Urokinase-mediated transactivation of the plasminogen activator inhibitor type 2 (*PAI-2*) gene promoter in HT-1080 cells utilises AP-1 binding sites and potentiates phorbol ester-mediated induction of endogenous PAI-2 mRNA

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Abstract Urokinase-type plasminogen activator (u-PA) bound to its receptor, u-PAR, initiates signal transduction pathways able to induce expression of the activator protein-1 (AP-1) family member *c-fos* [1]. Since transcription factors bound to AP-1 recognition sequences within the *PAI-2* gene promoter play a role in basal and phorbol ester-mediated induction of *PAI-2* gene expression, we hypothesised that u-PA/u-PAR-mediated modulation of AP-1 activity would in turn influence constitutive and inducible *PAI-2* gene expression. Treatment of HT-1080 or U-937 cells with high molecular weight u-PA (HMW u-PA) resulted in induction of nuclear proteins binding to a functional AP-1 element in the proximal PAI-2 promoter. This increase in AP-1 activity correlated with a transactivation of the *PAI-2* gene promoter in transiently transfected HT-1080 cells. We also demonstrate the u-PA treatment potentiated phorbol ester (PMA)-mediated induction of PAI-2 mRNA, indicating that u-PA binding produces a bone fide response in vivo.

Key words: Urokinase-type plasminogen activator; Urokinase-type plasminogen receptor ; Signal transduction; Activator protein-1; PAI-2; Gene expression

1. Introduction

Localised cell surface proteolysis forms the fundamental basis for cellular migration facilitating numerous biological and pathological processes including wound healing ([2], tissue modelling), and metastatic malignant disease [3]. The plasminogen activation enzyme cascade is one of the major proteolytic systems engaged at the cell surface. Urokinase-type plasminogen activator (u-PA) bound to its cell surface receptor (u-PAR) concentrates cell-surface plasmin generation, and thus degradation of the extracellular matrix (ECM), whilst plasminogen activator inhibitor type-2 (PAI-2), a serine protease inhibitor, is primarily responsible for inhibition of receptor-bound u-PA activity [4].

In addition to its integral role in restricting plasminogen activation to the cell surface, occupancy of u-PAR is associated with an increase in mitogenic activity [5]. u-PAR is a glycosyl-phosphatidylinositol (GPI)-linked cell-surface protein lacking both transmembrane and cytoplasmic domains [6]. Recent studies have identified putative transmembrane proteins and intracellular signal transduction cascades thought to be responsible for mediating the mitogenic effects of u-PAR.

The catalytic inactivation of receptor-bound u-PA upon binding its natural inhibitor PAI-2 is well documented [7]. Modulation of *PAI-2* gene and protein expression is a potential mechanism by which u-PA activity is controlled. Molecular regulation studies have demonstrated that *PAI-2* gene expression is maintained at low, even or undetectable levels in most cells but is highly induced in the presence of agents such as phorbol ester [8], tumour necrosis factor (TNF) [9], and okadaic acid [10]. Analysis of the proximal *PAI-2* gene promoter has identified at least three functionally relevant regulatory sites which are responsible for constitutive and inducible expression. Two of these sites are related to the AP-1 binding site consensus sequence, while the other site is related to the cyclic AMP response element [11].

Activation of signal transduction pathways together with an increase in the expression of the AP-1 component *c-fos* [1] as a result of u-PAR occupancy prompted us to hypothesise that u-PAR activation by its ligand u-PA would increase *PAI-2* gene promoter transactivation via an AP-1-dependent mechanism. Our results support this hypothesis and further suggest that u-PA-mediated induction of *PAI-2* gene expression is associated with pre-existing cellular activation and that this effect may contribute to a potential autoregulatory phenomenon modulating cell-surface plasminogen activation.

2. Materials and methods

2.1. Cell culture and materials

Human HT-1080 fibrosarcoma cells (American Type Culture Collection, Rockville, MD) were cultured to confluence at 37°C in Nunclon cell culture dishes according to standard techniques in 10 ml of Dulbecco's modified Eagles Medium (DMEM) supplemented with 2 mM glutamine and 10% heat-inactivated foetal calf serum (HI-FCS, Gibco BRL, Australia). Human U-937 histiocytic lymphoma cells (American Tissue Culture Collection, Rockville, MD) were grown in suspension in Nunclon cell culture flasks at 37°C according to standard techniques in 30 ml of RPMI-1640 medium (Gibco BRL, Australia) supplemented with 2 mM glutamine and 10% HI-FCS.

HMW-u-PA was kindly donated by ARES Serono, Australia. ATF was obtained from American Diagnostica (Greenwich, CT) and Phorbol 12-myristate 13-acetate (PMA) from Sigma (St. Louis, MO). Cleavage of GPI-linked proteins from the cell surface by phosphoinositol-specific phospholipase C (PI-PLC; Sigma, St. Louis, MO) was performed by incubating cells with 0.75 U/ml PI-PLC for 90 min prior to addition of subsequent stimulatory agents.

2.2. PAI-2 promoter deletion constructs

PAI-2 promoter constructs, fused to the chloramphenicol acetyl transferase (*CAT*) reporter gene, used in this study included PAI-2-1100-CAT, PAI-2-1100-CAT with either or both AP-1 sites mutated, and PAI-2-219-CAT. These constructs have been previously described

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[11]. The PAI-2 AP-1a site (5′-TGAATCAC-3′) was substituted with the sequence 5′-ACCGGGT-3′ whilst the PAI-2 AP-1b site (5′-CTGAGTAA-3′) was substituted with the sequence 5′-AGAATTCT-3′. The mutant constructs were generated in the PAI-2-CAT-1100 construct.

