PKC-dependent phosphorylation of the p97 repressor regulates the transcription of aldolase A L-type promoter

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Abstract Expression of mouse aldolase A L-type mRNA is negatively modulated by a *cis* element (AldA-NRE), located within the aldolase A distal promoter (pL). AldA-NRE interacts with a 97-kDa repressor protein (p97), which binds DNA in a cell cycle-dependent manner. We demonstrate that the binding between AldA-NRE and p97 decreases during differentiation of human Caco-2 cells and is inversely correlated with L-type mRNA expression. Phosphorylation of the p97 repressor weakened its DNA binding activity in differentiated Caco-2 cells, while dephosphorylation enhanced the binding in proliferating cells. Stimulation of protein kinase C (PKC) in vivo decreased the binding of p97 to AldA-NRE and stimulated transcription, while inhibition of PKC stimulated p97 binding and downregulated transcription. These findings suggest that PKC is a mediator of the binding and silencing function of the p97/AldA-NRE repressor complex.

Key words: Negative *cis* element; Protein kinase C; Phosphorylation; Cell cycle; Transcriptional regulation

1. Introduction

Transcription of the aldolase A gene is driven by three autonomous promoters – distal (pL), middle (pM) and proximal (pF) – and is modulated by interactions between nuclear proteins and DNA *cis* elements located within the promoter regions upstream of leader exons L1, M and F, depending on cell and stage specificity [1–8]. Within the human pL promoter we have identified a negative regulatory element (hAldA-NRE) that binds a nuclear protein and acts as a true 'silencer' because it represses transcription in its own configuration and transcription of a reporter gene driven by a heterologous promoter [6,8]. Another negative regulatory transcriptional element (reducing module) has been found to attenuate the proximal promoter-driven transcription in the rat thymocyte aldolase A gene [9]. However, this negative 'reducing module' is located in a position completely different from that of AldA-NRE. In mouse and rat cells we found that L-type mRNA expression is enhanced during growth arrest and at the onset of the differentiation programme [10]. Recently, within the mouse distal promoter we detected a negative regulatory element (mAldA-NRE), highly homologous to the human AldA-NRE, which binds a 97-kDa specific nuclear protein. We showed that an AGAGA DNA motif within the AldA-NRE is the target for binding of the repressor and that a dinucleotide (AA), adjacent to the GA-rich motif, is required for silencing activity. Interestingly, the amount of the silencer/repressor complex was greater in proliferating NIH 3T3 cells than in serum-deprived cells and it was inversely correlated with expression of the L-type promoter, whose transcription was downregulated in proliferating cells. These findings prompted the notion that AldA-NRE is involved in the regulation of aldolase A L-type promoter activity through its binding with a cell cycle-modulated repressor [11].

Here, we report that transcription of the human L-type promoter is regulated by interaction between AldA-NRE and a 97-kDa repressor (p97) in Caco-2 cells and that L-type mRNA levels increase during cell differentiation in correspondence with a drastic decrease of the p97/AldA-NRE complex. We demonstrate that a protein kinase C (PKC)-dependent phosphorylation of p97 causes binding activity to decrease. In fact, in cells stably expressing an AldA-CAT fusion promoter construct, downregulation of PKC increased p97 binding and drastically reduced transcriptional L-type promoter activity. Conversely, PKC activation decreased the binding and stimulated transcription of L-type promoter.

2. Materials and methods

2.1. Cell culture

The human colon carcinoma cell line Caco-2 was grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% foetal calf serum (FCS). Cells were seeded at a density of about 500 000/100 mm diameter dish, and routinely subcultured when the cells were about 80% confluent. The culture medium was changed every day. Caco-2 cells differentiate into enterocyte-like cells as a function of cell density alone, without changes in the culture conditions [12]. Confluence was reached within 6–8 days and the differentiate state on day 14–15. Mouse NIH 3T3 fibroblasts were grown in DMEM supplemented with 10% FCS. The medium was changed every 2–3 days, and the cultures were maintained strictly subconfluent. Cells were allowed to grow exponentially for 3 days. Thereafter cells were maintained for 48 h in DMEM medium supplemented with 0.5% FCS to achieve quiescence. Phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) was added to the cells at a concentration of 120 ng/ml for 10 min or at a concentration of 10 ng/ml for 1 h (PKC-stimulating doses) or at a concentration of 200 ng/ml for 24 h (PKC-inhibiting doses) [13,14].

