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Characterization of alpha-enolase as an interferon-alpha $_2$ alpha $_1$ regulated gene

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1. ABSTRACT

Interferons (IFNs) are multifunctional cytokines that after binding to the cell surface receptor induce the expression of a large number of genes, which in turn, mediate many biological processes including host defense, cell growth control, signaling, and metabolism. Here we show that IFN-alpha activates the mitogen-activated protein kinases (MAPK) ERK1/2 and the transcription factor CREB/ATF-1, which lead to the alpha-enolase (alpha-ENO) gene expression in fibroblasts. Alpha-ENO mRNA accumulation was apparent 6 h post-IFN stimulation and required both *de novo* protein synthesis and active gene transcription, which is typical of a secondary response gene. Alpha-ENO expression does not appear to be restricted to fibroblasts, since it was equally verified in peripheral blood mononuclear cells (PBMC). Furthermore, IFN-alpha stimulates the expression of the primary response genes *c-fos* and *egr-1*, which was followed by an increase in DNA binding activity of c-FOS and EGR-1 proteins, as verified by shift assays using the cis-acting elements AP-1 and EGR-1 localized at the alpha-ENO promoter. Finally, we also demonstrated that IFN treatment of PBMC cause an increase in both, alpha-ENO expression on the cell surface and plasmin generation followed addition of exogenous plasminogen.

2. INTRODUCTION

Interferons (IFNs) are a heterogenic family of cytokines that in vertebrates exert a broad spectrum of biological functions, such as, growth inhibition, regulation of the immune system and antimicrobial activity (1, 2). The recognition of pathogen-associated molecular patterns (PAMPs) by the Toll-like receptors (TLRs), engages downstream signaling pathways resulting in IFN production and secretion (3, 4). Humans IFNs, type I (mainly alpha, beta, and tau) and type II (IFN-gamma), once secreted, bind to specific cell surface receptors and activate a cascade of intracellular signaling pathways that results in the induction of hundreds of IFN-stimulated genes (ISGs), whose gene products are responsible for their biological activities (1, 5, 6). The function of some of the IFN-stimulated proteins is well described, whereas that of many others remains poorly characterized (7). Although the classical JAK/STAT pathway is well characterized for IFN signaling (1), recent studies indicate that IFN activates several other protein kinases, including the MAP kinase family and downstream transcription factors (8).

Alpha enolase (alpha-ENO) is a multifunctional protein firstly characterized by its enzymatic activity in the glycolytic pathway. Unlike other glycolytic enzyme genes,

which are continuously expressed, alpha-ENO is not a house-keeping gene, since its expression is regulated upon stimulation with mitogens (9-11), hypoxia (12, 13), cytokines (14, 15), phorbol ester and bacterial LPS (16). Alpha -ENO resides into the cytoplasm, where it is abundantly expressed. The enzyme also migrates, by an unidentified mechanism, to the cell surface, where it is able to bind to plasminogen/plasmin, therefore, acting as its receptor (17-19).

In addition to its classical role on glycolysis, alpha-ENO has several other cellular functions: it regulates c-myc expression (20, 21), to control thermal tolerance and growth in *S. cerevisiae* (22), to function as a structural component of the crystallin lens (23), to be associated with a variety of autoimmune diseases (24) and to serve as a plasminogen-binding protein both at the surface of bacterial (25) and mammalian cells (17-19), among others. Alpha -ENO acting as a plasminogen-binding protein was firstly demonstrated in monocytoid U937 cells (17), and soon thereafter it was also found at the surface of a variety of human hematopoietic cells such as lymphocytes T and B, monocytes, neutrophils, besides neuronal and endothelial cells (18, 19, 26).

IFNs have also been reported to exert some regulatory activities on components of the plasminogen system such as u-PA (urokinase plasminogen activator) and u-PAR (u-PA receptor) (27-30). Of note, under certain circumstances, the plasminogen system may also recruit common components of the IFN-regulated JAK/STAT pathway (31-33), suggesting that a cross-talk between the two systems may occur.

This study was undertaken to investigate whether IFN- alpha$_2$ alpha$_1$ regulates alpha-ENO expression. The results show that the levels of both alpha-ENO mRNA and protein are upregulated upon IFN stimulation, with a typical secondary response pattern of gene expression. We also showed that this regulation is not restricted to fibroblasts, since it was equally verified with PBMC. The accumulation of alpha-ENO on the cell surface of monocytes upon IFN stimulation was paralleled by cellular plasmin generation.

3. MATERIAL AND METHODS

3.1. Cell culture, probes, chemicals, and antibodies

A31 cells (a clone derived from mouse Balb/c 3T3) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and antibiotics in 5% CO_2 at 37^{o}C. Cells were grown until reached 70-80% of confluence and then starved with DMEM medium containing 1% FBS for 24h. Peripheral Blood Mononuclear Cells (PBMC) from heparinized blood of healthy donors were isolated by Ficoll-Paque (Amersham Biosciences, UK) density centrifugation as described (34). Alpha-enolase specific probe (10, 11) was kindly provided by Dra Louise C. Showe (Wistar Institute, Philadelphia, USA). *c-fos* (35) and *egr-1* (36) specific probes and anti FOS antibody were generously provided by Dr T. Curran (St Jude Children's Research Hospital, Memphis, TE, USA). Guanylate-Binding Protein-1 (GBP-1) probe and mouse recombinant IFN-gamma (R&D Systems, USA) were kindly provided by Dr L.F.L. Reis - Ludwig Institute for Cancer Research - São Paulo). Anti-phospho: MAPK ERK1/2 and CREB/ATF-1, total ERK1/2, anti beta-actin or secondary anti-rabbit or anti-mouse peroxidase conjugate antibodies were purchased from Cell Signaling Technology (Beverly MA, USA). Anti EGR-1 antibody was from Santa Cruz Biotechnology (Santa Cruz, EUA). Polyclonal rabbit antibody anti human alpha-enolase was as described (37,38). The pharmacological inhibitors: PD95098 (MEK), H89 (PKA), Genistein (tyrosine kinase) and Bisindolylmaleimide - Bis (PKC) were purchased from Calbiochem, La Jolla, CA, USA or Sigma-Aldrich. Human recombinant IFN-gamma was from Sigma-Aldrich and human recombinant IFN-alpha$_2$ alpha$_1$ was purified as described (39).

3.2. Cell treatment

Cells were serum-starved as described above and then incubated with IFN (1000UI/ml), for the times shown. When indicated, cells were treated with the inhibitor: H89 (PKA) (20μM), PD98059 (MEK) (50μM), Genistein (100μM), Bisindolylmaleimide (PKC) (150nM), Cycloheximide (50μg/ml) or Actinomycin D (5μg/ml) by 30 min prior to and throughout the IFN treatment. The drugs doses used throughout the experiments were established based on experimental observations, nonetheless, without cause any harm to the cells, given that no measurable effect on cell viability was verified by Trypan Blue dye exclusion. PBMCs were harvested, washed, suspended in RPMI 1640 at a concentration of 2x106 cells/ml and cultured for the indicated times, either in the absence or in the presence of IFN (1000UI/ml) and then properly prepared for specifics assays as indicated.