2.3. Transfection studies

Transfection of PAI-2 promoter constructs (2 μg) into 0.5×10^6 HT-1080 cells was performed by the DEAE-Dextran chloroquine method as previously described [12]. Briefly, 0.5×10^6 cells were plated on 60 cm^2 dishes in 4 ml of supplemented DMEM and grown overnight at 37°C. The following day, cells were transfected with 2 μg of plasmid DNA containing the PAI-2 promoter fused to the *CAT* reporter gene constructs (above) in 2 ml of serum-free DMEM and 10 μg/ml DEAE-Dextran. Two micrograms of pRSVCAT and $pBLCAT_3$ [13] were also transfected in parallel and used as positive and negative controls, respectively. After 2 h the medium was aspirated and the cells subsequently incubated for 5 h in serum-supplemented DMEM containing 100 μM chloroquine, aspirated, washed in PBS, subjected to 10% DMSO-PBS shock (2 min), rinsed twice with PBS and maintained overnight in fresh DMEM containing 10% serum. The following day transfected cells were first washed in PBS and then incubated for 24 h in either serum-free medium or serum-free medium supplemented with 100 nM or 1 μM HMW-u-PA, 25 ng/ml PMA or a combination of HMW-u-PA and 25 ng/ml PMA. Cells were harvested by trypsinization, washed in PBS, centrifuged, the supernatant removed and the cells finally resuspended in 100 μl of 0.25 M Tris-HCl buffer, pH 7.4, and disrupted by three cycles of freeze-thawing. Samples were centrifuged and cytoplasmic extracts prepared by three freeze-thaw cycles, collected, assessed for protein concentration of the cytoplasmic extracts was assessed using the BioRad dye reagent system (BioRad, Australia). Samples were either used immediately or stored at −80°C.

2.4. Chloramphenicol acetyl transferase (CAT) assay

Cytoplasmic extracts of transfected cells (usually 50–100 μg in 40 μl of 250 mM Tris-HCl, pH 7.4) were incubated with 5 μl of 4.4 mM acetyl coenzyme A (Boehringer Mannheim) and 1 μl of [^{14}C]chloramphenicol (DuPont) for 4 h at 37°C. The samples were processed by standard techniques and subjected to analysis by thin layer chromatography. The percentage conversion of [^{14}C]-chloramphenicol to its acetylated products was quantified by phosphorimaging using a Fujix BAS 1000 phosphorimager.

2.5. Preparation of nuclear proteins

The preparation of nuclear proteins from HT-1080 or U-937 cells was performed using the method described by Osborn et al. [14].

2.6. Preparation and labelling of oligonucleotides

Double-stranded oligonucleotides containing either the AP-1 binding consensus sequence and the AP-1a binding site within the *PAI-2* gene promoter were synthesised. The oligonucleotides were gel purified by electrophoresis through a 15% polyacrylamide gel containing 7 M urea and labelled with T4 polynucleotide kinase using standard techniques [12]. Annealing of complementary single stranded oligomers was performed as previously described [13]. The sequences of oligonucleotides used for this study were: 5′-GATTCAATGACTCACGGCTGTG-3′, (AP-1 consensus oligomer); 5′-AAAATGTTGAATCACTCAAAG-3′, (AP-1a oligomer, complementary to the region between -110 and -90 in the *PAI-2* gene promoter [11]). Underlined regions within the AP-1 and AP-1a oligonucleotides indicate the consensus AP-1 and AP-1a core elements, respectively.

The sequence of the unrelated oligomer used for the competition experiments was: 5′-CTGGGGCTGACAGATTTTAGCT-3′.

2.7. Protein/DNA binding reactions and electrophoretic mobility shift assay (EMSA)

Four microlitres of HT-1080 or U-937 nuclear protein extracts containing 4 μg of protein in Osborn buffer D [14] were incubated at 4°C for 15 min with 1 μl (500 ng to 1 μg) of poly d(I-C) (Boehringer Mannheim, Australia) and 3 μl of SMK buffer (12 mM spermidine, 1.2 mM $MgCl_2$ and 200 mM KCL) [13]. The concentration of poly d(I-C) required to reduce non-specific binding was determined empirically and depended on the cell source of nuclear proteins. Four microlitres of γ^{32}P-labelled probe (100 cps diluted in buffer D (above)) was then added and the mixtures incubated on ice for a further 15 min before being applied to a 5% polyacrylamide gel prepared in 0.25×Tris/boric acid/EDTA (TBE) buffer [12] and subjected to electrophoresis. The gels were dried and autoradiographed at −70°C overnight with an intensifying screen. For competition experiments, nuclear extracts were incubated with a 10–100-fold excess of unlabelled annealed oligonucleotides 15 min after addition of poly d(I-C) competitor.

