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Plasminogen/plasmin regulates *c-fos* and *egr*-1 expression via the MEK/ERK pathway☆

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Abstract

In this study, we showed that plasminogen (Plg) and plasmin (Pla) bind to lysine-binding sites on cell surface and trigger a signaling pathway that activates the mitogen-activated protein kinase (MAPK) MEK and ERK1/2, which in turn leads to the expression of the primary response genes *c-fos* and early growth response gene *egr-1*. Our data show that the Plg/Pla-stimulated steady-state mRNA levels of both genes reached a maximum by 30 min and then returned to basal levels by 1 h. The gene induction was sensitive to both pharmacological and genetic inhibition of MEK. Leupeptin, a serine protease inhibitor, suppressed Pla but not Plg-induced *c-fos* and *egr-1* expression, emphasizing the role played by the serine protease activity associated with Pla. Pre-incubation with cholera toxin completely blocked the Plg/Pla-induced gene expression, suggesting that another signaling pathway, which recruits G protein-coupled receptors, may also be involved. Furthermore, Plg/Pla also stimulated AP-1 and EGR-1 DNA-binding activities, which were abrogated by pharmacological inhibition of MEK. Altogether, these results suggest that Plg/Pla stimulates *c-fos* and *egr-1* expression via activation of the MEK/ERK pathway.

Keywords: Plasminogen; Plasmin; MAPK; ERK1/2; *c-fos*; *egr-1*

The plasminogen/plasmin (Plg/Pla) proteolytic system is associated with a variety of biological activities beyond the classical dissolution of fibrin deposits [1], such as cell migration, tissue repair, inflammation, and metastasis [2–5]. These activities result from the conversion of the zymogen Plg to the serine protease Pla owing mainly to the proteolytic activity of urokinase-type Plg activator (uPA) [6,7]. In addition, uPA has also been associated with the regulation of the primary response gene *c-fos* [8], or the transcriptional activator AP-1 (FOS/JUN), which in turn may regulate diverse components of the Plg system such as plasminogen activator inhibitor-1 and -2 (PAI-1 and PAI-2) [9], uPA [10], and uPA receptor (uPAR) [11]. The nuclear transcription factor namely early growth response gene (*egr-1*) is implicated in the regulation of a number of pathophysiologically important genes, including tissue factor (TF) [12], uPA [13], uPAR [14], transforming growth

☆ *Abbreviations:* Plg, plasminogen; Pla, plasmin; MAPK, mitogen-activated protein kinase; ERK, extracellular-signal regulated kinase; *egr*-1, early growth response gene-1; MEK, MAPK/ERK kinase; EBS, EGR-1 binding site, PAs, plasminogen activators.

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factor-β1 [15], and platelet-derived growth factor A [16] and B [17].

This study was aimed to investigate the control exerted by Plg/Pla over the expression of *c-fos* and *egr-1*, and to characterize the signaling pathway associated with their expression. Here we provide evidence that Plg/Pla stimulates their expression through the MEK/ERK pathway.

Materials and methods

Cell culture, 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 °C. In all experiments, cells were serum-starved in DMEM supplemented with 1% FBS for 24 h after reaching 70–80% confluence. Anti-phospho: MAPK ERK1/2 and total ERK1/2 or secondary anti-rabbit peroxidase conjugate antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-EGR-1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, EUA). Anti-FOS antibody was generously provided by Dr. T. Curran (St. Jude Children's Research Hospital, Memphis, TE, USA). The pharmacological inhibitors of MEK PD95098 and U0126, and human Plg were purchased from Calbiochem (La Jolla, CA, USA). Human plasmin (Pla) and urokinase (uPA), ε-aminocaproic acid (EACA), actinomycin D, leupeptin, and anti-β-actin antibody were from Sigma–Aldrich, São Paulo, Brazil.

Cells treatment. Cells were serum-starved as described above and then incubated with Plg or Pla, for the times shown. When indicated, cells were pre-incubated for 30 min with the following concentration of inhibitor prior to and throughout Plg/Pla treatment: PD98059 and U0126—50 μM (MEK), actinomycin D—5 μg/ml, cholera toxin—1 μg/ml (G protein), leupeptin—25 μg/ml (serine protease), and EACA—0.1 M (lysine binding). The drug doses used throughout the experiments were established based on experimental observations, without, nonetheless, causing any harm to the cells, given that no measurable effect on cell viability was verified by trypan blue dye exclusion.

MEK1 dominant-negative cell lines. To generate cellular clones stably expressing the MEK1 dominant-negative mutation, A31 cells were transfected with 10 μg plasmid DNA carrying either negative dominance for MEK1 or the wild-type MEK1 [18], by using standard calcium phosphate protocols [19]. Transfectants were ring-cloned after selection with G418 (800 μg/ml) (Geneticin, Invitrogen, São Paulo, Brazil) for at least 21 days and then tested for MEK1 unresponsiveness, as evaluated by ERK1/2 phosphorylation, after stimulation with epidermal growth factor (50 ng/ml) (Sigma–Aldrich, São Paulo, Brazil) or vaccinia virus infection [20]. Selected clones (MEK1-DN) presenting identical responses to the above stimuli were then used to carry out the experiments.