3.3. RNA isolation and Northern blot Analysis

Cells ($5x10^6$) were cultured in 75cm^2 tissue culture flasks, starved as above and then were treated with IFN or treated with different inhibitors prior to addition of IFN. At the indicated times, total RNA was isolated as described elsewhere (40) and 15μg RNA per sample were loaded, eletrophoresed on a 1.5% denaturing agarose-formaldehyde gel, transferred onto nylon membrane (Amersham Biosciences, UK), UV cross-linked for 5 minutes and hybridized with alpha-ENO, c-fos, egr-1 or GBP probe. Probes were labeled with alpha[32P] dCTP (Amersham Biosciences, UK) to a specific activity 1-5 x 10^8 cpm/μg DNA, by using a multiprime DNA labeling system from Amersham Biosciences, UK. Hybridizations and washing procedures were carried out as described (41). The membranes were stripped of the probe and re-probed with oligonucleotide for 18S rRNA labeled at 5´end with gammaATP (32P), by using T4 phage PNK (Promega, USA), which was used as an internal control for RNA loading.

3.4. Electrophoretic mobility shift assay (EMSA)

A31 cells were cultured and starved as above and then treated with IFN for the indicated times, or were incubated with PD98059 (50μM) for 30 min prior to and throughout IFN treatment. EMSA was carried out

essentially as described (42). Nuclear cell extracts were prepared as described elsewhere (43). Protein concentration was determined by the Bio-Rad assay. Ten µg of protein were pre-incubated with 1.2 µl poly (dI-dC) (5.4 mg/ml) (Amersham Biosciences, UK) at room temperature for 10 min, followed by the addition of a reaction mix containing 1.25 µg BSA, 0.125 µg of E. coli DNA, 0.25 µg yeast tRNA, 2% Ficoll 400 and 0.32 ng labeled probe (8.0 x 10^4 cpm). The reactions were incubated at room temperature for 30 min and then analyzed by 6% polyacrylamide gel electrophoresis. The 5' 32P-end-labeled double-stranded probes (only one strand is shown) corresponding to both the wild-type (WT) and the mutated (M) (underlined) alpha-ENO cis-acting elements (AP-1 or EGR-1) are as follows: WT-AP-1ENO: 5'-ATTTAGCTGCTGAGTCATGGGGC-3'; M-AP-1ENO: 5´- ATTTAGCTGCTGAGTTGTGGGGC–3´; WT-EGR-1ENO: 5' -AGCTGAGGGGGCGTGCCCC -3' and M-EGR-1ENO: 5´-AGCTGATAGGGCGTGCCCC–3´. Competition assays were performed with a 50-fold molar excess of cold oligonucleotide corresponding to c-fos SRE: 5'-GATGTCCATATTAGGACATC-3'. For super-shift assays, nuclear extracts were incubated with the specific antibody for 1 h at 4°C prior to incubation with the reaction mix for 30 min at room temperature.

3.5. Lysates preparation and Western Blot Analysis

After treatments, cells were washed with PBS and lysed on ice with lysis buffer (1% Triton X-100, 100mM Tris/HCl pH 8.0, 10% glycerol, 5mM EDTA, 200mM NaCl, 1mM DTT, 1mM PMSF, 2.5µg/ml leupeptin, 5µg/ml aprotinin, 1mM sodium orthovanadate). Lysates were scraped and collected into Eppendorf tubes and then centrifuged at 13,000 x g for 10 min at 4°C. Cell lysates samples (30µg) were separated by electrophoresis on a denaturing 10% polyacrylamide-SDS gel and transferred onto nitrocellulose membranes as described (40). Membranes were blocked overnight at 4°C with phosphate-buffered saline (PBS) containing 5% (w/v) nonfat dry milk and 0.1% Tween-20, washed three times with PBS containing 0.1% Tween-20 and then incubated with specific rabbit polyclonal anti-phospho-ERK1/2 antibody (1:1500), anti-phospho-CREB/ATF-1 antibody (1:1000), anti-ERK1/2 antibody (1:2000), anti-beta-actin antibody (1:3000) or anti-alpha-enolase antibody (1:20,000) in PBS containing 5% (w/v) BSA and 0.1% Tween-20. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary anti-rabbit or anti-mouse antibody (1:3000). Immunoreactive bands were visualized by using ECL detection system as described at manufacturer's instructions (Amersham Biosciences, UK)

3.6 - Flow Cytometry Analysis of Peripheral Blood Mononuclear Cells

Alpha-enolase expression on the cell surface was evaluated by flow cytometry analysis as described (17). After treatment or not, with IFN for 40h, PBMCs (1x10^6cells) were washed with PBS and incubated with 5% human serum in PBS for 15min at 37°C to block Fc receptors. Cells (2x10^5) were seeded in 96 wells plates and 0.5 µg of affinity-purified rabbit IgG specific for alpha-enolase (38, 44) in staining buffer (PBS 1x with 0,2% BSA and 0,01% NaN_3, pH 7.4), or staining buffer alone, was then added, and incubation was continued for 15 min on ice. The cells were washed again and 20 µl of fluorescein-conjugated goat anti-rabbit IgG (1/100) (Sigma-Aldrich) was added, and the reaction mixtures were further incubated for 15 min on ice in the dark. After being washed, cells were resuspended in 200 µL of PBS containing 2% paraformaldehyde. As negative controls, cells were always stained in parallel with the secondary antibody alone and with an irrelevant isotype-matched primary antibody. The cells were analyzed by flow cytometry using a Becton Dickinson FACS Vantage (Becton Dickinson Biosciences, San Jose, CA). Comparisons of fluorescence intensity between cells treated, or not, with IFN, were done with paired Student's t test.

3.7. Cell Surface-generated plasmin Assay

Cell-dependent plasminogen activation was determined indirectly by using the plasmin-specific chromogenic substrate H-D-Val-Leu-Lys-p-nitroanilide S-2251 (Sigma, Aldrich) as previously described (45). In brief, PBMCs seeded at 2x10^6 cells/ml in 24-well plates were treated or not with IFN (1000UI/ml) for 40 h. The cells were then washed two times with PBS and incubated in serum-free RPMI medium containing 20µg of human plasminogen (Calbiochem, USA) for 3 h. The cells were washed again with PBS and incubated in 0,1M Tris buffer, pH 8.0, containing the substrate S-2251 at 1.0mM. The amount of p-nitroanilide released after 2 h of incubation at 37°C was measured at 405nm using a plate reader (Start Fax-2100 Awareness Technology, INC). Each sample was also assayed without Plg to establish the Plg-dependence on the enzymatic activity. Absorbance rate values were used to estimate plasmin activation. Comparisons of the optical density (OD) between non-treated or IFN-treated cells were done with Student's *t* test.