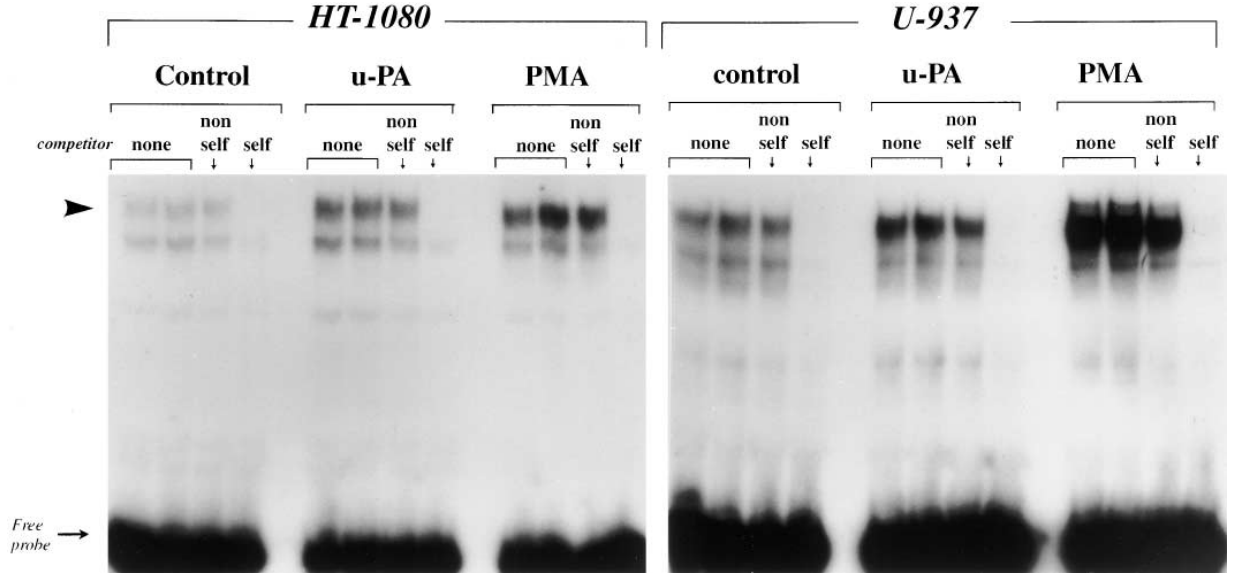

Fig. 1. Treatment of HT-1080 fibrosarcoma and U-937 histiocytic lymphoma cells with HMW u-PA increases binding activity of nuclear proteins to the AP-1 consensus binding site. Nuclear proteins extracted from both HT-1080 and U-937 cells treated for 6 h with normal medium or medium containing 1 μM HMW-u-PA were subjected to EMSAs using a labelled oligonucleotide harbouring the AP-1 binding site. As a positive control for inducible AP-1 binding activity, EMSAs were also performed using nuclear extracts prepared from cells treated for 6 h with 25 ng/ml PMA. Competition experiments using 100 ng of unlabelled unrelated (non-self) or identical (self) oligonucleotide, indicated that the binding activity was specific. The position of the AP-1 shifted complex is indicated by the arrowhead at the left of each panel. The unbound probe (free probe) is indicated at the bottom of the figure.

2.8. Gel super shift procedure

Monoclonal and polyclonal antibodies against the cAMP response element binding protein (CREB), the cAMP response element modulator (CREM), the CREB binding protein (CBP), activator transcription factor-2 (ATF-2), jun-D, c-jun and c-fos, specifically designed for supershift experiments, were obtained from Santa Cruz Inc. (USA). Supershifting was performed by the same procedure as the standard EMSA, except that 1 μl of specific antibody (1 μg total) was added to the nuclear extracts for 1 h before the addition of the labelled oligomer. Samples were then applied to a 5% native acrylamide gel, subjected to electrophoresis and processed as described for the EMSA procedure.

2.9. Northern blot analysis

Isolation of total RNA from HT-1080 cells was performed by the method of Chomczynski and Sacchi [15]. Ten micrograms per lane of RNA was loaded to each lane and electrophoresed through a 1% agarose gel containing 20% formaldehyde before being transferred to Hybond-N^+ membrane (Amersham, Australia). Filters were hybridised overnight at 42°C in a standard 50% formamide hybridisation buffer [16] containing ^{32}P-labelled cDNA inserts. The cDNAs used for this procedure were the *Eco*R1 fragment of plasmid pJ7 containing the full-length PAI-2 cDNA [8]; the 2.5 kb *Bgl*II fragment of tPA114B [17] containing the full-length t-PA cDNA, and the full-length cDNA for human *uPAR* [18]. After hybridisation, the membranes were washed by standard techniques and exposed to Kodak BioMax film (Eastman Kodak, Rochester, NY) at −80°C with an intensifying screen.

3. Results

3.1. Treatment of U-937 and HT-1080 cells with HMW-u-PA increases binding of nuclear proteins to the AP-1 consensus sequence

Nuclear extracts prepared from HT-1080 fibrosarcoma and U-937 histiocytic lymphoma cells treated for 6 h with 1 μM HMW u-PA were subjected to EMSA analysis using a labelled AP-1 consensus oligomer. Six hour stimulations were used as the AP-1 family of transcription factors are classed as immediate early genes and changes in the DNA binding activity of these proteins, mediated by phorbol esters and other agents, usually occur within this time frame.

A significant increase in binding of nuclear proteins to both labelled AP-1 probe oligonucleotides occurred in both HT-1080 and U-937 cells treated with HMW-u-PA over controls (Fig. 1). As a positive control for induction of AP-1 binding activity EMSA was also performed using extracts prepared from 6 h PMA-treated cells [19]. Increase in AP-1 binding as a consequence of PMA treatment was approximately 3-fold more effective than the induction seen with u-PA, indicating that PMA is a more potent activator of the signaling pathways which activate and/or phosphorylate components of the AP-1 family of proteins (Fig. 1). Competition experiments confirmed that binding of nuclear proteins to the AP-1 oligonucleotide from control, u-PA and PMA-treated cells was specific.