RNA isolation and Northern blot analysis. Cells (5×10^6) were cultured in 75 cm^2 tissue culture flask, starved as above, and then incubated with Plg/Pla for the times shown, or were incubated with different inhibitors as described above. At the indicated times, total RNA was isolated as described elsewhere [21], and 15 μg RNA per sample was loaded, electrophoresed on a 1.5% denaturing agarose–formaldehyde gel, transferred onto nylon membrane (Amersham Biosciences, UK), UV cross-linked for 5 min, and hybridized with the probes: *c-fos* [22] or *egr-1* [23]. Probes were labeled with [α-^{32}P]dCTP (Amersham Biosciences, UK) to a specific activity of $1–5 \times 10^8$ cpm/μg DNA, by using a multiprime DNA labeling system from Amersham Biosciences, UK. Hybridizations and the washing procedures were carried out as described [24]. The membranes were stripped of the probe and re-probed with oligonucleotide for 18S rRNA labeled at 5′ end with [γ-^{32}P]ATP, by using T4 phage PNK (Promega, USA), which was used as an internal control for RNA loading.

Lysate preparation and Western blot analysis. After treatments, cells were washed with PBS and lysed on ice with lysis buffer (1% Triton X-100, 100 mM Tris/HCl, pH 8.0, 10% glycerol, 5 mM EDTA, 200 mM NaCl, 1 mM DTT, 1 mM PMSF, 2.5 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 mM sodium orthovanadate). Lysates were scraped, collected into Eppendorf tubes, and then centrifuged at 13,000*g* for 10 min at 4 °C. Cell lysate samples (30 μg) were separated by electrophoresis on a denaturing 10% polyacrylamide–SDS gel and transferred onto nitrocellulose membranes as described [21]. Membranes were blocked overnight at 4 °C with phosphate-buffered saline containing 5% (w/v) nonfat dried milk and 0.1% Tween 20, washed three times with phosphate-buffered saline containing 0.1% Tween 20, and then incubated with specific rabbit polyclonal anti-EGR-1 antibody (1:2000), anti-phospho ERK1/2 antibody (1:2000), anti-ERK1/2 antibody (1:2000) or anti-β-actin (1:1000) in phosphate-buffered saline containing 5% (w/v) BSA and 0.1% Tween 20. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary anti-rabbit antibody (1:3000). Immunoreactive bands were visualized by using ECL detection system as described in manufacturer's instructions (Amersham Biosciences, UK).

Electrophoretic mobility shift assay. A31 cells were cultured, starved, and then treated as described above. Electrophoretic mobility shift assay (EMSA) was carried out essentially as described [25]. Nuclear cell extracts were prepared as described elsewhere [26]. Protein concentration was determined by Bio-Rad assay. Ten micrograms of protein was 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 *Escherichia coli* DNA, 0.25 μg yeast tRNA, 2% Ficoll 400, and 0.32 ng labeled probe (8.0×10^4 cpm). The reactions were incubated at room temperature for 30 min and then analyzed by 6% polyacrylamide gel electrophoresis. The 5′ ^{32}P-end-labeled double-stranded probes (only one strand is shown) corresponding to both consensus *cis*-acting elements (AP-1 or EGR-1) are as follows: AP-1: 5′-CGCTTGATGACTCAGCCGGAA-3′; EGR-1: 5′-GGATCCA GCGGGGGCGAGCGGGGGCGA-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.

Results

Plg/Pla stimulates c-fos and egr-1 mRNA expression

Previous studies reported that urokinase, a component of the Plg/Pla system, was able to induce the expression of the primary response gene *c-fos* [2,8]. This observation suggested that its gene product could be associated with the transactivation of other components of this system. In fact, it was verified that *c-fos* was directly involved with the expression of PAI-2, urokinase (auto-regulation), and uPAR [9–11]. Based on these observations, we decided to examine whether Plg and Pla were also able to regulate *c-fos* expression. Because another primary response gene, early growth response gene (*egr-1*), is frequently associated with the