4. RESULTS

4.1. Interferons-alpha and -gamma stimulate alpha-enolase mRNA accumulation

To investigate whether IFN regulates alpha-ENO expression, A31 cells were exposed to either IFN-alpha or IFN-gamma for the times shown and total RNA was isolated, blotted and probed. Figure 1A - upper panel - shows that both IFN-gamma (lanes 2-6) and IFN- $alpha_2$ $alpha_1$ (lanes 7-11) stimulate alpha-ENO mRNA expression in a time-dependent manner. α-ENO mRNA accumulation showed a pattern of secondary response gene, the message being detected 6 and 9 hours post-stimulation, peaked at 12 h, and prolonged up to 40 h (data not shown). GBP-1, a well characterized gene that responds to IFN-stimulation (46, 47), was included as a control (middle panel). In accordance with the mRNA accumulation, protein levels of alpha-ENO also increased upon IFN-alpha treatment (Figure 1B). We also demonstrated that alpha-ENO expression is dependent on *de novo* protein synthesis and active transcription (Figure 1C), since pre-incubation with cycloheximide (Figure 1C, lane 4) or actinomycin D (Figure 1C, lane 6), blocked the IFN-stimulated alpha-ENO mRNA expression. In addition, alpha-ENO expression seems to be mediated through a coordinated activation of a

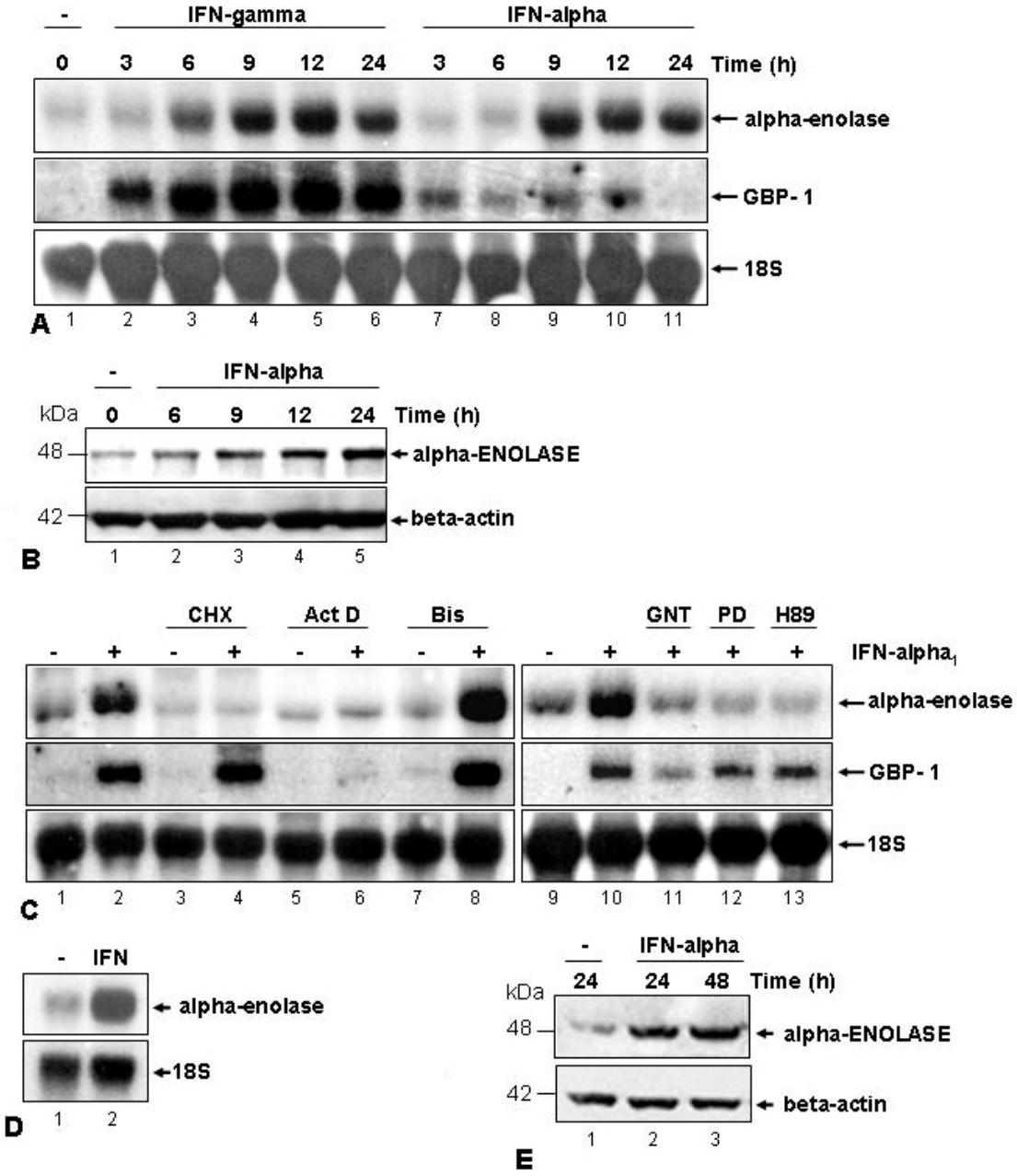

Figure 1. Interferon-alpha and -gamma stimulates alpha-ENO mRNA expression. Quiescent monolayers of A31 cells were treated with IFN as indicated and processed for Northern blot (A, C) or Western Blot (B) as described in Material and Methods. (A) *Kinetics of alpha-ENO mRNA expression*. Cells were either left untreated (lane 1) or treated with 1000UI/ml of IFN-gamma (lanes, 2-6) or IFN-alpha$_2$ alpha$_1$ (lanes 7-11) for the indicated times. (B) *The kinetics of alpha-ENO protein expression*. Cells were left untreated or were treated with 1000UI/ml IFN-alpha$_2$ alpha$_1$ for the indicated times. (C) *Alpha-ENO mRNA expression relies on both de novo protein synthesis and active transcription and is influenced by protein kinases inhibitors*. Cells were left untreated (lanes 1 and 9), treated with 1000UI/ml IFN-alpha$_2$ alpha$_1$ (lanes, 2 and 10) for 9 h. or incubated with the specific inhibitor for 30 min prior to IFN-alpha$_2$ alpha$_1$ (1000UI/ml) stimulation for 9 h (lanes 4, 6, 8, 11-13). Inhibitors were used at the following concentrations: cycloheximide (CHX) (50µg/ml); actinomycin D (Act D) (5µg/ml); bisindolylmaleimide (Bis) (150nM); genistein (GNT) (50µM); PD98059 (PD) (50µM), H89 (20µM). (D and E) *Northern and Western Blot of PBMCs, respectively.* Cells were left untreated or treated with IFN-alpha$_2$ alpha$_1$ (1000UI/ml) for 9 h (D) or treated with IFN for the times shown (E). 18S rRNA and beta-actin were used as an internal control for RNA and protein loading, respectively. Data are representative of three independent experiments.

phospho tyrosine-sensitive, MEK/ERK and PKA pathways, but not PKC (Figure 1C, lane 8), because pre-incubation with the pharmacological inhibitors genistein, PD98059, or H89 (Figure 1C, lanes 11-13), blocked the IFN-alpha stimulated alpha-ENO expression.