3.2. Addition of HMW-u-PA to HT-1080 and U-937 cells increases nuclear protein binding to an oligonucleotide containing the AP-1a binding site in the PAI-2 gene promoter

Two AP-1 binding sites exist in the *PAI-2* gene promoter: AP-1a and AP-1b [13]. Mutagenesis studies have indicated that both sites are functionally important, although the AP-1a site has been shown to play a more prominent role in constitutive expression and in the response of the *PAI-2* gene to PMA in transfected HT-1080 cells [11]. To determine

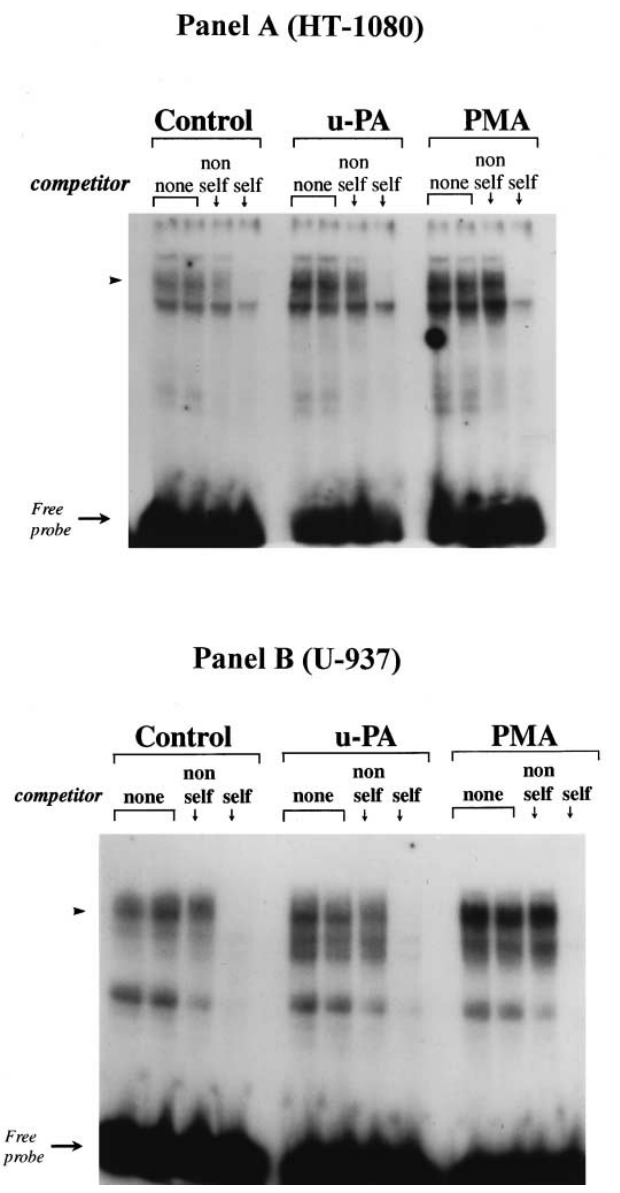

Fig. 2. Treatment of HT-1080 and U-937 cells with HMW-u-PA increases binding activity of nuclear proteins which recognise the AP-1a site in the PAI-2 promoter. To determine whether HMW-u-PA could also increase the binding activity of nuclear proteins to the AP-1a site in the *PAI-2* gene promoter, nuclear proteins extracted from both HT-1080 (A) and U-937 cells (B) treated for 6 h with 1 μM HMW-u-PA were subjected to EMSA using a labelled oligonucleotide harbouring the PAI-2 AP-1a binding site (see Section 2 for the AP-1a sequence). Extracts from PMA-treated cells were also included as a positive control. The position of the unbound oligonucleotide (free probe) is indicated at the bottom of the figure. The position of the AP-1a shifted complex is indicated by the arrowhead at the left of the figure. Competition experiments indicated that the binding activity was specific.

whether the binding activities of nuclear proteins which recognise the functional AP-1a site in the PAI-2 promoter were also modulated by treatment with u-PA, EMSAs were performed using a labelled AP-1a oligomer. Nuclear proteins extracted from u-PA-treated HT-1080 and U-937 cells produced a significant increase in AP-1a binding activity compared to untreated controls (Fig. 2A,B). This particular experiment was performed on four separate occasions using independently prepared nuclear protein extracts: u-PA treat-

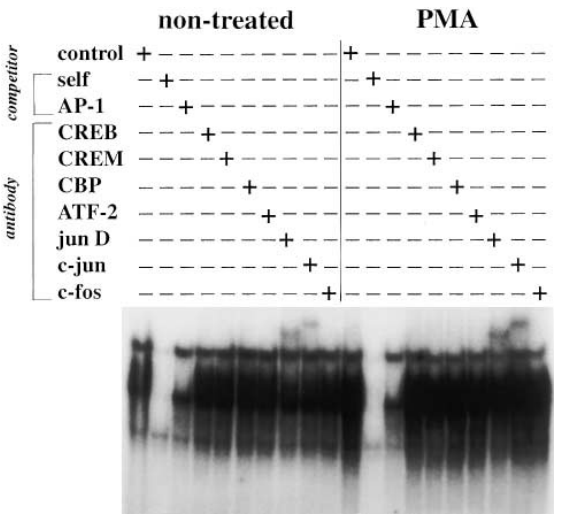

Fig. 3. Jun D and c-jun interact with the AP-1a site in the *PAI-2* gene promoter. Nuclear extracts prepared from non-treated and PMA-treated HT-1080 cells were used in a gel supershift experiment using a labelled oligonucleotide harboring the AP-1a site as a probe. Competition experiments were performed using 100 ng of unlabelled AP-1a or AP-1 consensus oligonucleotide in the absence of antibody, Specific monoclonal and polyclonal antibodies against CREB, CREM, CBP, ATF-2, jun-D, c-jun and c-fos included in the experiment are indicated by +.

ment produced a 2–3-fold increase in AP-1a binding in each experiment. As a positive control, EMSAs were also performed using nuclear extracts prepared from PMA-treated cells which demonstrated the expected increased in binding of nuclear proteins to the AP-1a oligonucleotide (Fig. 2A,B). Compared with shifts observed using the consensus AP-1 oligomer as a probe (Fig. 1), higher migrating complexes were also observed with nuclear extracts prepared from HT-1080 cells, suggesting the presence of additional protein factors binding to the AP-1a site.