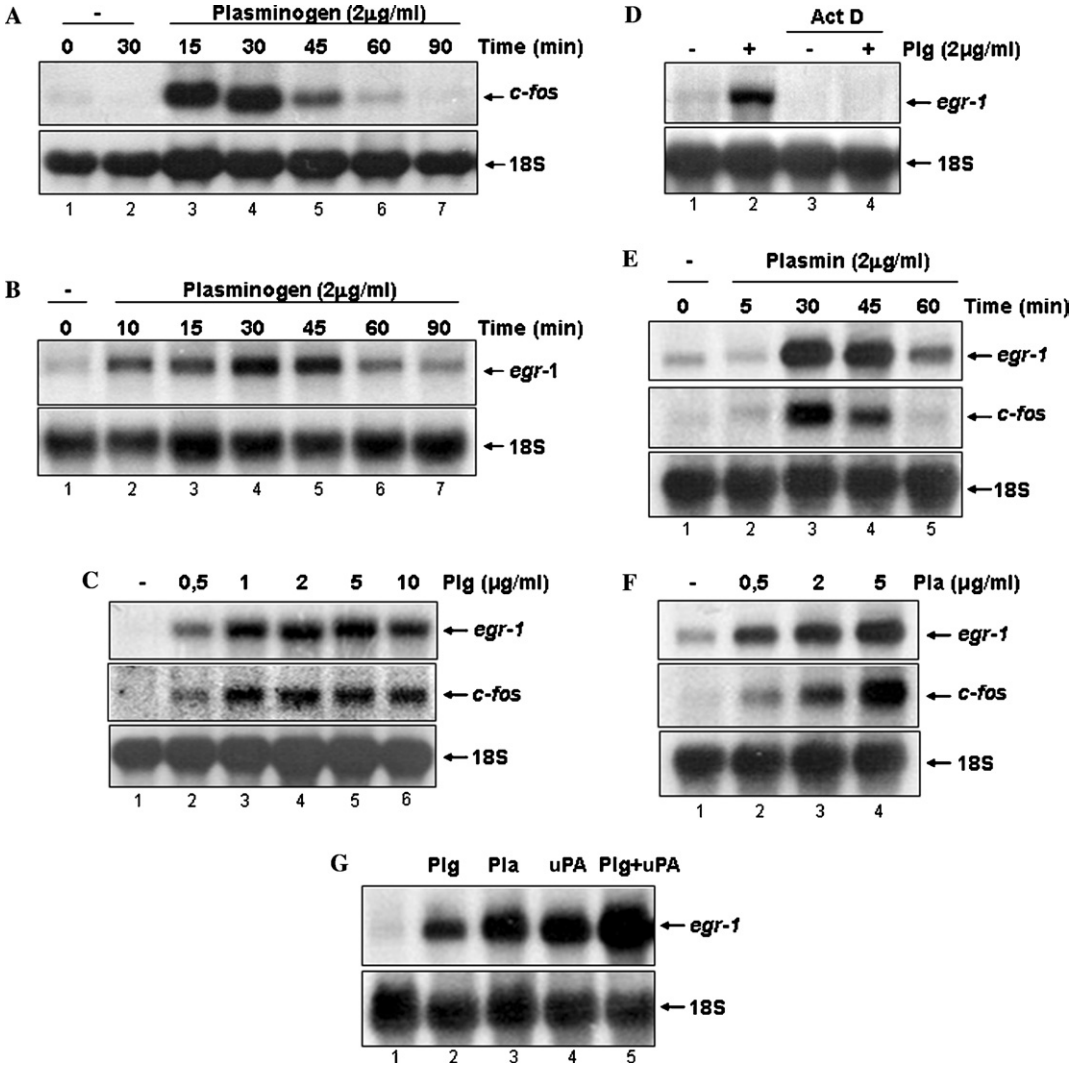

Fig. 1. Plg/Pla induces *c fos* and *egr-1* mRNA expression. Quiescent monolayers of A31 cells were incubated with Plg or Pla as indicated. Total RNA was collected and subjected to Northern blot analysis as described in Materials and methods. (A,B) Plg-stimulated *c-fos* and *egr-1* accumulation. Cells were either left untreated or treated with Plg for the times shown. (C) Plg-stimulated *c-fos* and *egr-1* expression is dose-dependent. Cells were cultured in the absence or in the presence of Plg, at the indicated concentration, for 30 min. (D) Pre-treatment with actinomycin D abolishes *egr-1* expression. Cells were pre-incubated with the transcription inhibitor Act D prior to Plg stimulation for 30 min. (E) Pla-stimulated *c-fos* and *egr-1* expression. Cells were either left untreated or treated with Pla for the times shown. (F) Pla-stimulated *c-fos* and *egr-1* expression is dose-dependent. Cells were cultured in the absence or in the presence of Pla, at the indicated concentration, for 30 min. (G) Effects of various fibrinolytic proteases on *egr-1* expression. Cells were either left untreated—lane 1 or were treated with: Plg (2 µg/ml)—lane 2; Pla (2 µg/ml)—lane 3; urokinase (10 nM)—lane 4; or Plg (2 µg/ml) pre-incubated with urokinase (10 nM) for 1 h at 37 °C before exposure to the cells for 30 min—lane 5. RNA loading was monitored by probing the membranes with 18S rRNA. These data are representative of three independent experiments.

