Daudi cells ( $5 \times 10^5$  cells/mL) were labeled for 18 h with [<sup>35</sup>S]methionine in RPMI containing 1/5 the usual methionine concentration in the presence or absence of 300 IU/mL IFN- $\alpha$ . Extracts were prepared and immunoprecipitations were performed as described (22).

## Results

**Isolation of a cDNA Clone Encoding gp96.** A cDNA library constructed in  $\lambda$ gt10 from Poly (A<sup>+</sup>) mRNA isolated from Daudi cells was screened with radiolabeled cDNA prepared from mRNA of untreated or IFN- $\alpha$ -treated Daudi cells. A clone,  $\lambda$ lx-1, was observed to generate a stronger signal when probed with cDNA prepared from mRNA of IFN- $\alpha$ -treated Daudi cells than with cDNA prepared from mRNA of untreated cells. Comparison of partial nucleotide sequences of  $\lambda$ lx-1 cDNA with other sequences present in GenBank (23) revealed that it is identical with the human homologue of the murine tumor rejection antigen and endoplasmic reticular hsp gp96 (Fig. 1).

**Analysis of IFN Induction of the mRNA Encoding gp96.** Northern blot analysis was performed on mRNA isolated from IFN- $\alpha$ -treated or untreated Daudi cells, employing a probe generated from the cDNA of clone  $\lambda$ lx-1 and gp96

cDNA probe pH48 (11). Both probes showed an identical pattern of hybridization. The results reveal that the IFN treatment of cells results in an enhanced accumulation of an mRNA species of  $\sim 3$  kb in length (Fig. 2 A). Quantitation of the 3-kb transcript showed an IFN-mediated enhancement of approximately fourfold. Northern blot analysis of untreated HeLa cells and HeLa cells incubated in the presence of IFN- $\alpha$  or IFN- $\gamma$  for 18 h demonstrates a similar IFN-mediated increase in accumulation of the 3-kb transcript (Fig. 2 A). An examination of the kinetics of accumulation of this RNA in Daudi cells after IFN treatment revealed that the level of this RNA slowly increases after incubation with IFN (Fig. 2 B).

To ascertain whether IFN treatment affects the transcription or stabilization of the gp96 mRNA, nuclear run-on transcription assays were performed on RNA prepared from IFN-treated and untreated Daudi cells. Nuclear RNA from IFN-treated or untreated cells was hybridized with cDNA for gp96, actin and the IFN-inducible tryptophanyl tRNA synthetase (WRS). The data presented in Fig. 2 C reveal a clear IFN-mediated elevation in the level of transcription of gp96 gene, comparable to the level of induction of the WRS gene. No significant differences are seen in the actin control.

To distinguish between the possibilities that the accumulation of the gp96 mRNA either occurs as a direct result of the IFN treatment or requires prior de novo protein synthesis, Daudi cells were incubated with cycloheximide (50  $\mu$ g/ml), a protein synthesis inhibitor, for 30 min before and 8 h after the addition of IFN- $\alpha$  and the level of the gp96 mRNA was determined as described earlier. No induction of the gp96 mRNA was observed in the cells incubated with cycloheximide and IFN (Fig. 2 D) demonstrating that the IFN-mediated induction of gp96 mRNA is dependent on cellular protein synthesis and does not occur as a direct result of IFN treatment.

**Lack of Inducibility of hsp70 by IFN.** The known inducibility of gp96 in response to stress (24, 25) prompted us to examine whether the IFN-mediated induction of gp96 was due to a general stress response by the cells. The effect of IFN on the inducibility of a classical stress-induced gene, hsp70, was therefore examined. Northern blots of IFN-treated and untreated Daudi cells were probed with the hsp70 cDNA probe and it was observed that IFN treatment failed to mediate an induction of hsp70 mRNA (Fig. 3). It would thus appear that the transcriptional upregulation of gp96 is not part of a general stress-induced response, but rather is specific to gp96.

