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Overexpression of a dominant negative CREB protein in HT-1080 cells selectively disrupts plasminogen activator inhibitor type 2 but not tissue-type plasminogen activator gene expression

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Abstract The tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor type 2 (PAI-2) genes are differentially regulated by 12-phorbol 13-myristate acetate (PMA) in HT-1080 fibrosarcoma cells. PMA transcriptionally down-regulates the t-PA gene in HT-1080 cells, while the PAI-2 gene is simultaneously induced by this agonist. The t-PA and PAI-2 gene promoters harbour a cAMP-response element (CRE) which influences the expression of both genes. We have compared the binding activity of nuclear factors that recognise these CRE sites. We show that CREB (CRE binding protein) recognises each CRE and that the degree of constitutive Ser119-phosphorylated t-PA CRE-bound CREB was greater than for PAI-2 CRE bound CREB. Stable transfection of HT-1080 cells with a plasmid containing a CREB that could not be phosphorylated on Ser119 (pCI-CREB$_{ala119}$) did not influence PMA-mediated suppression of t-PA mRNA, but markedly impaired PMA-mediated induction of PAI-2 mRNA. Our results demonstrate that the Ser119 residue of CREB plays a crucial role in PMA-mediated induction of PAI-2 gene expression, whereas PMA-mediated suppression of t-PA in HT-1080 cells requires a different process.

Key words: Tissue-type plasminogen activator; Plasminogen activator inhibitor type 2; cAMP-response element binding protein; Transcription

1. Introduction

Extracellular proteolysis mediated by the plasminogen activating system is crucial for many biological phenomena. It is widely accepted that the primary role of this system concerns its fibrinolytic function, particularly in the maintenance of vascular patency. However, this proteolytic cascade is also implicated in other processes, including embryonic development, cell movement, metastatic spread of some malignancies [1] and, more recently, in synaptic plasticity [2,3] and neurodegeneration [4]. Given the broad action of the plasminogen activating system, it is not surprising that the genes encoding its individual protein components are highly regulated by various agents, including cytokines, hormones and excitotoxins. In some cases, the regulation pattern of some members of this system is cell-type dependent. For example, the tumour promoter 12-phorbol 13-myristate acetate (PMA) induces tissue-type plasminogen activator (t-PA) gene expression in HeLa cells and in endothelial cells, yet suppresses t-PA expression in HT-1080 fibrosarcoma cells [5–7]. Interestingly, a *cis*-acting DNA element related to the cAMP responsive element (CRE) in the t-PA gene promoter (tPACRE; TGACATCA) is reported to play a role in PMA-modulated induction of t-PA expression in endothelial cells and in HeLa cells [5,8]. In HT-1080 cells, constitutive t-PA gene expression is relatively high and mutation of the tPACRE substantially reduces basal t-PA promoter activity. However, it remains to be determined whether this element is also required to convey PMA-mediated suppression. Taken together, these observations led to the idea that the assembly of transcription factors associating with the tPACRE orchestrates the cell-specific response of the t-PA gene to PMA [9]. The plasminogen activator inhibitor type 2 (PAI-2) gene, on the other hand, is not at all expressed or regulated in HeLa cells, but is strongly induced by PMA in HT-1080 cells and in endothelial cells. DNA mutagenesis and transfection assays of the PAI-2 gene promoter have also revealed that a CRE-like element located at position −90 to −80 (PAI-2 CRE; TGACCTCA), is an absolute requirement for basal and PMA inducible expression of the PAI-2 gene at least in transfected HT-1080 cells [10]. The cell-specific regulation of the t-PA and PAI-2 genes and a common requirement for a functional CRE in their respective gene promoters lends additional circumstantial support that the factors interacting with the t-PA and PAI-2 CRE sites influence the tissue-specific expression pattern of these two genes.