3.3. *Proteins recognising the AP-1a site belong to the fos/jun family of transcription factors*

Competition experiments indicated that HT-1080 nuclear proteins which recognised the AP-1a sequence were completely inhibited by inclusion of the unlabelled AP-1a oligomer. Interestingly, addition of the unlabelled AP-1 consensus sequence did not completely compete for AP-1a binding: activity: binding activity of the slowest and fastest migrating protein/AP-1a complexes were not competed by the consensus AP-1 site, suggesting that non-AP-1 proteins were generating these particular shifts. Cross-competition EMSA experiments indicated that proteins recognising the consensus AP-1 binding site were in fact completely competed by unlabelled AP-1a oligomers, indicating that the proteins which recognise the AP-1 consensus site also interact with the AP-1a site.

To confirm further that the proteins which were competed by the consensus AP-1 oligomer were indeed members of the AP-1 family of proteins, supershift experiments were performed using antibodies which recognise various members of the AP-1 family of factors (jun-D, c-jun and c-fos) as well as members of the CREB/ATF family (CREB, CREM, CBP, ATF-2). Antibodies specific for members of the CREB/ATF family of proteins were included as they have been demonstrated to heterodimerise with components of the AP-1 (fos/jun) family of proteins. Results indicated specific supershifting with antibodies which recognised Jun D and c-jun in both untreated and PMA-treated cells (Fig. 3). Antibodies against the CREB/ATF proteins failed to produce supershifts, suggesting that these particular proteins are not associating with the AP-1a site. Despite the report of Dumler [1] of an increase in *c-fos* in ovarian carcinoma cells upon u-PA treatment, we found no evidence for *c-fos* activity in non-treated, u-PA- or PMA-treated HT-1080 cells. This negative result is consistent with a recent report [20] which indicated that HT-1080 cells are devoid of *c-fos* indicating that the distribution of *c-fos* is cell-type specific.

Taken together, these findings suggest that the AP-1a site binds the AP-1 members jun D and c-Jun and additional, as yet unidentified, nuclear proteins.

3.4. *Aminoterminal fragment of HMW-u-PA induces AP-1 binding in U-937 cells*

To determine whether u-PA-mediated induction of AP-1 binding was independent of the catalytic activity of u-PA, we assessed the effect of the aminoterminal fragment (ATF) of the HMW-u-PA molecule which binds efficiently to u-PAR but is devoid of catalytic activity [21]. EMSAs using nuclear extracts prepared from U-937 cells treated for 6 h with 1 μM

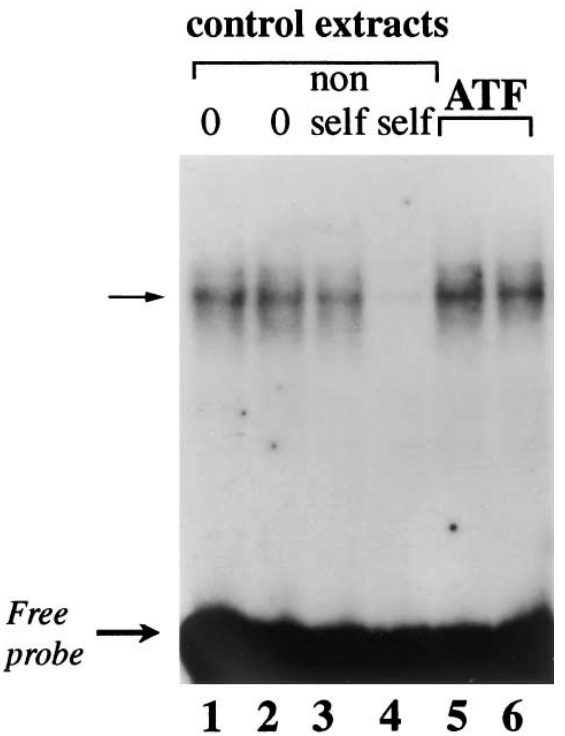

Fig. 4. Treatment of U-937 cells with the ATF of u-PA increases AP-1 binding activity. Nuclear extracts prepared from U-937 cells treated for 6 h with 1 μM of the ATF of u-PA were subjected to EMSA using the labelled AP-1 oligomer as a probe. Results indicated that ATF-treatment of cells produced an increase in AP-1 binding activity compared with non-treated cells (lanes 1-2 compared with lanes 5-6). Competition experiments (lanes 3 and 4) using 100 ng of unlabelled self and unrelated competitor, indicated that the AP-1 binding activity was specific. The position of the AP-1 complex is indicated by the arrow to the left of the figure whilst the position of the unbound oligonucleotide (free probe) is indicated at the bottom of the figure.