2.2. RNase protection analysis

Total RNA was isolated by the method of Chomczynski and Sacchi

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Abbreviations: CAT, chloramphenicol acetyltransferase; AldA-NRE, aldolase A negative regulatory element; EMSA, electrophoretic mobility shift assay; TPA, phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate; H7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine; 4NPP, 4-nitrophenylphosphate; PAP, potato acid phosphatase

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[15]. RNase protection assays were performed as previously described [10]. The human aldolase A L-type probe was obtained by transcribing with T7 RNA polymerase, in the presence of [α^{32}P]CTP (400 Ci/mmol; Amersham Corp.), a 150-bp fragment, which includes L1 and L2 exons, cloned in the pGem4Z vector (Promega). The protected fragments generated from this probe are 126 and 72 bp long.

2.3. Southwestern, EMSA and phosphatase assay

Nuclear protein extracts from NIH 3T3 and Caco-2 cells were prepared according to Dignam et al. [16] modified as previously described [11]. Southwestern experiments [11] and EMSA [1] were performed as described. In phosphatase assay nuclear extracts (4 μg) were incubated with or without potato acid phosphatase (PAP) 0.06 and 0.12 U. for 20 min at 37°C in Parker buffer 1× (glycerol 10%, HEPES 10 mM (pH 7.9), KCl 100 mM, EDTA 0.1 mM, DTT 0.25 mM) prior to the EMSA experiments. The phosphatase inhibitor 4-nitrophenylphosphate (4NPP) was added at concentrations of 1, 5 and 10 mM for 10 min prior to the addition of PAP. The specific inhibitor of PKC 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H7) was added to the cell cultures at a concentration of 50 μM for 20 min prior to the addition of TPA (120 ng/ml for 10 min). TPA, PAP, H7 and 4NPP were purchased from Sigma-Aldrich.

2.4. Isolation of stable transfectants and CAT assays

To obtain NIH 3T3 cells permanently transfected with the L-type-CAT fusion gene, 3×10^5 cells were cotransfected with 20 μg of AldALΔ-555 plasmid or 20 μg of AldALΔ-555MutN1 plasmid, previously described [8,11], and 2 μg of CMV-NEO plasmid by the calcium phosphate technique [17]. After transfection, cells were cultured for 72 h in DMEM supplemented with 10% FCS; thereafter cells were grown in selective medium containing G418 Geneticin (Gibco-BRL) at a concentration of 800 μg/ml. Selection continued for 3 weeks until G418-resistant colonies were established. Several G418-resistant colonies were selected and expanded in complete medium containing 300 μg/ml of G418. One G418-resistant colony, clone 5, derived from transfection with AldALΔ-555, and one colony, clone MN1, derived from transfection with AldALΔ-555MutN1, were treated with TPA, as described above, and used in CAT assays, performed according to Gorman et al. [18].

3. Results

3.1. The binding efficiency of the 97-kDa repressor to AldA-NRE decreases during differentiation of Caco-2 cells

We previously demonstrated that the expression of L-type mRNA in NIH 3T3 cells is modulated by the interaction between a cell cycle-dependent DNA binding protein of about 97 kDa and the mouse AldA-NRE [11]. Using EMSA and the Southwestern technique we investigated whether a similar DNA binding protein (p97) binds to the human AldA-NRE and regulates L-type mRNA expression in Caco-2 cells (Fig. 1). We incubated the oligonucleotide Neg1 probe, which covers the sequence of AldA-NRE from −510 to −485 bp upstream of the initiation transcription site of L-type mRNA [8], with nuclear extracts from undifferentiated and differentiated Caco-2 cells. These cells differentiate spontaneously into en-