CRE motifs provide specific binding sites for transcription factors that are members of the CREB/ATF or the *fos*/*jun* families [11]. Although PAI-2 CRE binding factors have yet to be identified, tPACRE binding proteins in the nuclei of primary human umbilical vein endothelial cells have been identified as CREB, CREM, ATF-2 and c-*jun* [8]. Interestingly, CREB was also found be a prominent tPACRE binding protein in nuclear extracts prepared from HT-1080 cells [9]. The opposite regulation of the t-PA gene by PMA in endothelial cells and in HT-1080 cells raised the possibility that CREB may play a dual role in the regulation of t-PA and contribute to the positive and negative effects of PMA on the t-PA gene in these cell types.

The transcriptional effects of CREB depend to some extent on its association with other factors in a process that requires phosphorylation of a serine residue located at position 119 (Ser119) [11]. Although CREB is implicated in the regulation of t-PA gene regulation, the phosphorylation status of CREB associating with the tPACRE in any of the cell systems

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studied to date and the significance of this remains to be determined.

In this study, we have assessed the role of CREB in the regulation of the t-PA and PAI-2 genes in HT-1080 cells. Our results demonstrate that the PAI-2 CRE site provides a binding site for various members of the CREB/ATF family of proteins, including c-*jun* and CREB. Importantly, we show that the degree of Ser119-phosphorylated CREB bound to both tPACRE and PAI-2 CRE sites differs. Using a dominant negative approach to explore the role of CREB in the regulation of the t-PA and PAI-2 genes, our data indicate that the Ser119 residue in the CREB protein is required to convey PMA-mediated induction of PAI-2 expression in HT-1080 cells yet has little, if any, influence on PMA-mediated suppression of the t-PA gene. Hence, this study indicates for the first time the functional role of CREB in PMA-mediated induction of PAI-2 gene expression.

2. Materials and methods

2.1. Cell culture

Human HT-1080 fibrosarcoma cells were maintained in Dulbecco's modified Eagle's medium as previously described [9]. Where indicated, cells were treated for up to 48 h with 25 nM PMA (Sigma).

2.2. Northern blot procedure

Total RNA was isolated from cells [12] and 10 μg of total 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 50% formamide hybridisation buffer as previously described [13]. ^{32}P-Labeling of DNA was performed by the random priming procedure using the Prima-gene labeling kit (Promega, Australia). cDNA inserts used for this study included the *Bgl*II fragment of the plasmid p114B containing the t-PA cDNA [14], the *Eco*R1 fragment of plasmid pJ7 containing the 1.9-kb cDNA insert of PAI-2 [15] and the *Bgl*II insert of type 1 collagenase (1.8 kb, kindly provided by Dr Peter Angel, Germany). Plasmids harbouring the full-length mouse β-actin cDNA were denatured at 100°C, and directly labeled. After hybridisation, the membranes were washed by standard techniques and exposed to Kodak BioMax film (Eastman Kodak, Rochester, NY, USA) at −70°C with an intensifying screen.

2.3. Preparation and labeling of oligonucleotides

The labeling of gel purified oligomers (100 ng) was performed using T4 polynucleotide kinase and ^{32}P γ-ATP [9]. Annealing was performed by adding a four-fold molar excess of the complementary strand to the kinase-treated, heat-inactivated mixture [9].

2.4. Electrophoretic mobility shift assay (EMSA) and supershift procedure

Four μg of nuclear proteins extracted from HT-1080 cells, were prepared in 4 μl of Osborne buffer D [16] and EMSAs were performed [9]. The sequences of the labeled oligomers are provided in the figure legends.

Commercial monoclonal or polyclonal antibodies against ATF-1, ATF-2, CREB, CREM, c-*fos*, c-*jun* and *jun*-D specifically designed for supershift experiments were obtained from Santa Cruz Inc. (USA). Commercial antibody against Ser119-phosphorylated CREB was obtained from Signal Transduction laboratories (USA). Supershifting was performed using the same procedure as for standard EMSA except that specific antibody (1–3 μg total) was added to the nuclear extracts for 1 h after the addition of the labeled oligomers [9].