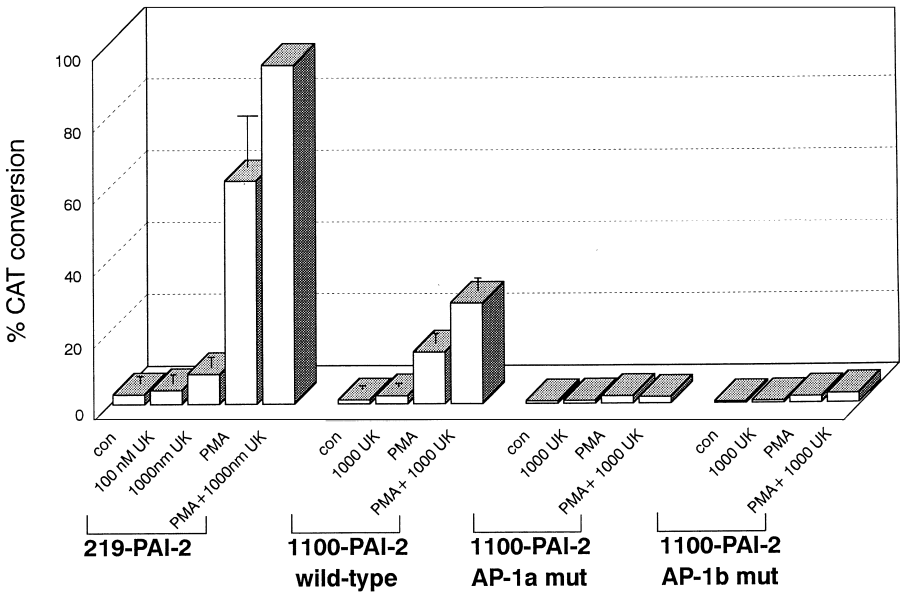

Fig. 5. HMW-u-PA transactivates the *PAI-2* gene promoter in transfected HT-1080 cells. HT-1080 cells were transiently transfected in duplicate with the wild-type -219 and wild-type -1100 bp PAI-2 promoter-CAT constructs. To assess the functional role of the AP-1a and AP-1b binding sites in u-PA-mediated induction, cells were also transfected with a -1100 PAI-2 CAT construct containing mutations within either the AP-1a or AP-1b binding sites. As indicated, transfected cells were stimulated for 24 h with 100 nM or 1 μM HMW-u-PA, 25 ng/ml PMA or a combination of 1 μM u-PA and 25 ng/ml PMA. CAT activities were quantitated by phosphorimaging of the TLC plates and results expressed in arbitrary units. This experiment was performed in duplicate on two (AP-1a mut, AP-1b mut) or three (-219, 1100 wild-type) separate occasions. Error bars represent SEM of samples with three data points.

ATF demonstrated an increase in binding of nuclear proteins to the labelled AP-1 consensus oligonucleotide, confirming that the proteolytic activity of u-PA is not necessary for increased binding activity of nuclear proteins to the AP-1 site (Fig. 4).

3.5. *HMW-u-PA transactivates the PAI-2 gene promoter in transfected HT-1080 cells*

To determine whether the u-PA-mediated increase in binding of nuclear proteins to the AP-1a binding site would activate the *PAI-2* gene promoter, HT-1080 cells were transiently transfected with a -219 bp *PAI-2* gene promoter construct fused to the *CAT* reporter gene. The -219 PAI-2 promoter harbours essential regulatory elements including the two AP-1 binding sites (AP-1a and AP-1b). Transfected cells were treated with HMW-u-PA (100 nM or 1 μM), 25 ng/ml PMA, or a combination of both agents. As shown in Fig. 5, stimulation of transfected cells with 100 nM or 1 μM HMW-u-PA alone resulted in a 2- and 4.5-fold increase in CAT activity, respectively, as determined by phosphorimaging analysis, while PMA-treatment resulted in a 22-fold increase in CAT activity. A synergistic increase in CAT activity (38-fold over basal levels) was observed in cells treated with a combination of 1 μM HMW-u-PA and PMA. Addition of 100 nM u-PA also potentiated PMA-mediated induction of CAT activity (data not shown). Taken together, these data indicate that the *PAI-2* gene promoter is a downstream target for signals initiated by u-PA bound u-PAR.

3.6. *Role of the AP-1a and AP-1b binding sites in the PAI-2 gene promoter in mediating u-PA-mediated induction of PAI-2 promoter activity*

A series of transient transfection experiments were performed to determine whether u-PA-mediated transactivation of the PAI-2 promoter was dependent on either or both of the AP-1 binding sites in the *PAI-2* gene promoter. HT-1080 cells were transfected with a construct harbouring the first 1100 bp of the PAI-2 promoter fused to the *CAT* reporter gene. In parallel, cells were transfected with the -1100 construct containing mutations of either or both the AP-1a or AP-1b binding sites. Transfected cells were treated for 24 h with 1 μM u-PA, 25 ng/ml PMA, or a combination of both agents. Results of CAT activity assays revealed that u-PA and PMA treatment alone produced a reproducible 2-fold and 14-fold increase in CAT activity, respectively, in cells transfected with the wild-type -1100 PAI-2 CAT construct. Consistent with the results observed with the -219 PAI-2 construct, u-PA potentiated PMA-mediated induction (28-fold over basal levels) (Fig. 5). Mutagenesis of the AP-1a site weakened basal, u-PA- and PMA-mediated induction of CAT activity and significantly abrogated the potentiation by u-PA- of PMA-mediated induction of CAT activity (Fig. 5). Mutagenesis of the AP-1b site also produced a similar effect (Fig. 5), indicating that this site also plays a role in mediating this effect. Mutagenesis of both AP-1 sites in combination abolished all detectable basal and inducible activity (data not shown). Taken together, these data indicate that both the AP-1a and AP-1b sites influence basal and PMA-mediated induction of promoter activity, and are likely to be involved in conveying u-PA- and u-PA-mediated potentiation of the PMA effect.

Basal and inducible CAT activity generated in cells transfected with the -219-PAI-2-CAT construct was significantly greater than observed with the -1100-PAI-2-CAT construct. The difference seen between these two constructs is consistent with previous reports of a repressor element located between positions -219 and -1100 [22,23].