We have also investigated alpha-ENO expression after IFN-alpha$_2$ alpha$_1$ stimulation in human PBMC and found that the cytokine induces both mRNA and protein accumulation in these cells (Figure 1 D and E).

4.2. Early response genes associated with alpha-ENO expression

Based upon the pattern of secondary response gene presented by alpha-ENO and its sensitivity to PD98059 and genistein, we searched for an early response gene(s) that could be associated with alpha-ENO expression and, at the same time, could be sensitive to those pharmacological inhibitors. Since the 5' upstream regulatory sequence of alpha-ENO has potential binding sites for AP-1 and EGR-1, we started by investigating whether IFN-alpha$_2$ alpha$_1$ regulates both *c-fos* and *egr-1* mRNA expression in A31 cells, and if their expression were blocked by pre-incubation with PD98059 and genistein. Our findings provide evidence that IFN- alpha$_2$ alpha$_1$ not only stimulates the expression of both genes (Figure 2A, upper and middle panels), but that their expression also relied on MEK and phospho-tyrosine pathways (Figure 2B, upper and middle panels). Fig 2C shows that consistent with *egr-1* mRNA accumulation, EGR-1 protein is transiently expressed. Similar results were also obtained with c-FOS (data not shown).

4.3. DNA-protein complexes formed upon IFN-alpha$_2$ alpha$_1$ stimulation

Based on the observation that: a)- IFN induces the primary response genes *c-fos* and *egr-1* through MEK- and tyrosine kinase-dependent pathways, b)- AP-1 (FOS/JUN) and EGR-1 may act as transcriptional activators involved with the regulation of a number of genes (48-52), and c)- there are putative AP-1 and EGR-1 binding sites in alpha-ENO promoter, we hypothesized whether FOS and EGR-1 could bind to their cognate sequences at the alpha-ENO promoter.

Figure 3A shows a schematic representation of the promoter region of the alpha-ENO gene with the potential DNA binding sites for AP-1, EGR-1, CREB, HIF-1 and Sp-1. We designed double-stranded oligodeoxynucleotides corresponding to the AP-1 and EBS (Egr-1 Binding Site) of alpha-ENO, to use as probes in gel shift-assays. Our data show that, upon IFN- alpha$_2$ alpha$_1$ stimulation, an increased AP1-ENO-binding activity was verified, from 30 min (Figure 3B, lane 2), which reached maximal DNA-protein interaction at 2 h (lane 4), and was prolonged up to 24 h. The specificity of the interaction was verified by competing the labeled probe with molar excess of either cold AP1-ENO (lane 11) or consensus AP-1 (lane 12) oligodeoxynucleotides. The probe, however, did not compete with the unrelated SRE oligo (lane 13). In the attempt to characterize the proteins involved with the IFN-stimulated complex, cell extract was pre-incubated with anti-c-FOS antibody prior to addition of the labeled probe. As shown (Fig 3B, lane 14) c-FOS was super-shifted, which identify the protein as a component of this complex. We also provide evidence that the binding of FOS to AP1-ENO is dependent on MEK/ERK pathway, since PD98059 completely abrogated this interaction (Figure 3B, lane 18).

Analysis carried out with the EGR1-ENO probe demonstrated that IFN-alpha$_2$ alpha$_1$ was also able to stimulate the formation of two specific complexes with this cis-acting element. As shown in Figure 3C, Slower migrating complex (SMC) was increased over basal levels post-IFN-stimulation (lanes 2-8), and the faster migrating complex was formed after 1-2 h post-IFN-stimulation (lanes 4-5). The SMC was detected until 24 h post-IFN-stimulation (data not shown). Competition experiments performed either with molar excess of cold related oligo (Figure 3C, lane 9), or with unrelated SRE oligo (lane 10), confirmed the binding specificity. Pre-incubation with anti-EGR-1 antibody abolished EGR-1/ENO complex formation, present at the faster migrating complex that was formed after 1-2 h of IFN treatment (Figure 3C, lanes 4-5), but was not able to super-shift the SMC (compare lane 4 with 11), although competition assay carried out with molar excess of unlabeled EGR-ENO disrupted the formation of both complexes (Fig 3C, lane 9). Pre-incubation of the cell extracts with the MEK inhibitor PD98059 drastically reduced both complexes formation (Figure 3C, lane 15).

Of note, the above DNA-protein complexes were not formed any more, when both wild-type sequences were substituted to the mutated AP-1ENO and EGR-1ENO counterparts, as shown in Figures 3B and 3C, (lanes 20 and 17), respectively.

4.4. IFN-alpha$_2$ alpha$_1$ stimulates both ERK1/2 and CREB/ATF-1 phosphorylation

In order to investigate the signaling pathways involved with the IFN-alpha$_2$ alpha$_1$ stimulated alpha-ENO expression and based upon the influence exerted by the specific MEK inhibitor PD98059 and PKA inhibitor H89 on alpha-ENO mRNA accumulation, western-blot analysis was carried out to analyze the status of ERK 1/2 and CREB/ATF-1 phosphorylation under this circumstance. Figure 4A shows that ERK1/2 was phosphorylated upon IFN-alpha$_2$ alpha$_1$ stimulation. The kinetic of ERK activation in A31 cells, showed a biphasic pattern of phosphorylation, with the first wave occurring between 0.5 h (stronger activation was observed at 15 min - data not shown) and 1.0 h of stimulation and decreasing by 2 h (lanes 3-5), followed by a second phase of activation starting after 6 h and prolonging up to 11 h after IFN treatment (lanes 7-10). Consistent with the inhibition caused by PD98059 on alpha-ENO mRNA accumulation, the same effect was also observed with ERK1/2 phosphorylation, whereas no inhibition was observed with the PKA inhibitor (H89) (Figure 4B, lanes 3 and 4), respectively.