2.5. Mutagenesis

2.5.1. Generation of a dominant negative CREB. Site-directed mutagenesis of the human CREB cDNA sequence was performed using the Transformer DNA kit (Clontech, USA), following the manufacturers instructions. The mutagenic primer (5′-AGGAGGCCTGCC-TACAGGAAA-3′) was designed to replace the codon TCC (a serine residue) at position 119 with GCC (underlined in primer sequence), thereby creating an alanine residue at this position ($CREB_{ala119}$). The human CREB cDNA (kindly provided by Dr Gerard Waeber, Lausanne, Switzerland) was obtained as an *Eco*RI fragment in plasmid pGEM-7Zf(+).

The selection primer (5′-GAAATCGATCATATGGGATCCGGA-GAGC-3′) was designed to substitute the *Hin*dIII site in plasmid pGEM 7Zf(+) with *Nde*I. The wild-type and mutated CREB cDNAs were removed from the pGEM-7Zf(+) plasmid by *Eco*RI digestion and inserted into the *Eco*RI site of pCI-neo (Promega) following standard techniques, creating plasmids pCI-$CREB_{wt}$ and pCI-$CREB_{ala119}$, respectively. The orientation of the inserted CREB cDNAs was confirmed by sequencing.

2.6. Transfection studies

Stable transfection of pCI-neo, pCI-$CREB_{wt}$ or pCI-$CREB_{ala119}$ expression plasmids into HT-1080 cells was performed by the Qiagen Superfect reagent (Qiagen) following the manufacturer's instructions. Stably transfected cells were selected on the basis of resistance to G418 (600 μg/ml).

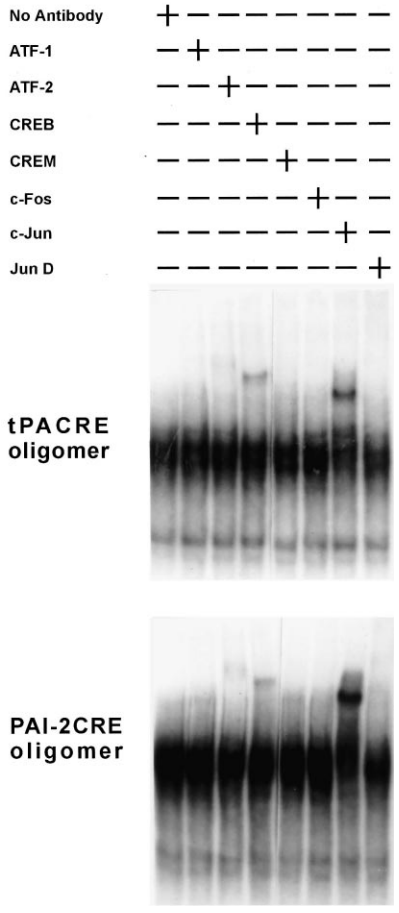

Fig. 1. Identification and comparison of tPACRE and PAI-2 CRE binding proteins in HT-1080 cells. A supershift experiment was performed to identify the tPACRE and PAI-2 CRE binding proteins in HT-1080 cells. Nuclear extracts prepared from non-treated HT-1080 cells were incubated with a labeled double-stranded 22-bp oligonucleotide harbouring the region between positions −119 and −98 in the t-PA promoter (5′-ATTCAATGACATCACGGCTGTG-3′) which contains tPACRE (underlined) (top panel). An identical experiment was performed using a double-stranded 22-bp oligomer homologous to the region between positions −196 and −175 of the PAI-2 gene promoter (5′-TTCAGAGTGACCTCATCCTCC-3′) which contains the PAI-2 CRE [10] (bottom panel). Extracts were incubated with antibodies specific to ATF-1, ATF-2, CREB, CREM, c-*fos*, c-*jun* or *jun*-D as indicated (+). Migration profiles were then compared on 5% native polyacrylamide gels as described in Section 2.

2.7. Preparation of nuclear proteins

The preparation of nuclear proteins from HT-1080 cells was performed as previously described [9].