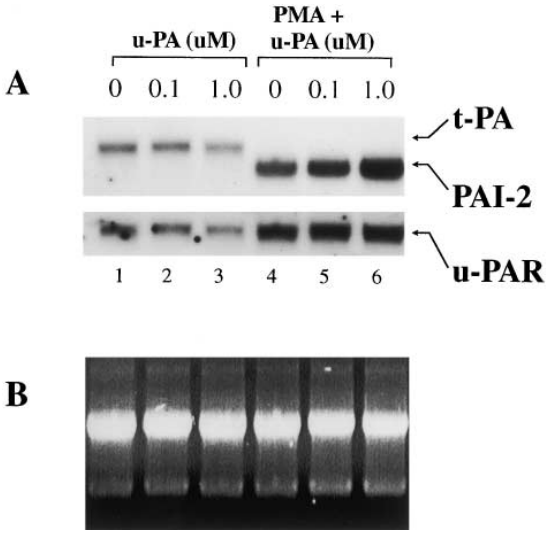

Fig. 6. HMW-u-PA potentiates PMA-mediated induction of endogenous PAI-2 mRNA expression. A: Total RNA was extracted from HT-1080 cells untreated or treated for 24 h with 100 nM or 1 μM HMW-u-PA, 25 ng/ml PMA or a combination of PMA with 100 nM or 1 μM u-PA, and subjected to Northern blotting. The filter was first co-hybridised with labelled cDNA probes complementary to PAI-2 and t-PA mRNA. The position of the 1.9 kb PAI-2 mRNA and the 2.5 kb t-PA mRNA are indicated. The filter was subsequently stripped then hybridised with a labelled cDNA for the 1.4 kb urokinase receptor (u-PAR) as indicated. B: Relative intensity of the ribosomal RNA bands in each lane as assessed by ethidium bromide staining of the gel.

3.7. *HMW-u-PA potentiates PMA-mediated induction of PAI-2 mRNA expression*

To determine whether the u-PA-mediated activation of the PAI-2 promoter was associated with a concomitant increase in endogenous PAI-2 mRNA expression, Northern blot experiments were performed using RNA extracted from HT-1080 cells treated with HMW-u-PA alone or in combination with PMA for a period of 24 h. Despite the previous results which demonstrated an increase in AP-1a binding and PAI-2 promoter transactivation, treatment of HT-1080 cells with u-PA did not produce a visible increase in PAI-2 mRNA (Fig. 6), possibly due to limitations in the sensitivity of the Northern blotting procedure. However, 1 μM HMW-u-PA potentiated PMA-mediated PAI-2 mRNA at least 3-fold. Potentiation of u-PA-mediated induction of PAI-2 mRNA by PMA was also observed using 100 nM u-PA, consistent with results obtained from the transfection experiments. This experiment was performed on three separate occasions with a near identical pattern of response being observed. To determine the specificity of the potentiation of PAI-2 mRNA by co-treatment with PMA+u-PA, the filter was co-hybridised with a labelled cDNA fragment of the human *t-PA* gene. The high basal levels of t-PA mRNA in HT-1080 cells were unaffected by treatment with u-PA. PMA treatment potently suppresses t-PA mRNA [10] and this suppressive activity was not noticeably affected by u-PA, although we note that the marked suppressive effect of t-PA by PMA would make minor effects of u-PA difficult to determine. However, subsequent hybridisation of the stripped filter with a labelled DNA fragment complimentary to the human u-PAR, also indicated that basal and PMA-mediated inductions of u-PAR [24] were not altered by u-PA. Despite the presence of AP-1 sites in the u-PAR promoter [25]; u-PA failed to alter u-PAR expression substantiating the selectivity of our effect. Although circumstantial, this also suggests that the AP-1-dependent mechanisms which operate to regulate both PAI-2 and u-PAR expression are likely to be different, most likely as a result of different AP-1 components associating with the AP-1 sites in the PAI-2 and *u-PAR* gene promoters. Nonetheless, these data indicate that addition of u-PA to HT-1080 cells produces a selective potentiation of PMA-mediated induction of endogenous PAI-2 mRNA.

4. Discussion

We have demonstrated that addition of u-PA to either HT-1080 or U-937 cells increases binding of nuclear proteins to both a consensus AP-1 binding site and the functional AP-1a binding site in the *PAI-2* gene promoter. u-PA-dependent AP-1a binding activity further correlated with transactivation of the *PAI-2* gene promoter. At the level of endogenous PAI-2 mRNA expression, u-PA treatment alone did not increase PAI-2 mRNA, but produced a significant and reproducible potentiation of PMA-mediated induction of PAI-2 mRNA.

Results of supershift experiments identified c-jun and Jun D (but not jun B) as part of the assembly of factors which recognised the AP-1a site under both basal and PMA-treated conditions, establishing that at least some of the proteins assembling on this site were members of the AP-1 family. Although members of the CREB/ATF family of transcription factors are known to associate with members of the fos/jun family of proteins, we found no evidence for CREB, CREM, CPB or ATF-2 among the proteins binding to this site. Based on the results of competition experiments, it is also evident that additional proteins, probably not related to the AP-1 family, also recognise this site. The identity of these proteins and their role in *PAI-2* gene activation remains to be determined.

To address the possibility that u-PA was indirectly inducing AP-1 binding by a plasmin-mediated effect, we tested whether the ATF of u-PA would also increase AP-1 binding. The ATF is devoid of catalytic activity and is known to interact with u-PAR. Our results indicated that addition of ATF to U-937 cells induced AP-1 binding indicating that the catalytic activity of u-PA was not required to mediate this effect. We note, however, that the level of induction of AP-1 binding was not as intense as that produced when using the complete HMW-u-PA molecule. Recent evidence indicates that u-PA, upon binding u-PAR, cleaves domain 1 of u-PAR from domains 2 and 3, unmasking previously cloaked u-PAR epitopes which are thought to enhance transmembrane signal transduction [26]. Since the ATF of u-PA does not possess the catalytic domain of u-PA, cleavage of domain 1 from domains 2 and 3 of u-PAR cannot occur, which may explain, at least in part, this discrepancy.