pathophysiology of the Plg/Pla system [7,17], we included it in our study. Treatment of A31 fibroblast cells with Plg/Pla leads to the mRNA accumulation of *c-fos* and *egr-1*, as shown in Figs. 1A–G. Their steady-state mRNA levels peaked in about 30 min and then returned to basal levels by 60 min (Figs. 1A, B, and E), their induction being dose-dependent (Figs. 1C and F). Actinomycin D completely inhibited the Plg-induced *egr-1* mRNA accumulation (Fig. 1D), indicating that the gene needs to be constantly transcribed. The same was also observed with Pla stimulation (data not shown). We also demonstrated that urokinase is able to induce *egr-1* gene expression (Fig. 1G), as compared to other fibrinolytic components. The levels of *egr-1* expression were similar to those caused by Pla treatment (Fig. 1G, lanes 3–4) and higher when compared with Plg stimulation (Fig. 1G, lanes 2 and 4). Pre-incubation of Plg with urokinase, before treating the cells, had an additive effect over *egr-1* mRNA expression (Fig. 1G, lanes 2, 4, and 5). Figs. 2A and B show that *egr-1* message is then translated into its gene product with a kinetic that parallels that observed for mRNA accumulation, i.e., it was

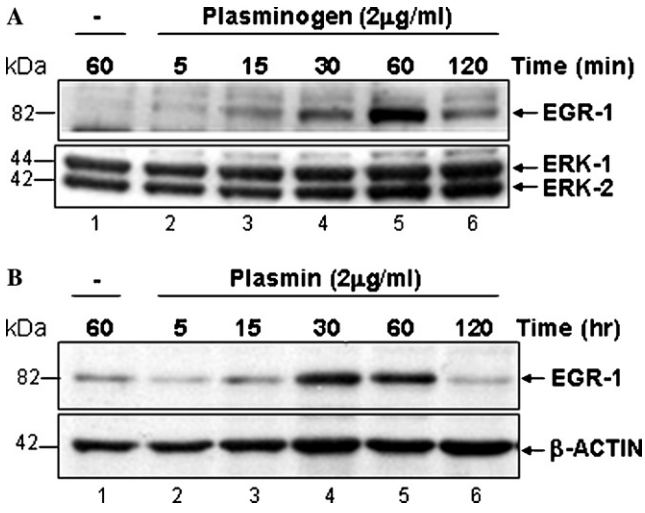

Fig. 2. Plg/Pla induces EGR-1 protein expression. (A,B) Western blot analysis of EGR-1 expression upon Plg- or Pla-stimulation, respectively. Cells were incubated with Plg or Pla for the times shown and the lysates were prepared as described in Materials and methods. Proteins were fractionated on SDS–PAGE, transferred onto nitrocellulose membrane, and then probed with anti-EGR-1 antibody (upper panel) or with anti-total ERK1/2 antibody or anti-β-actin (lower panel), for loading control. These results were consistently repeated in three independent experiments.

apparent 30 min post-stimulation, reached its maximum at 1 h, and declined thereafter. The same was also verified with the c-FOS gene product (data not shown).

Plg/Pla-induced c-fos and egr-1 expression is MEK and serine protease dependent

The MAPK ERK1/2 plays a critical role on the Plg/Pla-stimulated *c-fos* and *egr-1* expression, since pre-incubation of the cells with the specific MEK inhibitor, PD98059, abrogated the expression of both genes (Figs. 3A and B, lane 4). In line with the pharmacological inhibition of Plg-stimulated *c-fos* and *egr-1* mRNA accumulation, the same inhibition was verified with a cell line expressing a MEK dominant-negative mutation (Figs. 3C and D).

To verify whether Plg-induced gene expression is due to Plg-binding to a receptor directly and initiating intracellular events or if there is pericellular activation by secreted Plg activators (PAs) leading to proteolytically active Pla, that in turn may exert effect independently of Plg, experiments were carried out using leupeptin, a Pla serine protease inhibitor. We found that *c-fos* and *egr-1* mRNA was no longer expressed in the presence of Pla pre-incubated with leupeptin (Fig. 3E, lane 6), contrasting with the Plg pre-treated with leupeptin (Fig. 3E, lane 5), because the zymogen Plg does not have proteolytic activity. These results suggest that Plg is converted into Pla, which then stimulates *c-fos* and *egr-1* expression through its serine protease activity. In this regard, A31 cells pre-incubated with leupeptin for 1 h (for serine protease inhibition) and then treated with Plg no longer induce *c-fos* and *egr-1* mRNA expression (data not shown). Of note, the Plg/Pla-induced *egr-1* and *c-fos* expression also appears to be dependent on G protein-coupled receptors, because incubation with cholera toxin (a G protein signaling modulator) prior to treatment with Plg/Pla abrogated both gene expression (Fig. 3E, lanes 8–9) and EGR-1 protein expression (Fig. 3G, lane 6).