3. Results

3.1. Identification of tPACRE and PAI-2 CRE binding proteins in HT-1080 cells

A major tPACRE binding protein identified in HT-1080 cell nuclei has previously been found to be CREB (CRE binding protein). We used an extensive range of antibodies in supershift experiments to identify additional HT-1080 nuclear factors associated with the tPACRE and the PAI-2 CRE sites under basal conditions. Antibodies specific for ATF-1, ATF-2, CREB-1, CREM, c-*fos*, c-*jun* or *jun*-D were used for this purpose. As shown in Fig. 1, the predominant tPACRE binding protein in HT-1080 cells was c-*jun*. CREB was also clearly part of the assembly of tPACRE binding proteins (as previously described). Anti-ATF-2 antibodies produced a weak (and inconsistent) supershifted complex, while antibodies against ATF-1 and c-*fos* failed to generate supershifted complexes. *jun*-D- and CREM-specific complexes became apparent only after longer exposure of the gel to X-ray film.

An identical experiment performed using the PAI-2 CRE oligomer as a probe indicated that the most predominant PAI-2 CRE binding protein in the same extracts was also c-*jun*, with clear supershifted complexes attributed to CREB and ATF-2, and a weaker shift attributed to *jun*-D.

CREB-mediated transactivation of promoters has been shown to be dependent on the phosphorylation status of Ser119 [11,17]. We performed experiments to determine whether there was any difference in the degree of Ser119-phosphorylated CREB bound to either the tPACRE or the PAI-2 CRE and also whether this could be influenced by PMA treatment. To this end, nuclear proteins were extracted from cells treated with PMA for 1, 6 and 24 h and assessed for Ser119-phosphorylated CREB by EMSA supershift experiments using an antibody that specifically recognised the Ser119-phosphorylated form of CREB (anti-p-CREB). As shown in Fig. 2, CREB associating with both the tPACRE and the PAI-2 CRE was constitutively phosphorylated on Ser119. Furthermore, the results clearly show that the degree of phosphorylated CREB was substantially stronger while bound to the tPACRE than the PAI-2 CRE. PMA treatment reduced the degree of Ser119-phosphorylated CREB bound to the tPA-CRE. PMA treatment also appeared to slightly reduce the levels of Ser119-phosphorylated CREB bound to the PAI-2 CRE.

To determine whether PMA treatment resulted in a more pronounced change in the Ser119 phosphorylation status of total CREB within the nuclear compartment, we performed Western blot experiments using nuclear extracts prepared from HT-1080 cells following a PMA time course. Unfortunately, the p-CREB antibody was not suited to this approach and the results were inconclusive (data not shown). Nonetheless, the most important parameter in this context is the degree of Ser119 phosphorylation of *CRE-bound* CREB, which we have attempted to demonstrate in this study.

Taken together, results of these supershift experiments indicate that there are a number of common nuclear proteins that associate with the tPACRE and PAI-2 CRE sites, the most obvious being c-*jun* and CREB. The relative abundance of c-*jun* bound to both CRE sites is suggestive of a role for this transcription factor in the regulation of t-PA and PAI-2 expression. However, since there was a marked difference between the relative levels of Ser119-phosphorylated CREB constitutively bound to these sites, we focused our attention on the role of Ser119-phosphorylated CREB in the differential control of the t-PA and PAI-2 gene expression. Furthermore, the phosphorylation status of Ser119 is well known to modulate the ability of CREB to transactivate gene promoters [11,17].