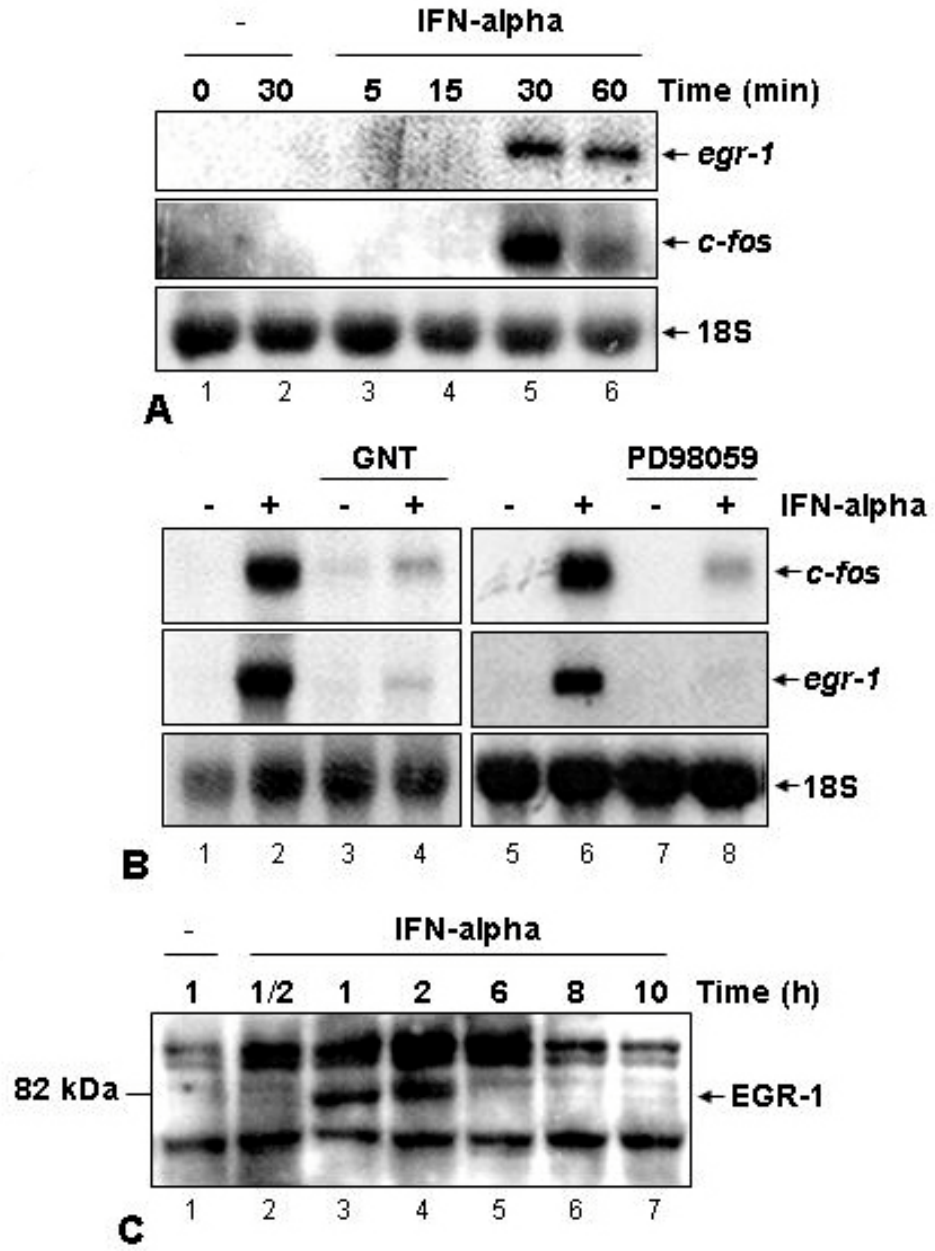

Figure 2. IFN-alpha stimulates *c-fos* and *egr-1* expression. Starved A31 cells were treated with 1000UI/ml IFN-alpha$_2$ alpha$_1$ and then assayed by Northern blot as described in Material and Methods. (A) *IFN-alpha$_2$ alpha$_1$ stimulated c-fos and egr-1 expression*. Cells were either left untreated or treated with IFN-alpha$_2$ alpha$_1$ for the times shown. (B) *Pre-treatment with PD98059 and Genistein abolishes egr-1 and c-fos mRNA expression*. Cells were incubated with the inhibitor prior to IFN-alpha$_2$ alpha$_1$ stimulation for 30 min. (C) *IFN-alpha$_2$ alpha$_1$ -stimulated EGR-1 protein expression*. Cells were either left untreated or treated with IFN-alpha$_2$ alpha$_1$ (1000UI/mL) for the times shown, and then probed with anti-EGR-1 antibody. RNA loading was monitored by probing the membranes with 18S rRNA. Data are representative of three independent experiments.

Our data also demonstrated that cells exposure to the IFN-alpha$_2$ alpha$_1$ led to the phosphorylation of the transcription factors CREB and ATF-1 with a kinetic of activation presenting a biphasic pattern. Figure 4C shows that CREB/ATF-1 were phosphorylated to the same extent between 15-30 min and decreased by 1-2 h (lanes 3-6), whereas only ATF-1 was phosphorylated between 4 and 5 h (lanes 9-10), while CREB phosphorylation was only observable after 6 h of IFN treatment (lane 11). Pre-incubation with H89 or PD98059 inhibited CREB/ATF-1 phosphorylation (Figure 4D, lanes 3 and 4), respectively.

4.5. IFN-alpha$_2$ alpha$_1$ -upregulated alpha-enolase expression at the surface of monocytes

Alpha-enolase is a multifunctional protein that may act as plasmin/plasminogen receptor at the cell surface