**Induction of gp96 Protein by IFN.** Induction of gp96 in IFN-treated cells was tested at the protein level. IFN-treated and untreated Daudi cells were metabolically labeled with [<sup>35</sup>S]methionine and extracts prepared as described in Materials and Methods. The extracts were immunoprecipitated with a rat anti-human gp96 monoclonal antibody or with unrelated rat immunoglobulins. A clearly enhanced synthesis of gp96 is observed in the IFN-treated cells (Fig. 4). A number of nonspecific bands are also seen in the immunoprecipitations; it may be noted that the levels of the nonspecifically precipitating bands are identical in IFN-treated and untreated cells. The only exception to this is an  $\approx 56$ -kD band, which is present at elevated levels in extracts of IFN-

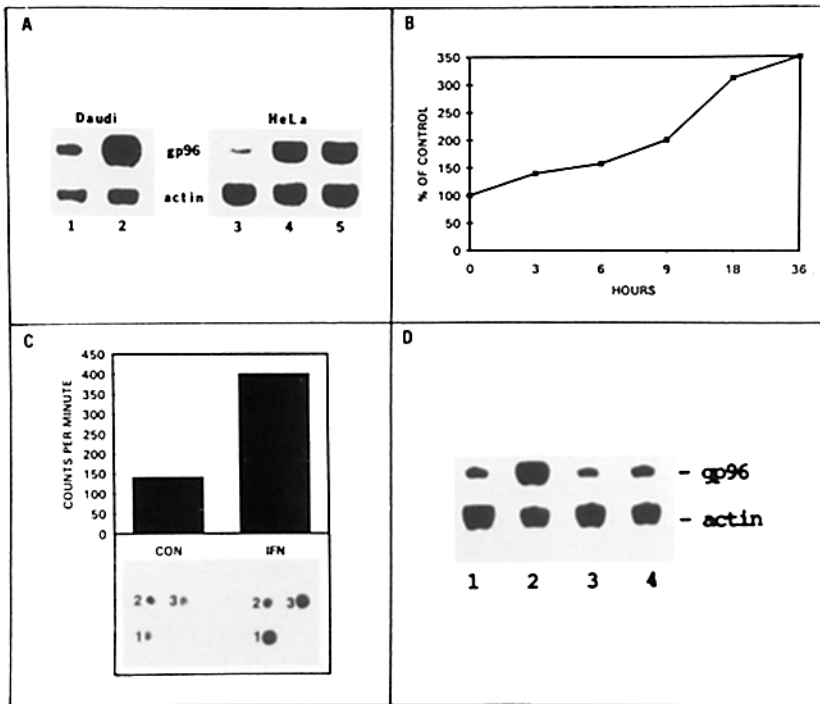
**A Nucleotide sequence at 5' end of  $\lambda$ IX-1**

$\lambda$ IX-1	GTGGCGGAC	CGCGCGGCTG	GAGGTGTGAG	GATCCGAACC	CAGGGGTGGG	GGGTGGAGGC	60
	.....	.....	.....	.....	.....	.....	
HUMTRAI	GTGGCGGAC	CGCGCGGCTG	GAGGTGTGAG	GATCCGAACC	CAGGGGTGGG	GGGTGGAGGC	60
$\lambda$ IX-1	GGCTCCTGCG	ATCGAAGGGG	ACTTGAGACT	CACCGGCCGC	ACGCCATGAG	GGCCCTGTGG	120
	.....	.....	.....	.....	.....	.....	
HUMTRAI	GGCTCCTGCG	ATCGAAGGGG	ACTTGAGACT	CACCGGCCGC	ACGCCATGAG	GGCCCTGTGG	120
$\lambda$ IX-1	GTGCTGGGCC	TCTGCTGCGT	CCTGCTGACC	T	151		
	.....	.....	.....	:			
HUMTRAI	GTGCTGGGCC	TCTGCTGCGT	CCTGCTGACC	T	151		

**B Nucleotide sequence at 3' end of  $\lambda$ IX-1**

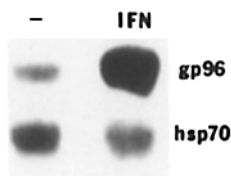
$\lambda$ IX-1	AATCTCGTCA	AAAAATATTC	ACAGTTCATA	AACITTCCTA	TTTATGTATG	GAGCAGCAAG	60
	.....	.....	.....	.....	.....	.....	
HUMTRAI	AATCTCGTCA	AAAAATATTC	ACAGTTCATA	AACITTCCTA	TTTATGTATG	GAGCAGCAAG	960
$\lambda$ IX-1	ACTGAAACTG	TTGAGGAGCC	CATGGAGGAA	GAAGAAGCAG	CCAAAGAAGA	GAAAGAAGAA	120
	.....	.....	.....	.....	.....	.....	
HUMTRAI	ACTGAAACTG	TTGAGGAGCC	CATGGAGGAA	GAAGAAGCAG	CCAAAGAAGA	GAAAGAAGAA	1020
$\lambda$ IX-1	TCTGATGATG	AAGCTGCAGT	AGAGGAAGAA	GAAGAAGA		158	
	.....	.....	.....	.....			
HUMTRAI	TCTGATGATG	AAGCTGCAGT	AGAGGAAGAA	GAAGAAGA		1058	