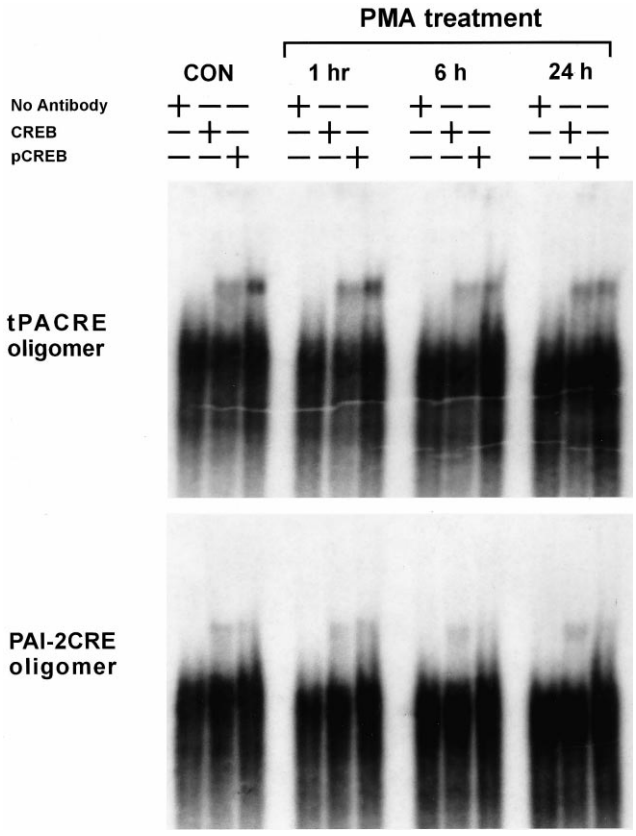

Fig. 2. Time-dependent changes in the Ser119 phosphorylation status of tPACRE and PAI-2 CRE bound CREB following PMA treatment. Supershift experiments were performed to assess changes in the degree of Ser119-phosphorylated CREB bound to the tPA-CRE and PAI-2 CRE sites following PMA treatment. Nuclear proteins were harvested from HT-1080 cells treated with 25 nM PMA for 0, 1, 6 or 24 h. A supershift experiment was performed using labelled tPACRE and PAI-2 CRE oligonucleotide probes and antibodies specific for CREB and the Ser119-phosphorylated form of CREB (p-CREB) as described in the legend to Fig. 1. Migration profiles were then compared on 5% native polyacrylamide gels.

3.2. $CREB_{ala119}$ overexpressed in HT-1080 cells binds to the t-PA and PAI-2 CRE sites and selectively disrupts PAI-2 gene expression

To address the role of Ser119-phosphorylated CREB in the control of t-PA and PAI-2 gene expression we generated a plasmid expressing a dominant negative CREB (pCI-$CREB_{ala119}$) that could not be phosphorylated at Ser119. HT-1080 cells were transfected with pCI-$CREB_{ala119}$ and G418 resistant clones (about 200) were pooled. In this series of experiments, pooled populations of HT-1080 cells transfected with wild-type CREB ($CREB_{wt}$) and the empty pCI-

neo vector were also generated. Nuclear extracts prepared from these pooled populations of transfected cells were assessed for total CREB (phosphorylated and non-phosphorylated) and Ser119-phosphorylated CREB by supershift assays using labeled oligomers harbouring the tPACRE and PAI-2 CRE as probes.

As shown in Fig. 3, the transfection of cells with either pCI-$CREB_{wt}$ or pCI-$CREB_{ala119}$ resulted in a marked increase in CREB binding to both the tPACRE and the PAI-2 CRE sites. Interestingly, there was no concomitant increase in Ser119-phosphorylated CREB binding to either the tPACRE or the PAI-2 CRE sites in cells overexpressing $CREB_{wt}$. This may suggest that there is a limitation to the extent by which CREB can be phosphorylated at this residue or that a phosphatase exists to limit the degree of CREB phosphorylation.

Cells overexpressing $CREB_{ala119}$, although displaying abundant CREB supershifts, still produced a supershifted complex when using the anti-p-CREB antibody, but the intensity of this complex was weaker compared to the supershift produced in cells overexpressing $CREB_{wt}$. The reason for the background Ser119-phosphorylated CREB activity in the pooled population of cells expressing pCI-$CREB_{ala119}$ is most likely due to differences in the degree of $CREB_{ala119}$ expression within the individual clones of the pooled population.