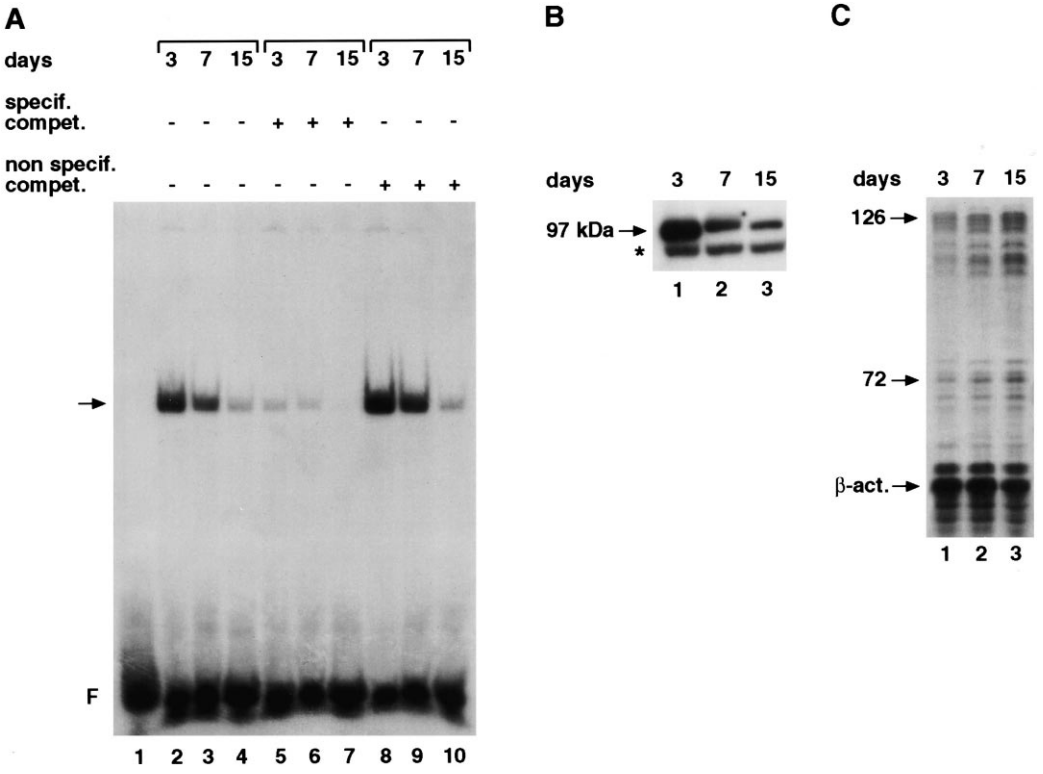

Fig. 1. Regulation of L-type mRNA expression in Caco-2 cells. A: Gel shift assay of nuclear extracts from undifferentiated Caco-2 cells at day 3 (lanes 2, 5 and 8), and differentiated cells at day 7 (lanes 3, 6 and 9) and at day 15 (lanes 4, 7 and 10) of culture, using the 5′-end-labelled probe Neg1. Specific competition (lanes 5–7) was performed with a 200-fold molar excess of Neg1 oligonucleotide; non-specific competition (lanes 8–10) was performed with a 200-fold molar excess of unrelated oligonucleotide. Lane 1: the Neg1 probe alone. The arrow indicates the specific DNA-protein complex. F indicates the free probe. B: Southwestern assay of nuclear extracts from Caco-2 cells at day 3 (lane 1), day 7 (lane 2) and day 15 (lane 3), hybridised with probe Neg1. On the left is the molecular mass indicator. The asterisk indicates another complex of lower molecular size that has a similar intensity in both undifferentiated and differentiated cells. C: RNase protection assay on total RNA from Caco-2 cells at day 3 (lane 1), day 7 (lane 2) and day 15 (lane 3), hybridised with riboprobe L. Two fragments of 72 and 126 bp, corresponding to different initiation transcription sites of L-type mRNA, were protected by RNase digestion in the presence of L-type specific probe. The β-actin probe was added to each sample as internal control of the amounts of RNA used.