In order to demonstrate that the gene induction was dependent on Plg/Pla interaction with its receptors through carboxy-terminal lysines, we incubated Plg and Pla with ε-aminocaproic acid (EACA) prior to treating the cells. EACA is a lysine analog that reversibly interacts and blockades the lysine-binding sites on Plg molecules. Under this circumstance, the Plg/Pla-induced *egr-1* expression was inhibited at both mRNA accumulation and protein levels (Figs. 3F and G, lanes 3 and 4), respectively. Similar results were also obtained with *c-fos* mRNA (data not shown). We also tested another Plg-lysine binding site inhibitor, tranexamic acid, with identical results (data not shown).

Plasminogen stimulates ERK1/2 phosphorylation

We then investigated whether the MAPK ERK1/2 could be, in fact, associated with the transmission of signals initiated upon Plg/Pla binding to the cell surface and leading to *c-fos/egr-1* expression. We showed that Plg stimulation leads to strong ERK1/2 phosphorylation in a time-dependent manner. Increased phosphorylation of p44/42 MAPK was evident at 5 min of exposure to Plg with maximum levels. The phosphorylation levels, although smaller, were maintained until 45 min and declining to basal levels after 60 min (Fig. 4A). Similar results were also verified with Pla (data not shown). ERK phosphorylation was completely blocked by pre-incubation with the specific MEK inhibitor (PD98059) (Fig. 4B). This effect seems to be specific, since MAPK phosphorylation was not affected by pre-incubation with the PKC inhibitor—bisindolylmaleimide (data not shown). In accordance with the pharmacological inhibition of Plg-stimulated ERK phosphorylation, a significant decrease was also observed with the cell line expressing a MEK1 dominant-negative form—MEK1-DN (Fig. 4C). Similar result was also found when using another MEK1-DN clone (data not shown).

Plg/Pla increases AP-1 and EGR-1 DNA-binding activity

Because AP-1 and EGR-1 response elements are found in the promoter regions of *c-fos* and *egr-1* gene, we examined whether Plg/Pla could activate AP-1 and EGR-1, two downstream targets of MEK. By using probes corresponding to the consensus AP-1 and