To assess the effect of the overexpressed wild-type and mutant CREB proteins on the expression of endogenous t-PA and PAI-2 mRNA, the pooled populations of HT-1080 cells stably transfected with pCI-neo, pCI-$CREB_{wt}$ and pCI-

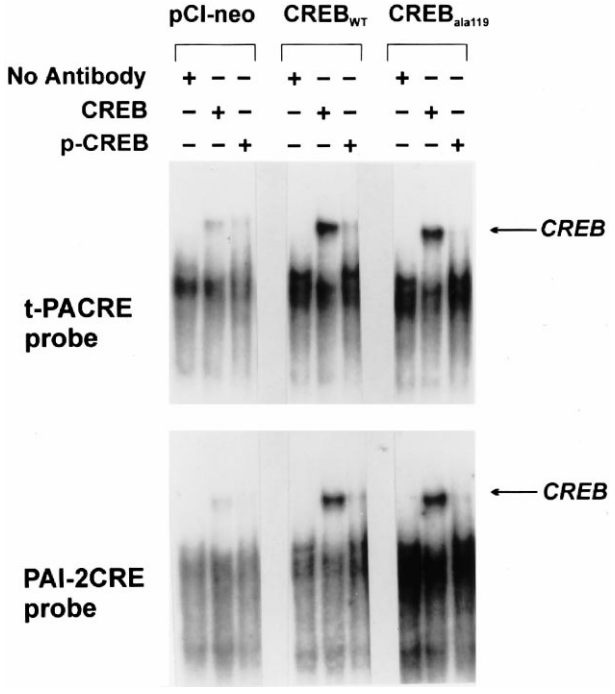

Fig. 3. $CREB_{ala119}$ overexpressed in HT-1080 cells binds to the t-PA and PAI-2 CRE sites. Nuclear extracts prepared from pooled populations of HT-1080 cells stably transfected with pCI-neo, pCI-$CREB_{wt}$ or pCI-$CREB_{ala119}$ were incubated with labeled double-stranded oligomers harbouring either the tPACRE (top panel) or the PAI-2 CRE (bottom panel), followed by addition of antibodies against CREB or p-CREB as described in Fig. 1. Migration profiles were then compared on 5% native polyacrylamide gels. The position of the complex supershifted by the anti-CREB antibodies is indicated to the right of the figure.

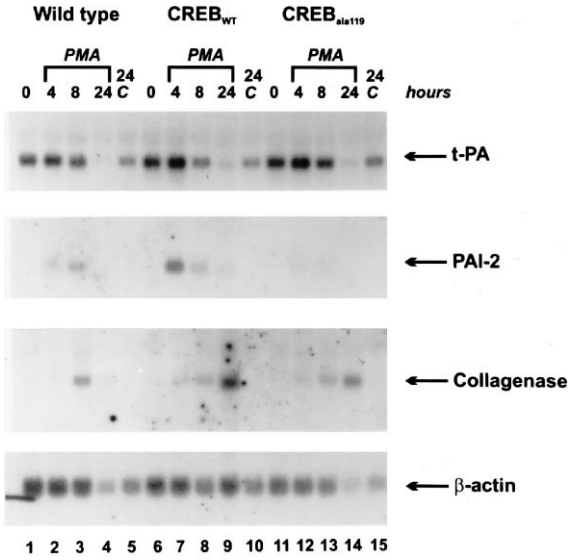

Fig. 4. PAI-2 gene expression is selectively disrupted in pooled populations of HT-1080 cells overexpressing $CREB_{ala119}$. Pooled populations of HT-1080 cells stably transfected with either pCI-neo (wild-type), pCI-$CREB_{wt}$ or pCI-$CREB_{ala119}$ were treated with 25 nM PMA for various times up to 24 h. Total RNA was prepared and electrophoresed through a 1% agarose gel containing 20% formaldehyde, then transferred to a Nylon membrane. The filter was sequentially hybridised with ^{32}P-labeled cDNA inserts specific for t-PA, PAI-2, collagenase and β-actin. This experiment was performed on at least three separate occasions with similar results being obtained each time. Lanes 1–5: pCI-neo (wild-type) transfected cells; lanes 6–11: pCI-$CREB_{wt}$ transfected cells; lanes 11–15: pCI-$CREB_{ala119}$ transfected cells.