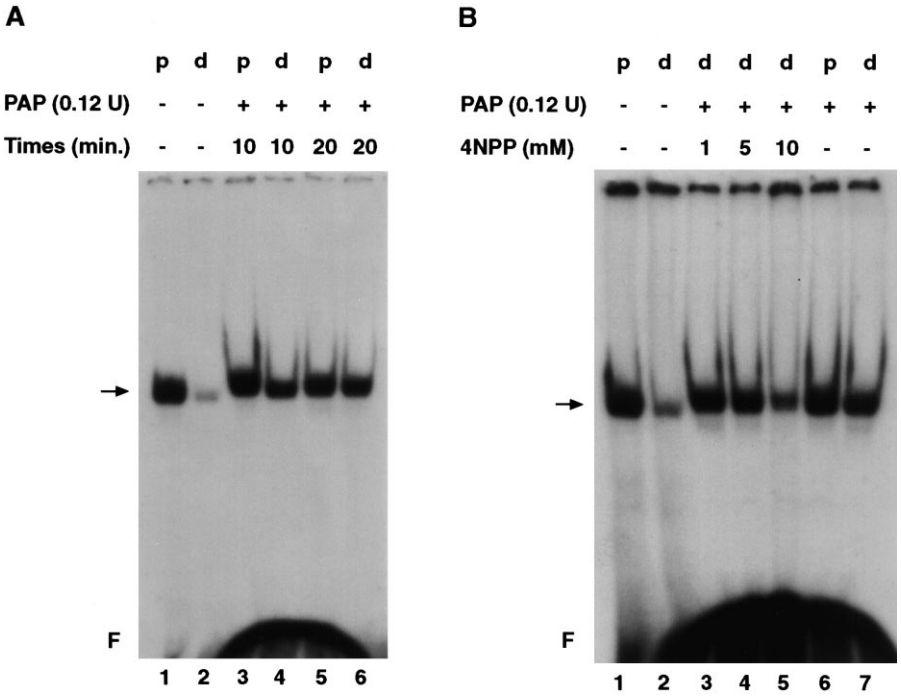

Fig. 2. Effect of phosphatase treatment on the binding activity in nuclear extracts from Caco-2 cells. A: Nuclear extracts from proliferating (lanes 3 and 5) and differentiated (lanes 4 and 6) Caco-2 cells were treated with PAP (0.12 U) for 10 min (lanes 3 and 4) or for 20 min (lanes 5 and 6) at 37°C, prior to EMSA. Nuclear extracts from proliferating (lane 1) and differentiated (lane 2) Caco-2 cells were incubated without PAP at 37°C for 20 min prior to EMSA, as control. The arrow indicates the specific DNA-protein complex. F indicates the free probe; p indicates proliferating cells; d indicates differentiated cells. B: Nuclear extracts from differentiated Caco-2 cells were incubated with the phosphatase inhibitor 4NPP at concentrations of 1, 5 and 10 mM (lanes 3, 4 and 5, respectively) for 10 min, prior to the addition of PAP. Lanes 6 and 7: nuclear extracts from proliferating and differentiated Caco-2 cells, respectively, treated with PAP at 37°C for 20 min without the phosphatase inhibitor 4NPP. Nuclear extracts from proliferating (lane 1) and differentiated (lane 2) Caco-2 cells were incubated without PAP at 37°C for 20 min prior to EMSA, as control. The arrow indicates the specific DNA-protein complex. F indicates the free probe; p indicates proliferating cells; d indicates differentiated cells.

terocyte-like cells under standard culture conditions, depending on cell density [12]. A retarded DNA-protein complex was found in both undifferentiated (Fig. 1A, lane 2) and differentiated cells at 7 and 15 days (Fig. 1A, lanes 3 and 4), although the amount of DNA-protein complex decreased progressively during Caco-2 cell differentiation. The same amount of nuclear extracts tested for Sp1 DNA binding activity gave retardation signals of the same intensity in both undifferentiated and differentiated cells (data not shown). Binding of undifferentiated and differentiated cells was prevented by a 200-fold molar excess of Neg1 oligonucleotide as specific competitor (Fig. 1A, lanes 5–7), whereas it was not affected by a non-specific competitor (Fig. 1A, lanes 8–10). In Southwestern experiments, using the same nuclear extracts as in EMSA, there was a severe decrease of p97/DNA interaction in differentiated cells at 7 and 15 days (Fig. 1B, lanes 2 and 3) compared with undifferentiated cells (Fig. 1B, lane 1).