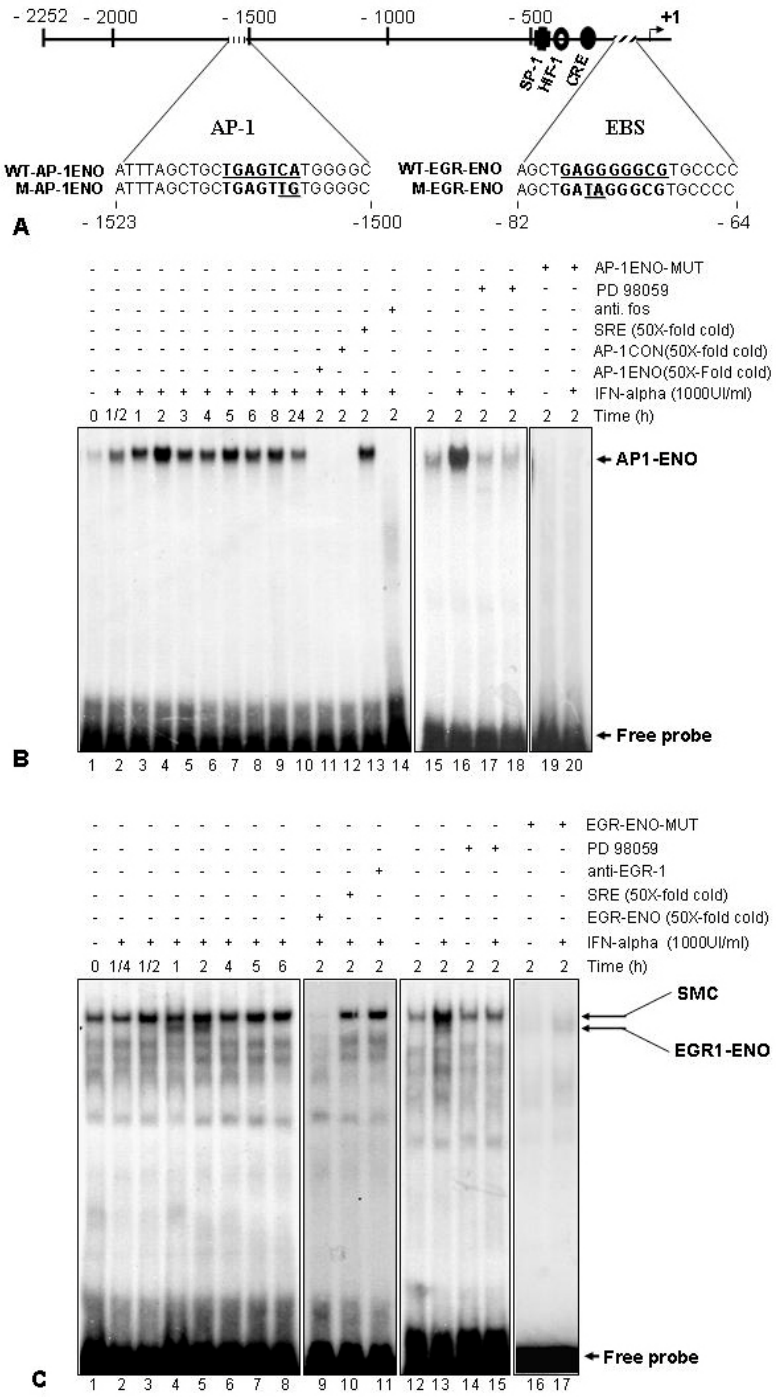

Figure 3. Analysis of IFN-alpha-stimulated DNA-protein complexes formation. (A) *Schematic representation of the 5' regulatory sequence of the alpha-ENO gene*. The DNA-binding sites for AP-1, EBS (EGR-1 binding site), CRE and NF-kB are shown. (B, C) *Transcriptional complexes formed with AP1-ENO or EGR1-ENO*. EMSA was carried out after treating the starved cells with IFN-alpha$_2$ alpha$_1$ (1000UI/mL) for the indicated times. Ten µg of nuclear protein were incubated with an end-labeled oligodeoxynucleotide probe containing the alpha-ENO cis-acting elements: AP-1 or EBS. DNA-protein complexes formed are indicated by arrows on the right. The specificity of the interactions was confirmed by competing the probe with 50-fold molar excess of the indicated cold oligo (Figs. 3B and C, lanes 11-13 and 9-10), respectively. c-FOS and EGR-1 were super-shifted by specific antibodies (B and C, lanes 14 and 11). Pre-incubation with PD98059 disrupted the formation of the DNA-protein complexes (B and C - lanes 18 and 15), respectively. Mutations mapping at the DNA binding sites of AP-1 or EGR-1 abolished the complexes formation (B and C- lanes 20 and 17), respectively. Figure 3C - SMC - slower migrating complex. These data were repeated in three independent experiments with identical results.

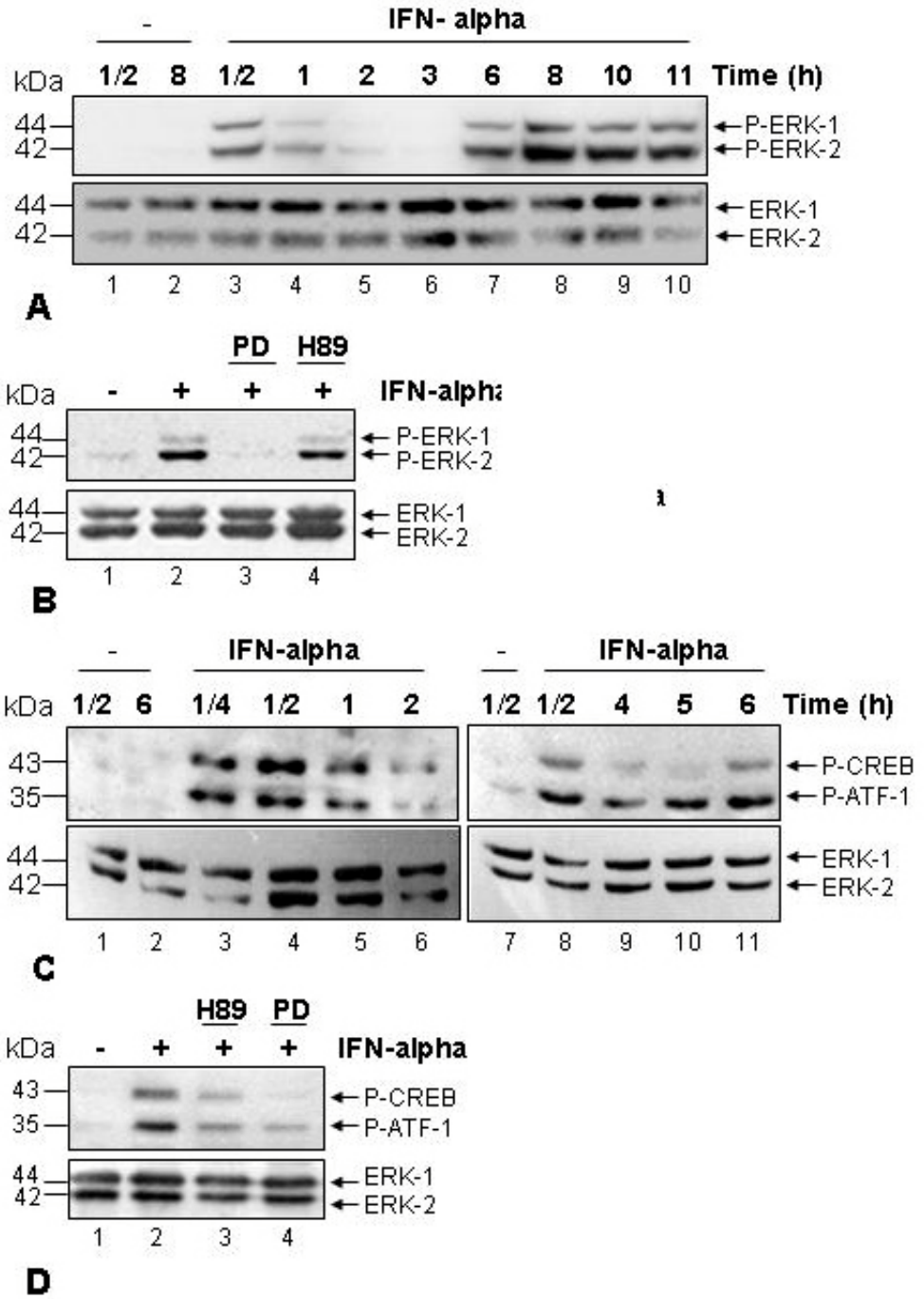

Figure 4 . ERK1/2 and CREB/ATF-1 are phosphorylated upon IFN-alpha-stimulation. Western blot analysis performed with cell lysates prepared after treating or not the starved cells with IFN-alpha$_2$ alpha$_1$ (1000UI/mL) for the indicated times (A and C) or for 30min (B and D). Proteins were fractionated on 10% SDS-PAGE, transferred onto nitrocellulose and then probed with anti phospho ERK1/2 or CREB/ATF-1 antibodies. (A) *Kinetics of ERK1/2 phosphorylation*. (B) *PD98059 inhibits the IFN-stimulated ERK1/2 phosphorylation*. Cells were incubated with PD98059 or H89 for 30min prior to IFN stimulation. (C) *Kinetics of CREB/ATF-1 phosphorylation*. (D) *PD98059 and H89 inhibit the IFN-stimulated CREB/ATF-1 phosphorylation*. Cells were incubated with PD98059 or H89 for 30min prior to IFN stimulation. The positions of proteins are indicated in kilodaltons (kDa) on the left. These data were consistently repeated in at least three independent experiments.