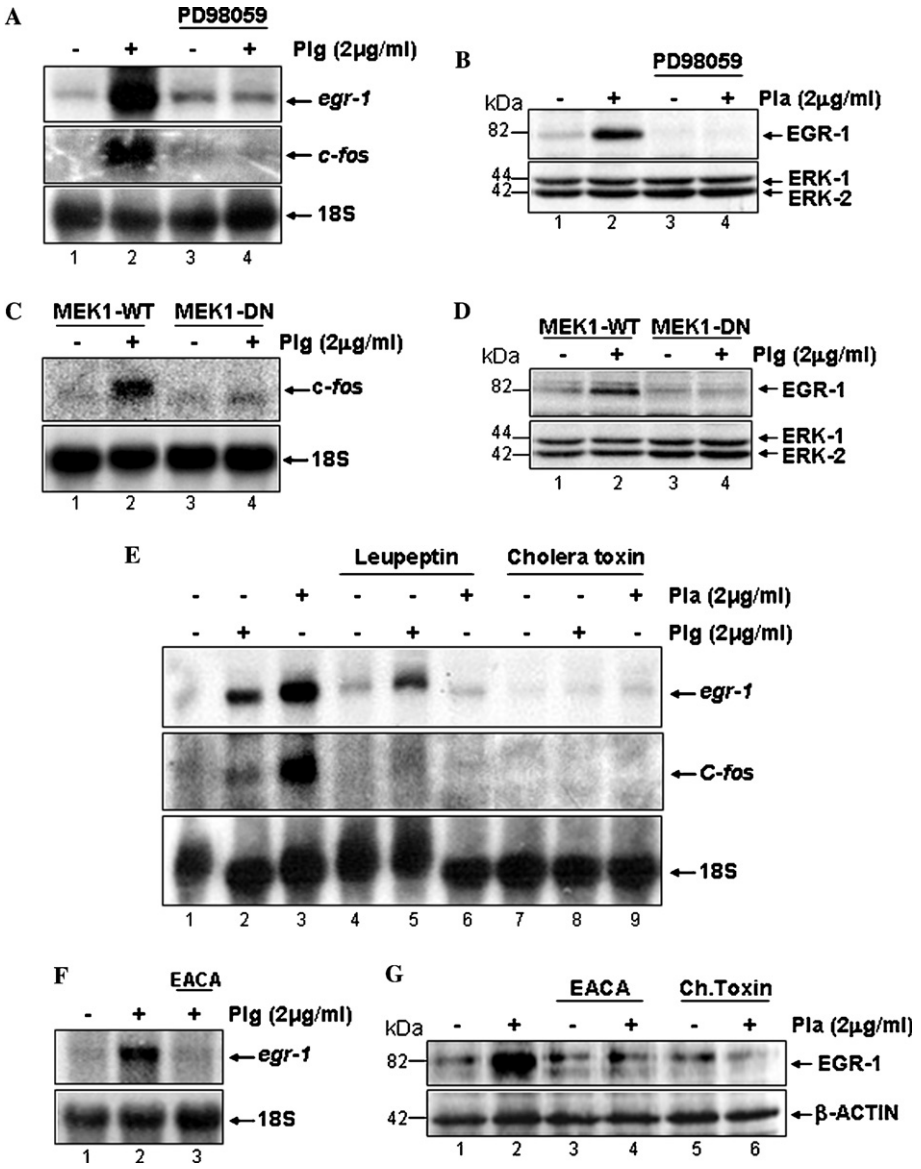

Fig. 3. Effect of PD98059, leupeptin, cholera toxin, and EACA on Plg/Pla-induced *c-fos* and *egr-1* expression. (A) Pre-treatment with PD98059 abolishes Plg-induced *egr-1* and *c-fos* mRNA expression. Cells were incubated with PD98059 (50 μM) prior to Plg stimulation for 30 min. (B) Pre-treatment with PD98059 abolishes Pla-induced EGR-1 protein expression. Cells were incubated with the inhibitor (50 μM) prior to Pla stimulation for 30 min. (C,D) ERK1/2 plays an essential role in Plg-stimulated *c-fos* and EGR-1 expression. Upper panels: cell lines expressing either wild-type (lanes 1 and 2) or dominant-negative MEK1 (lanes 3 and 4) were exposed to Plg for 30 or 45 min and then probed with radiolabeled *c-fos* or with anti-EGR-1 antibody, respectively. Lower panels: membranes were probed with 18S rRNA or with anti-total ERK1/2 antibody for loading controls. (E) Serine protease inhibitor and G protein signaling modulator abolish Pla-induced expression of *c-fos* and *egr-1* mRNA. Plg and Pla were pre-incubated with the serine protease inhibitor leupeptin for 1 h at 37 °C and then added to the cells for 30 min (lanes 5 and 6), or the cells were pre-incubated with cholera toxin (1 μg/ml) for 1 h and then stimulated with Plg or Pla for 30 min (lanes 8 and 9). RNA loading was monitored by probing the membranes with 18S rRNA. (F) EACA inhibits Plg-induced *egr-1* mRNA expression. Plg was pre-incubated with the lysine analog EACA (0.1 M) for 1 h at 37 °C and then added to the cells for 30 min (lane 3). (G) EGR-1 protein expression upon Pla-stimulation is dependent on Pla binding to the cell surface and G protein-coupled receptors. Pla was pre-incubated with lysine analog EACA (0.1 M) for 1 h at 37 °C and then added to the cells for 45 min (lanes 3 and 4) or the cells were pre-incubated with cholera toxin (1 μg/ml) for 1 h and then stimulated with Pla for 45 min (lanes 5 and 6). These data are representative of at least two independent experiments with quite similar results.

EGR-1 binding sites (EBS) in the shift assays, we found that Plg/Pla increased both AP-1 and EGR-1 DNA-binding activity. These complexes were visualized at maximum levels at 30 min after Plg/Pla treatment, declining thereafter (Figs. 5A and B). We verified that the DNA-binding activity induced by Pla was a little stronger than that observed with Plg, confirming the data concerning the gene induction (Fig. 1G). The specificity of these interactions was verified by competing the labeled oligodeoxynucleotides with 50-fold molar excess