**Figure 1.** The nucleotide sequence of the  $\lambda$ IX-1 cDNA and a comparison with the nucleotide sequence of gp96 mRNA (HUMTRAI). The nucleotide sequence of ~150 bases at the 5' end of the  $\lambda$ IX-1 cDNA and ~150 bases at the 3' end was determined as described in the Materials and Methods. The optimized nucleotide sequence alignment was performed as described (23). The right column numbers refer to the last nucleotide in each line.



**Figure 2.** Induction of gp96 mRNA in response to IFN treatment. Daudi or HeLa cells were incubated for 18 h in the presence of no IFN (lanes 1 and 3), 300 IU/mL IFN- $\alpha$  (lanes 2 and 4), or 300 IU/mL IFN- $\gamma$  (lane 5). Poly (A<sup>+</sup>) RNA from these cells was purified and Northern blot analysis performed as described in the Materials and Methods. Positions of RNA molecular weight markers (not shown) run in parallel were used to determine the size of the gp96 mRNA. (B) The kinetics of accumulation of the gp96 mRNA in response to IFN treatment. Daudi cells were incubated for the time periods indicated in the presence of no IFN or 300 IU/mL IFN- $\alpha$ . Poly (A<sup>+</sup>) RNA from these cells was isolated and purified, then Northern blot analysis was performed as described in the Materials and Methods. The hybridization signals generated by mRNA from IFN-treated cultures are presented as a percentage of the hybridization signal generated by mRNA from untreated cultures. (C) Nuclear run-on transcriptional analysis of the IFN-induced gp96 mRNA in response to IFN treatment. Daudi cells were incubated for 14 h in the presence of either no IFN (con) or 300 IU/mL IFN- $\alpha$  (IFN). The nuclei were isolated and their mRNA isolated as described in the Materials and Methods. The RNA was hybridized to a membrane spotted with cDNAs for gp96 (1), actin

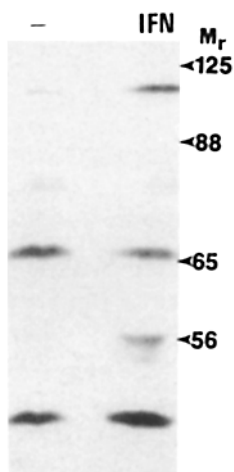
(2), and WRS (3). The amount of radioactivity associated with the gp96 cDNA was determined as described in the Materials and Methods and is presented as a histogram. (D) Lack of induction of gp96 mRNA by IFN in the presence of a protein inhibitor. Daudi cells were treated in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of cycloheximide and in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of IFN- $\alpha$  as described in the text. Poly (A<sup>+</sup>) mRNA from these cells was purified and northern blot analysis performed as described in the Materials and Methods.



**Figure 3.** Lack of induction of a stress-related mRNA in response to IFN- $\alpha$ . Daudi cells were incubated for 18 h in the presence (IFN) or absence (-) of 300 IU/mL IFN- $\alpha$ . Poly (A<sup>+</sup>) RNA from these cells was isolated, purified, and subjected to Northern blot analysis using a probe for hsp70 as described in the Materials and Methods.

treated cells. This band is a result of autodegradation of gp96 as reported earlier (22).

**Induction of gp96/ATPase Activity by IFN.** The effect of IFN on the cellular level of ATPase was examined. Soluble extracts from IFN-treated and untreated Daudi cells were prepared and assayed for total ATPase activity. A significantly enhanced ATPase activity was observed in IFN-treated cells. To determine if this increase was due to gp96, a monoclonal



**Figure 4.** Induction of gp96 protein in response to IFN treatment. Daudi cells were incubated with [<sup>35</sup>S]methionine for 18 h in the absence (-) or presence (IFN) of 300 IU/mL of IFN- $\alpha$ . Extracts were prepared and immunoprecipitations performed as described in the Materials and Methods.