$CREB_{ala119}$ were treated with for 4, 8 or 24 h with PMA. Total RNA was extracted and levels of endogenous t-PA and PAI-2 mRNA assessed by Northern blot analyses. As shown in Fig. 4, constitutive levels of endogenous t-PA mRNA were similar between the cell groups. PMA treatment, however, still suppressed levels of t-PA mRNA in cells transfected with the empty pCI-neo plasmid as well as in cells overexpressing either $CREB_{wt}$ or $CREB_{ala119}$. In contrast, PMA treatment did not result in an increase in PAI-2 mRNA levels in cells overexpressing $CREB_{ala119}$. This was not a consequence of a generalised increase in CREB protein, because PAI-2 mRNA levels in HT-1080 cells overexpressing $CREB_{wt}$ were increased by PMA in a manner similar to that seen in cells transfected with pCI-neo. To confirm the selectivity of these observations, PMA treatment resulted in a similar increase of collagenase mRNA in pCI-neo, pCI-$CREB_{wt}$ and pCI-$CREB_{ala119}$ expressing cells. Taken together, these data indicate that induction of PAI-2 mRNA by PMA is dependent upon a functional CREB protein.

4. Discussion

The functional importance of the cAMP responsive element binding sites in the t-PA and PAI-2 gene promoters is well documented [5,10]. It is also clear that, despite the sequence similarity between these elements, their relative contribution to the regulation of t-PA and PAI-2 gene expression is quite different. For the t-PA gene, mutagenesis of the CRE site in the t-PA promoter, reduces basal expression by 50% and weakens, but does not completely inhibit, PMA-mediated in-

duction in transfected HeLa cells [5] and in endothelial cells [9].

For the PAI-2 promoter, the role of the CRE site is particularly important, as mutagenesis of this motif in the absence of other promoter mutations completely abolishes both basal activity and PMA-mediated induction in transfected HT-1080 cells [10]. The aim of this study was, firstly, to identify and compare the tPACRE and PAI-2 CRE binding proteins and, secondly, to determine whether differences in the binding of these proteins contributed to the opposite regulation of these two genes in HT-1080 cells.

In our previous reports, we described CREB as a major tPACRE binding protein in HT-1080 cells. Here we show that tPACRE-bound CREB is constitutively phosphorylated on Ser119, although the percentage of phosphorylation cannot be accurately determined. c-*jun* was shown to be the singly most prominent tPACRE binding protein in these cells. Experiments performed using the PAI-2 CRE oligomer as a probe indicated that some of the HT-1080 cell-derived factors that recognise the tPACRE also recognise the PAI-2 CRE, namely c-*jun* and CREB. Our results also demonstrated that the degree of Ser119-phosphorylated CREB bound to the PAI-2 CRE was notably weaker than that seen for the tPA-CRE. We therefore focused our attention towards determining whether the Ser119 residue of CREB influenced expression of the t-PA and PAI-2 genes since the phosphorylation status of the Ser119 residue is known to greatly influence the ability of CREB to associate with co-factors and subsequently transactivate gene promoters [11,17–20]. Furthermore, the role of this residue on gene expression can also be cell-type specific. For example phosphorylation of the Ser119 counterpart in rat CREB (Ser133) was shown to inhibit hepatic cell proliferation [21], whereas expression of a mutant CREB that could not be phosphorylated on Ser119 results in defective thymocyte proliferation in transgenic mice [22]. Interestingly, circumstantial data suggests that CREB may regulate t-PA expression in vivo. CREB is strongly expressed in the brain [23,24] and is implicated in the *trans*-activation of immediate early genes involved in late phase long-term potentiation (L-LTP), since transgenic mice over-expressing a dominant negative mutant form of CREB have impaired L-LTP [25,26]. Interestingly, like CREB −/− mice, t-PA −/− mice also display impaired in L-LTP [23,27].