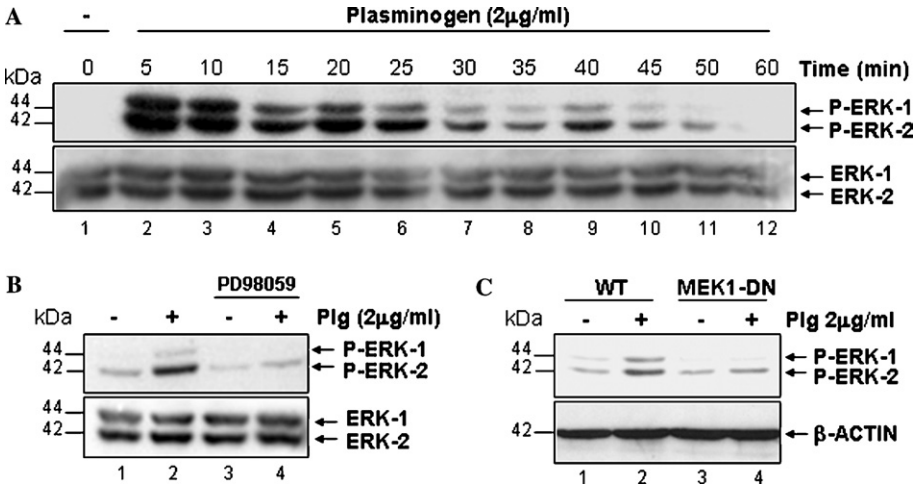

Fig. 4. ERK1/2 is phosphorylated in response to Plg stimulation. Western blot analysis performed as described in Materials and methods. Starved cells were treated with Plg (2 μg/ml) for the indicated times (A) or for 30 min (B). Blots were then probed with anti-phospho-ERK1/2 antibody. (A) Upper panel: Kinetics of ERK1/2 phosphorylation, (B) Upper panel: PD98059 inhibits the Plg-stimulated ERK1/2 phosphorylation. Cells were incubated with PD98059 (50 μM) for 30 min prior to Plg stimulation (lane 4). (A,B) Lower panels: blots were probed with anti-total ERK1/2 antibody for loading control. (C) Upper panel: Plg-stimulated phosphorylation relies on MEK1 activity. Cell lines expressing either the wild-type MEK1 (lanes 1 and 2) or dominant-negative MEK1 (lanes 3 and 4) were left untreated or treated with Plg for 30 min and then probed with anti-phospho-ERK1/2. Lower panel: blot was probed with anti-β-actin for loading control. The protein positions are indicated in kilodaltons (kDa) on the left. These data are representative of three independent experiments with identical results.

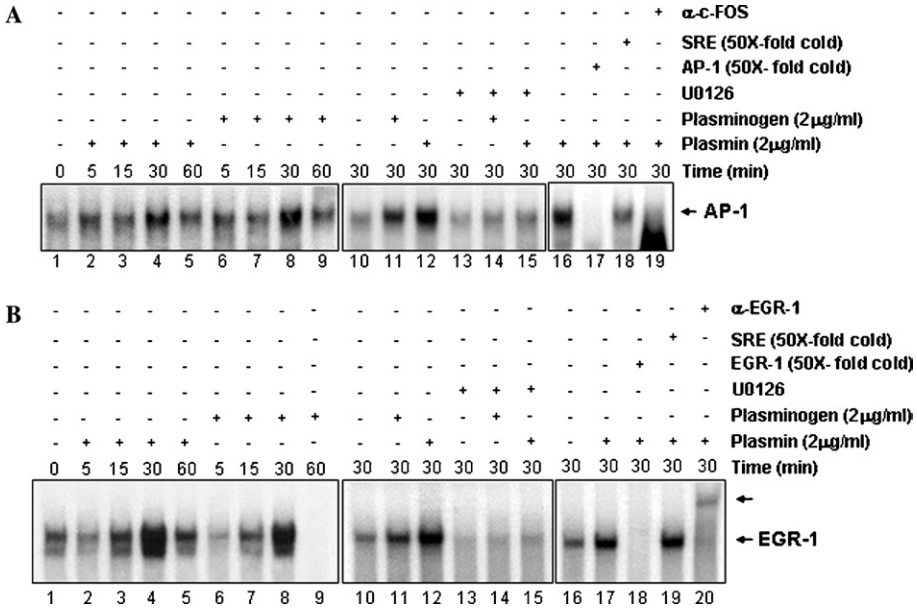

Fig. 5. Analysis of Plg/Pla-stimulated DNA–protein complex formation. EMSA was carried out after treating the starved cells with Plg or Pla (2 μg/ml) for the indicated times. Ten micrograms of nuclear protein was incubated with an end-labeled oligodeoxynucleotide probe containing the consensus AP-1 or EGR-1 *cis*-acting elements. (A,B) Transcriptional complexes formed with AP-1 or EGR-1, respectively. DNA–protein complexes are indicated by arrows. The specificity of the interactions was confirmed by competing the probe with 50-fold molar excess of the indicated cold oligo (A and B, lanes 17,18 and 18,19, respectively). c-FOS and EGR-1 were super-shifted by specific antibodies (A and B, lanes 19 and 20), respectively. U0126 disrupted the formation of the DNA–protein complexes (A and B, lanes 14 and 15). These data are representative of three independent experiments.

of the corresponding unlabeled oligodeoxynucleotides (AP-1 or EGR-1), or by competing with an unrelated oligodeoxynucleotide (SRE) (Figs. 5A and B, lanes 17,18 and 18,19, respectively). In order to better characterize the proteins that recognize these regulatory elements, cell extracts were pre-incubated with anti-c-FOS or anti-EGR-1 antibodies and then mixed with the specific labeled oligo. The antibodies recognized (super-shifted) both, c-FOS and EGR-1, as the proteins that interact with the AP-1 and EBS sites,

respectively (Figs. 5A and B, lanes 19 and 20). We also examined whether AP-1 and EGR-1 activation was regulated by MEK. We found that U0126 (a specific MEK inhibitor) abolished Plg/Pla-induced AP-1 and EGR-1 binding activity (Figs. 5A and B, lanes 14 and 15), respectively. The same results were found with PD98059 (data not show). These results suggest that Plg/Pla stimulates AP-1 and EGR-1 DNA-binding activities via the MEK/ERK pathway.