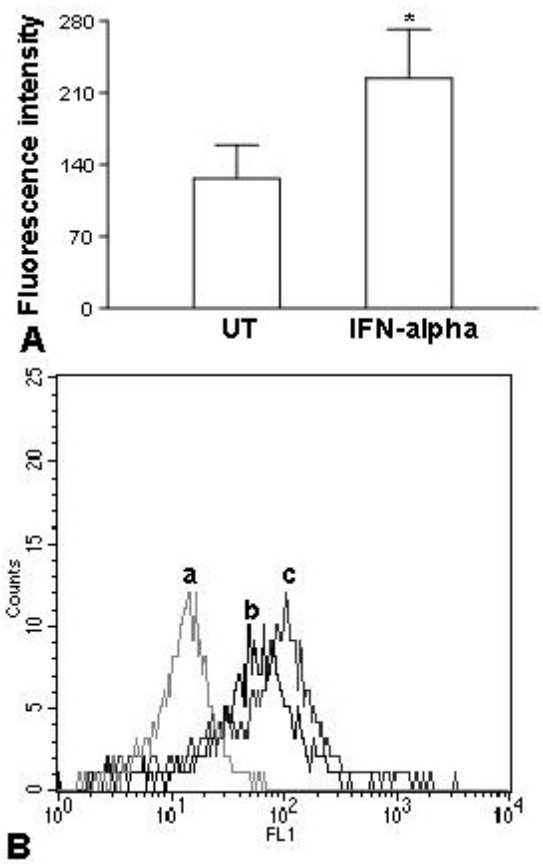

Figure 5. Interferon-alpha increases alpha-ENO expression at cell surface. PBMCs were either left untreated (UT) or treated with 1000UI/ml IFN-alpha$_2$ alpha$_1$ for 40 h and then incubated with 0.5 μg of anti-human alpha-ENO, followed by incubation with FITC labeled-goat anti-rabbit IgG and flow cytometry analysis. (A) *Mean Fluorescence intensity of PBMCs treated or not with IFN*. The data are expressed as mean of the absolute values of untreated or IFN-treated PBMC (Mean ± SD, n = 3 for each group). (*) for $P < 0.05$ compared to control. (B) *Histograms of one representative experiment.* a) Isotype control, b) and c) untreated and IFN-alpha$_2$ alpha$_1$ treated cells incubated with anti-human alpha-ENO, respectively.

(17-19, 26). To get some insights whether the IFN-stimulated expression was followed by its accumulation at the cell surface, flow cytometry analysis was carried out. PBMC were left untreated or incubated with IFN-alpha for 40 h and then stained for alpha-enolase. The cells were selected based on size and granularity, allowing for separate analysis of lymphocyte and monocyte populations. Figure 5A shows that the mean fluorescence intensity of surface-associated alpha-enolase increased 1.8-fold (about 89% of control) (P <0.05), and percent of positive cells increased about 76% of control (P <0.01) (data not show) in the IFN-alpha stimulated monocytes as compared to untreated control cells. Our preliminary data also shows that IFN-gamma caused a similar effect (data not show). Irrelevant antibodies, at the same concentration as that of anti-alpha-ENO, did not react with the cells. Fluorescence histograms of untreated (Figure 5B, histogram b) and IFN-alpha stimulated cells (Figure 5B, histogram c) show that fluorescence intensity was normally distributed, defining a single population of cells with relatively uniform staining characteristics, consistent with signals routinely observed with anti-alpha-ENO antibody. IFN-alpha induced a uniform upward shift in the fluorescence intensity of the entire population (Fig 5B, compare histogram b and c). As a control for alpha-ENO expression, PBMCs were treated with PMA (Phorbol Miristate Acetate) (16). We found that the mean fluoresce intensity of surface-associated alpha-enolase was increased about 2.1 fold as compared to untreated cells, and the fluorescence profile indicated that the alpha-ENO-positive cell population was heterogeneous (data not shown). A positive signal also was obtained for the lymphocyte population, but there were no significant differences between treated or untreated cells with both IFNs (data not shown).

4.6. IFN-alpha increased cell surface plasmin generation

Previous studies have shown that IFN-gamma increased cell surface plasmin generation in pulmonary epithelial cells (53) and mononuclear phagocytes (54). In order to investigate whether IFN-alpha actually is involved with the regulation of cell surface plasmin generation, followed the addition of exogenous plasminogen to PBMCs, we used a plasmin activation assay. Cells were incubated with IFN-alpha for 40 h and then incubated with plasminogen before the addition of the plasmin specific substrate (S-2251). We found that IFN-alpha caused a statistically significant ($p \leq 0.01$) increase in plasmin activity (about 64%), measured by the increase in absorbance after cleavage of S-2251, when compared to non-stimulated cells (Figure 6). IFN-gamma caused a similar effect (data not shown).

5. DISCUSSION

The diverse biological activities of IFNs reflect their abilities to induce a set of genes whose codifying proteins are the primary effectors of the cytokines actions. Although many ISGs have unknown functions, some of them play a key role in host defense (1, 4, 7, 55-56). In this sense, to identify new ISGs and to find out their functions, may contribute to increase our understanding of the IFN system.