We speculated that differences in the degree of the Ser119 phosphorylation status of CREB might influence constitutive expression directed by the t-PA and PAI-2 gene promoters and/or their abilities to respond to certain agonists. To address this possibility, we constructed a dominant negative CREB protein, $CREB_{ala119}$ and overexpressed this protein in HT-1080 cells. Constitutive and PMA-modulated expression of the endogenous t-PA and PAI-2 genes in both individual clones (data not shown) and in pooled populations of HT-1080 cells expressing the mutant CREB protein was assessed by Northern blotting. Results indicated that PMA-mediated suppression of t-PA mRNA was essentially unaltered in cells expressing $CREB_{ala119}$, whereas PMA-mediated induction of PAI-2 mRNA was greatly impaired. Furthermore, the fact that overexpression of $CREB_{wt}$ in pooled populations of cells did not influence the PMA-modulated response of either gene highlights the selective effect of the dominant negative form of CREB. Taken together, these findings demonstrate that the Ser119 residue of CREB is critical for the regulation of the PAI-2 gene. The fact that PMA treatment resulted in only a minor change in the degree of Ser119 phosphorylation raises the possibility that the marked disruption of the PAI-2 gene due to the expression of the CREB mutant may not be related to the inability of CREB to be phosphorylated at position 119, and may indicate a different role for Ser119 in the CREB protein. The mechanism by which CREB conveys its influence on the PAI-2 gene in HT-1080 cells is presently unknown. The kinase inducible domain of the CREB protein harbours many phosphoacceptor sites that are subject to phosphorylation by various kinases including PKA, PKC and CK II [18,28,29] the most important being Ser119 as we have focused on in this study. Phosphorylation of this residue permits CREB to associate with the CREB-binding protein (CBP) which in turn can acetylate histones and initiate relaxation of DNA to allow other essential transcription factors to engage and activate transcription [30]. Also, the phosphorylation of Ser119 can also create new phosphorylation sites for other kinases that in turn can influence the transactivation potential of CREB [31]. Whether these events, or a Ser119-phosphorylation-independent process, occurs during PMA-mediated induction of the PAI-2 gene remains to be determined.

We were surprised to observe that overexpression of the CREB mutant protein did not influence t-PA mRNA expression since the degree of Ser119-phosphorylated CREB bound to the tPACRE was greater than that seen for the PAI-2 CRE and the fact that PMA treatment significantly reduced the levels of Ser119-phosphorylated CREB bound to the tPA-CRE. The lack of an effect of $CREB_{ala119}$ on PMA-mediated suppression of t-PA mRNA in HT-1080 cells presents a number of other possibilities. Indeed, this study has not completely excluded a role for CREB in the control of the t-PA gene in HT-1080 cells as other phosphorylation sites on the CREB protein may be required to convey PMA-mediated downregulation of the t-PA gene in HT-1080 cells. In this context, it would be interesting to determine whether an inactive CREB, for example one that possesses a mutation in either the DNA binding or transactivation domain, would interfere with the regulation of the t-PA gene in these cells. Quite apart from CREB itself, other tPACRE binding proteins in HT-1080 cells need to be considered. c-*jun* is an interesting candidate as a possible regulatory molecule for t-PA and possibly for PAI-2 gene regulation, as it is an abundant tPACRE and PAI-2 CRE binding factor. It is also reasonable to consider the roles of other regulatory sites within the t-PA promoter. The Sp1 site located approximately 165 bp downstream from the tPACRE may be relevant as it has been shown to cooperate with the tPACRE in the maintenance of basal expression in HT-1080, HeLa and human endothelial cells [5,8]. Clearly, much work is needed to understand the basis of PMA-mediated suppression of t-PA in HT-1080 cells and the role of CREB and the other tPACRE binding proteins in this process.

In summary, this study is the first to demonstrate an important role for CREB in the induction of the PAI-2 gene in HT-1080 cells. This effect is dependent on the presence of Ser119 in the CREB protein. We also show that PMA-mediated suppression of the t-PA gene in HT-1080 cells does not require Ser119-phosphorylated CREB, suggesting that the suppression of the t-PA gene under these conditions requires a different process.

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