Discussion

The results presented in this study show that the binding of Plg/Pla to its cell surface receptor triggers intracellular signaling events leading to the expression of the primary response genes *c-fos* and *egr-1* (Figs. 1A–F) through the MEK/ERK activation pathway as demonstrated by both pharmacological and dominant-negative approaches (Figs. 3A–D). FOS and EGR-1 proteins could then act as transcription factors, as verified by their ability to bind to *cis*-acting elements (Figs. 5A–B), and demonstrated in several experimental settings involving components of the Plg/Pla system. For instance, uPA induces *c-fos* expression via a tyrosine-kinase sensitive pathway [8], and AP-1 (FOS/JUN) has been associated with the transcriptional regulation of various components of the Plg system such as plasminogen activator inhibitor-1 and -2 (PAI-1 and PAI-2) [9], uPA [10], and uPAR [11]. In addition, AP-1 is also involved with the plasmin-stimulated IL-1β and tissue factor expression [27,28]. EGR-1 in turn regulates the secretion of transforming growth factor (TGF-β) [29], which then leads to the expression of PAI-1 and fibronectin, contributing to enhancing cell attachment [30]. EGR-1 is also directly involved in the regulation of some Plg/Pla system elements such as uPA, uPAR, and TF genes [12–14] contributing to the functional maintenance of the Plg/Pla system.

ERK1/2-mediated Pla-induced gene expression was previously reported for the *Cyr61* gene, a growth factor-like gene that has been implicated in cell proliferation, adhesion, and migration [31]. Although exposure of human monocytes to Pla led to p38 MAPK activation [28], we neither investigate p38 MAPK nor JNK activation in our experimental setting. Therefore, we cannot exclude the contribution of these MAPKs for Plg/Pla-induced *c-fos* and *egr-1* expression in fibroblasts.

Plg is a 92 kDa zymogen present at plasma in a concentration of 200 μg/ml and needs to be activated to Pla upon cleavage by Plg activators (PAs) [6,7]. In accordance with the lack of protease activity associated with Plg, we found that Pla- but not Plg-induced *c-fos* and *egr-1* expression was blocked in the presence of protease inhibitor (Fig. 3E). Our data also show that both Pla and Plg induce the primary response genes with the same time course. Since PAs are also expressed in fibroblasts [32], and Plg pre-incubated with uPA induces *egr-1* mRNA accumulation to a higher extent than that observed with Plg alone (Fig. 1G), we propose that PAs primarily convert Plg to Pla, which then stimulates *c-fos* and *egr-1* mRNA expression. Although uPA was also able to stimulate *egr-1* mRNA accumulation in fibroblasts (Fig. 1G, lane 4), we did not investigate the involvement of a protease activity in such induction. Our unpublished observations showed that pretreatment with tranexamic acid abolished the uPA-induced *egr-1* mRNA expression, suggesting that Pla activation is indeed required.

In accordance with the need of lysine-binding sites for the interaction to the cell surface receptor [33], our data show that the Pla/Plg-induced gene expression was inhibited in the presence of the lysine analog EACA (Figs. 3F and G). In addition, we found that *c-fos* and *egr-1* expression occurred in a G protein-coupled receptor-dependent manner (Figs. 3E and G), similar to what has been observed for the expression of *Cyr61* gene [31]. Thus, our data share with the *Cyr61* gene both the requirement of p44/42 MAPK and the involvement of a G protein-coupled receptor to transmit the signals that will culminate with their increase in gene expression.

Consistent with the notion that MEK/ERK is required to the Plg/Pla-stimulated *c-fos* and *egr-1* expression, we present evidence that the regulatory sequences AP-1 and EBS found in the promoter regions of *c-fos* and *egr-1* genes may play a critical role in their expression, because incubation with the specific MEK inhibitor prior to Plg/Pla exposure caused a complete block in both DNA-binding activity (Figs. 5A and B), mRNA and protein accumulation (Figs. 3A–D).

Studies conducted by others [34,35] have also demonstrated the association of MEK/ERK with the transcriptional activator AP-1 and EGR-1, though as far as the Plg/Pla system concerns, only urokinase has been investigated [2,4,9].

Taken together, our data indicate that Plg/Pla induces the expression of the primary response genes *c-fos* and *egr-1* through MEK/ERK activation. The involvement of a G protein-coupled receptor also seems to be required. To our knowledge, this is the first report that associates Plg/Pla treatment to the induction of the primary response genes *egr-1* and *c-fos* in fibroblasts. We speculate that FOS (AP-1) and EGR-1, in turn, could potentially act as the putative transcriptional regulators that control secondary response gene expression of the Plg/Pla system acting as an auto-regulatory circuit.

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