In this study, we demonstrate that IFN-alpha activates MAPK ERK1/2 and CREB/ATF-1 in fibroblasts (Figure 4) leading to alpha-ENO mRNA expression, whose steady-state levels showed a pattern consistent with a secondary-response gene, i.e. it was delayed and dependent on *de novo* protein synthesis (Figs. 1A and C). By using the pharmacological inhibitors PD98059, H89, Genistein and Bisindolylmaleimide, we show that the gene expression seems to be coordinated by the simultaneous activation of MEK/ERK1/2, PKA and a tyrosine kinase-sensitive pathways, probably the JAK/STAT pathway (57) (Figure 1C, lanes 11-13), but it does not require PKC (Fig 1C, lane 8). These findings contrast with the regulation exerted by IFN-alpha over GBP-1 expression, since it occurred

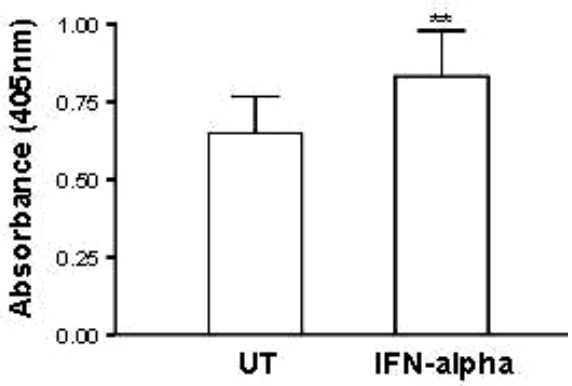

Figure 6. Effect of IFN-alpha on cell-surface plasmin generation. PBMCs were either left untreated (UT) or treated with 1000UI/ml IFN-alpha$_2$ alpha$_1$ for 40 h and then assayed for cell-surface plasmin generation as described in Material and Methods. Plasmin generation was detected as the OD increase at 405 nm, due to S-2251 hydrolysis. The results are Mean ± SD, n = 9 for each group), (**) for $P \leq 0.01$ compared to control.

independently of protein synthesis (Figure 1C, lane 4) (46) and relied upon tyrosine kinase (JAK/STAT pathway) (Figure 1C, lane 11) (58), but not MEK/ERK (Figure 1C, lane 12). Our data also show that the IFN-regulated alpha-ENO expression is not restricted to fibroblast cells since it was verified with PBMC as well (Fig.1 D and E).

Although alpha-ENO is one of the best characterized proteins, little is know about its regulation. It was recently demonstrated, by using MEK-dominant negative strategy, that ERK1/2 is involved with the regulation of alpha-ENO expression in cardiomyocytes submitted to ischemic hypoxia and reoxygenation (13). Other reports include: a) Alpha-ENO is induced by hypoxia on HIF-1 alpha-dependent manner in Hep3B cells (12), b) c-Myc directly transactivates alpha-ENO in Rat-1 fibroblasts (59), c) enolase neuron-specific is an EGR-1 target gene in prostate carcinoma cells (60), d) alpha-ENO expression is upregulated in *c-jun* transformed rat fibroblast (61), and e) MBP-1 (Myc-binding protein) shares 3´ end homology with alpha-ENO (20,21). On the other hand, IFN-induced gene expression via MAPK activation, was previously demonstrated (62). These authors showed that ERK1/2 is necessary for IFN-gamma-induced p48 gene transcription through C/EBP-beta and that ERK1/2 activation was sustained until 8 h, similar to what we observed in our experimental setting.

Consistent with the requirement of MEK and a tyrosine-kinase sensitive pathway for alpha-ENO expression after stimulation with IFN, the expression of the primary response genes *c-fos* and *egr-1* was also dependent on both pathways (Figure 2B). Furthermore, the requirement of the MEK pathway was equally true for the binding capability of these transcriptional activators to the 5´regulatory elements found at the alpha-ENO promoter (Figs. 3B-C). Our observation that incubation with anti-EGR-1 antibody blocked the DNA-protein interaction only of the faster migrating complex, but not the slower migrating one (Figure 3C, lane 11), could be explained as follows. Even though both complexes have the capacity to be formed with the same oligodeoxynucleotide, a GC-rich sequence, (Figure 4C, lanes 4-5), we believe that Sp-1 might be the protein associated with the SMC, because it competes with EGR-1 for binding to the same GC-rich sequence (63), though this issue remains to be further investigated. EMSA analysis was also carried out using the consensus EBS sequence. It was observed the formation of only one complex, after IFN treatment, which was super-shifted by pre-incubation with anti-EGR-1 antibody (data not shown), suggesting a more complex regulation of the alpha-ENO gene upon IFN treatment. Although our data support the notion that c-FOS and EGR-1 might be involved with alpha-ENO expression, additional experiments, nonetheless, should be necessary to definitively prove their implication with the gene expression.

Our findings also provide evidence that the transcription factors CREB/ATF-1 might play a role on alpha-ENO expression too, because, incubation with the pharmacological inhibitor of PKA (H89) prior to IFN treatment also led to dramatic decrease in CREB/ATF-1 phosphorylation (Figure 4D, lane 3) and a remarkable blockade in transcript accumulation as well (Figure 1C, lane 13). Since CREB/ATF-1 phosphorylation relies either on PKA or MEK/ERK pathways through the downstream kinases RSK2 (64, 65) and/or MSK1 (66, 67), that gives support to our finding that the IFN-stimulated CREB/ATF-1 activation results from the combined activation of both signaling pathways (Figure 4D, lanes 3-4).

Based on the observation reported by others, on the regulation exerted by IFN over some components of the Plasminogen/Plasmin fibrinolytic system (27-30, 53), and that alpha-ENO, under certain circumstances, can accumulate at the cell surface acting as a Plasminogen receptor (17-19, 26), the data presented at Figure 5A-B, shed some light on this matter and provide evidence that IFN is indeed able to regulate this component of the fibrinolytic system and promotes its increase at the surface of monocyte. Although the increase of alpha-ENO on the cell surface was followed by an augment in Plasmin proteolytic activity (Figure 6), we cannot directly associate this activity with a second role of alpha-ENO as a Plg receptor. Even though alpha-ENO represents more than 70% of Plg receptor in monocytes (26), a minor-represented Plg receptor, other than alpha-ENO, could be involved. Additional data, however, should be pursued to firmly establish the biological consequences of alpha-ENO upregulation by IFN. Recently, it was reported that MBP-1 (myc-binding protein-1), a shorter version of alpha-ENO with homology at 3´end and associated with down-regulation of Myc (21), was identified as an putative IFN-regulated gene by microarray analysis (56).

Although the regulation of alpha-ENO is under control of diverse cytokines (14, 15), to the best of our knowledge, this is the first report that shows a detailed characterization of alpha-ENO as a new ISG. The increased

levels of alpha-ENO observed at the cell surface correlate with an augment in localized pericellular plasmin generation and proteolysis. Since low doses of plasmin were shown to stimulate cytokine expression, the release of lipid mediators and chemotaxys in monocytes (68-70), we believe that the pericellular plasmin generation observed may be of biological relevance for both extracellular proteolysis and intracellular signaling.

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Abbreviations: alpha-ENO: alpha-enolase; IFN: interferon; ISG: Interferon-stimulated gene; MAPK: mitogen-activated protein kinase; ERK: extracellular-signal regulated kinase; MEK: MAPK/ERK kinase; AP-1: activator protein-1; EGR-1: early growth response-1 gene product; PBMC: peripheral blood mononuclear cells.

Key Words: Alpha-Enolase, Interferon, ISG, Plasminogen receptor, MAPK-ERK1/2, c-fos, *egr*-1, CREB/ATF-1

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