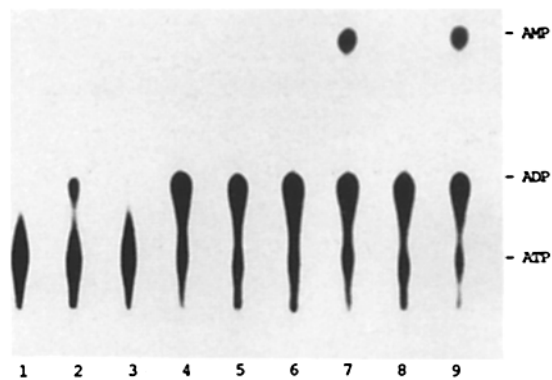
anti-gp96 antibody was included in the assay. It was observed (Fig. 5) that the IFN-induced increase in ATPase activity could be neutralized by anti-gp96 antibody. In contrast, unrelated rat immunoglobulins failed to neutralize this ATPase activity.

### Discussion

IFN treatment of cells has been shown to result in the enhanced synthesis of several proteins and mRNAs (1, 2, 4, 5). In this report, we show that the major endoplasmic reticular protein gp96, which is also a peptide-binding hsp and an ATPase (12, 26), belongs to the group of IFN-inducible proteins. The enhancement in expression of gp96 is mediated at the transcriptional level and requires de novo protein synthesis. Consistent with the ATPase activity of gp96, IFN-treated cells show a significantly enhanced ATPase activity and a substantial proportion of this increment is neutralized by anti-gp96 monoclonal antibody.

In light of the IFN-mediated inducibility of gp96, we examined the 5' regulatory region of the gene encoding gp96 for the presence of elements resembling the IFN-stimulated response element (ISRE) observed to be present in the 5' untranslated region of many of the IFN-inducible genes. We have observed that the sequence located between nucleotides -518 and -506 (GTTTCTTCTTCC), relative to the transcription start site, is highly homologous to the ISRE consensus sequence.

gp96 is a major component of the endoplasmic reticulum (27, 28) and has been suggested to be an accessory to antigen presentation by MHC class I molecules (26, 29): it accepts peptides transported into the lumen of the endoplasmic reticulum by transport-associated proteins (TAP) (30) and transfers



**Figure 5.** ATPase activity in extract of IFN-treated cells. Daudi cells were treated in the absence or presence of IFN- $\alpha$  (300 IU/mL) and extracts were prepared for ATPase assays as described in the Materials and Methods. ATPase assays were performed on samples containing no cell extract (lanes 1-3), extract from untreated cells (lanes 4-6) or extract from IFN- $\alpha$ -treated cells (lanes 7-9). Samples were preincubated with no antibody (lanes 1, 4, and 7), anti-gp96 antibody (lanes 2, 5, and 8) or normal rat IgG (lanes 3, 6, and 9), as described in the Materials and Methods.

them to MHC class I molecules in an ATP-dependent manner (31). This suggestion is supported by the following observations: (a) gp96 is found associated with a wide array of cellular peptides (12 and our unpublished observations); (b) gp96 possesses Walker A and B consensus sequences, binds ATP in vitro and in vivo and is an ATPase (12), permitting a mechanism for active acceptance and transfer of peptides; (c) gp96 associates with the MHC class I molecules and the calnexin/p88 molecule (Srivastava, P. K., manuscript submitted for publication); (d) immunization of mice with gp96 obtained from a particular tumor or virus-infected cell elicits antigen-specific T cell responses against that particular cell type, although gp96 sequences, per se, show no polymorphism (32). It is also interesting to note that all of the molecules known so far to be involved in antigen presentation by MHC class I have been shown to be upregulated by IFN. This includes selected subunits of the proteasome (33), the peptide transporters (34), and the MHC class I molecule itself (35). Our demonstration that gp96 is upregulated in the same manner, in conjunction with its other properties, puts it squarely in the pathway of antigen presentation. Increase of body temperature (heat shock) and release of IFNs are among the earliest and the most universal responses to infection and it appears reasonable that a molecule that possibly plays a key accessory role in antigen presentation (and hence in combatting viral infections) is upregulated by both heat shock and IFNs.

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