To explore whether the decrease in the amount of the DNA-protein complex in differentiated cells at 7 and 15 days correlates with L-type mRNA expression, we performed RNase protection experiments on total RNA from undifferentiated (Fig. 1C, lane 1) and differentiated Caco-2 cells (Fig. 1C, lanes 2 and 3). The levels of L-type mRNA increased significantly during cell differentiation. These data indicate that in the human system the enhanced expression of L-type mRNA during cell differentiation reflects the decreased binding of p97 to AldA-NRE, as previously shown in the murine system during cell growth arrest [11].

3.2. *Phosphatase treatment of nuclear extract from differentiated Caco-2 cells restores the binding efficiency of p97 to AldA-NRE*

To determine whether the different DNA binding efficiency of p97 to AldA-NRE, observed in proliferating and differentiated cells, is caused by phosphorylation of the protein, we treated nuclear extracts from Caco-2 cells with PAP prior to the addition of labelled Neg1 probe. As shown in Fig. 2A,the low binding activity of nuclear extracts from differentiated cells (lane 2) is remarkably increased after PAP treatment for 10 and 20 min (lanes 4 and 6). The restored binding activity of the differentiated Caco cells after PAP treatment is comparable to that of nuclear extracts from PAP-untreated proliferating cells (lane 1) and from PAP-treated proliferating cells (lanes 3 and 5). Furthermore, as shown in Fig. 2B, the increased binding activity of nuclear extracts from differentiated Caco-2 cells after PAP treatment (compare lanes 7 and 2) is abolished by inhibition of phosphatase activity with 4NPP. The phosphatase inhibition by 4NPP is dose-dependent (1, 5 and 10 mM in lanes 3–5, respectively), and the highest concentration of 4NPP (lane 5) completely prevents the effect of PAP treatment (compares lanes 5 and 7).

Taken together these results demonstrate that phosphorylation of p97, occurring in differentiated Caco-2 cells, decreases its DNA binding activity.

3.3. *Phosphorylation of p97 is mediated by protein kinase C*

To verify whether PKC is involved in phosphorylation of

p97, we treated proliferating NIH 3T3 cells with phorbol ester TPA, which specifically stimulates PKC activity at a concentration of 120 ng/ml for 10 min, and downregulates it at a concentration of 200 ng/ml for 24 h [13]. In experimental conditions of PKC stimulation the p97 binding activity was significantly decreased (Fig. 3, lane 3) compared with the binding of NIH 3T3 cells not treated with TPA (lane 1). Conversely, TPA-induced downregulation of PKC caused an increase of p97 binding activity (lane 2). The presence of H7, an inhibitor of PKC, caused an increase of p97 binding activity in NIH 3T3 cells treated with TPA in conditions of PKC stimulation (compare lane 4 versus lane 3).

Next, we demonstrate that p97 binding activity in proliferating NIH 3T3 cells (Fig. 4, lane 1) was inhibited by PKC (Fig. 4, lane 2) and recovered by increasing amounts of PAP (0.06 U in lane 4, and 0.12 U in lane 6); this is suggestive of regulation of the DNA binding protein by phosphorylation. Finally, we observed that PAP treatment of nuclear extracts from proliferating NIH 3T3 cells leads to an increased binding activity (compare lanes 3 and 5 versus lane 1); this suggests that a fraction of repressor might be phosphorylated in proliferating NIH 3T3 cells.