To further support the notion that u-PAR was indeed required for these effects, PI-PLC pretreatment of U-937 cells, which removes all GPI-linked proteins from the cell surface, resulted in complete abrogation of u-PA-mediated induction in AP-1 binding activity (data not shown).

The dilemma faced by investigators of u-PAR-mediated signalling (as with all GPI-linked receptor-mediated signalling) is reconciling how transmembrane signal transduction is possible given the absence of transmembrane and cytosolic

domains in u-PAR. So-called 'adaptor proteins' harbouring transmembrane and cytoplasmic domains are thought to relay signals initiated by u-PAR to the intracellular space. Adaptor molecules considered to be involved in mediating u-PAR-dependent signal transduction include caveolin, a 22 kDa transmembrane phosphoprotein [27]. Caveolin has been detected in Triton X-100 insoluble complexes together with GPI-anchored proteins, G-proteins and non-receptor tyrosine kinases including *c-Yes* [28]. Phosphorylation of caveolin on tyrosine residues both in vitro and in vivo has recently been demonstrated [29]. Stahl and Muller [30] have identified caveolin as a putative adaptor molecule in u-PAR-mediated signalling in M24met human melanoma cells. The presence of caveolin in transformed fibroblasts [27] is consistent with a role for this molecule in the signalling events associated with u-PAR-mediated induction of PAI-2. In addition to caveolin, other molecules known to associate with u-PA/u-PAR complexes may also play a facilitatory role in mediating transmembrane signalling initiated by u-PAR. The α_2-macroglobulin receptor/Ldl receptor-related protein (Lrp) is capable of internalising u-PAR [31] and while no direct evidence has linked u-PAR-mediated signal transduction with Lrp, this transmembrane protein complex could act as an 'adaptor molecule' facilitating u-PAR-mediated signal transduction.

The intracellular signalling pathways responsible for u-PA-mediated induction of *PAI-2* gene expression are currently being investigated. Previous studies have identified the production of second messengers in response to u-PA and ATF bound to the u-PAR [32], together with the association of u-PA bound u-PAR with PKC [33], non-receptor tyrosine kinases pp60fyn, pp53/56lyn and pp58/64hck and integrins in Triton X-100 insoluble complexes [34]. These findings suggest that these molecules act as intracellular mediators of u-PAR-dependent signal transduction events. The previously described association of u-PAR with protein kinase C (PKC), together with the capacity of activated PKC to initiate AP-1 transcription factor complex binding to DNA [35] implicate this pathway in u-PA-mediated induction of *PAI-2* gene expression. The synergistic effect of u-PA on PMA-mediated induction of the PAI-2 promoter suggests that signalling pathways in addition to PKC are recruited for this response. Signal transduction pathways, including those capable of activating the mitogen-activated protein (MAP) kinase and Jun N-terminal kinase [36], enhance binding and transactivation potential of the AP-1 transcription factor complex suggesting that these kinases may be involved in mediating the synergism seen upon costimulation of cells with PMA and u-PA.

Our data indicates that the increased binding of transcription factors to the AP-1a and presumably to the AP-1b sites is involved in conveying uPA/uPAR-dependent induction of *PAI-2* gene expression as mutagenesis of these sites abolished the u-PA-mediated increase in CAT activity. The PAI-2 promoter was rendered completely non-functional when both AP-1 sites were mutated, indicating that these sites have a role in controlling basal transcription. We also observed complete loss of the synergistic effect of u-PA on PMA-mediated induction of CAT activity indicating that these sites play a crucial role in mediating the synergistic effect. EMSA analysis utilising a labelled oligonucleotide harbouring the PAI-2 AP-1b site is currently being performed to ascertain modulation of transcription factor binding to this site during stimulation with u-PA.

Our demonstration that u-PA treatment of HT-1080 cells, although producing no detectable changes in expression of endogenous PAI-2 mRNA, resulted in a 3-fold potentiation of PMA-mediated increase in PAI-2 mRNA, indicates that the in vitro effects of u-PA-on AP-1a binding and PAI-2 promoter transactivation produces a bone fide biological response.

Whether the u-PA-mediated potentiation of PMA induced PAI-2 mRNA levels results in an increase in either intracellular, secreted or cell-associated PAI-2 protein is unknown and remains the subject of ongoing investigation. If so, we speculate that binding of PAI-2 to receptor-bound u-PA would promote receptor/complex internalisation [37] terminating the signal, and inhibiting downstream induction of *PAI-2* gene expression. This would establish the basis for a potential autoregulatory mechanism involved in modulating cell-surface plasminogen activation. The notion of autoregulation of components of the plasminogen-activating system has been previously explored. Autocrine saturation of prourokinase receptors has been described in human A431 cells [38] whilst constitutive PAI-1 biosynthesis is thought to be maintained by an autocrine factor in human Hep G2 hepatoma cells [39]. The underlying molecular mechanisms governing these observations have not been fully elucidated although documentation of u-PA/u-PAR-mediated modulation of gene expression including the *c-fos* [1], fibronectin [40] and *u-PA* genes [41] suggests that u-PAR-mediated signal transduction events may contribute to the autoregulatory effects previously identified. That plasminogen activators are capable of modulating the expression of their cognate inhibitors has been recently described with the identification of tissue-type plasminogen activator (t-PA)-mediated induction of PAI-1 antigen and mRNA expression [42] and establishes a precedent for our observations.

In summary, our data identify induction of *PAI-2* gene expression by its receptor bound, cognate protease u-PA, and molecular mechanisms responsible for this effect. The effect of u-PA is highly reproducible and evident at the level of nuclear protein-binding activity, promoter transactivation and potentiation of PMA-mediated induction of endogenous PAI-2 mRNA and suggests the presence of a potential novel autoregulatory mechanism involved in the regulation of cell-surface plasminogen activation.

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