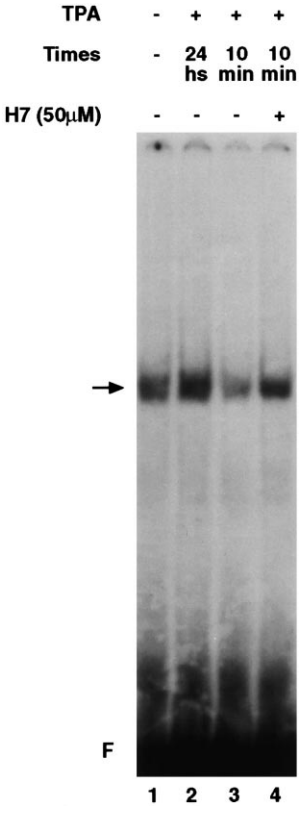

Fig. 3. Effect of TPA treatment on the binding activity in NIH 3T3 cells. The 5′-end-labelled Neg1 probe was incubated with nuclear extracts from NIH 3T3 cells (lane 1), from NIH 3T3 cells treated with TPA (200 ng/ml) for 24 h (lane 2), from NIH3 T3 cells treated with TPA (120 ng/ml) for 10 min (lane 3) and from NIH 3T3 cells treated with H7 at a concentration of 50 μM for 20 min prior to the addition of TPA (120 ng/ml for 10 min) (lane 4). The arrow indicates the specific DNA-protein complex. F indicates the free probe.

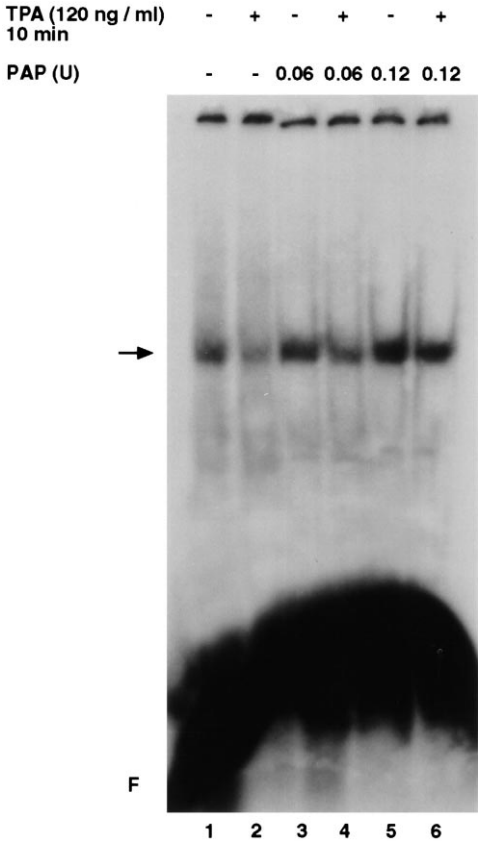

Fig. 4. Effect of phosphatase on the binding activity in NIH 3T3 cells treated with TPA. Nuclear extracts from NIH 3T3 cells (lanes 3 and 5) and from NIH 3T3 cells treated with TPA (120 ng/ml for 10 min) (lanes 4 and 6) were incubated with 0.06 U of PAP (lanes 3 and 4) or with 0.12 U of PAP (lanes 5 and 6) for 20 min at 37°C, prior to EMSA. Lanes 1 and 2: nuclear extracts from NIH 3T3 cells untreated and TPA-treated (120 ng/ml for 10 min), respectively, without PAP. The arrow indicates the specific DNA-protein complex. F indicates the free probe.

3.4. Transcriptional regulation of CAT reporter constructs is affected by phosphorylated/dephosphorylated forms of p97

To demonstrate that phosphorylation of p97 is involved in L-type mRNA expression, we analysed NIH 3T3 mouse cells constitutively expressing a chimeric L-type-CAT mRNA. We generated several stably transfected clones, using the LΔ-555 construct, previously described [8]. In transient transfection experiments, this construct exhibited reduced transcriptional activity, due to the silencing effect of AldA-NRE [11]. To modulate PKC activity, we treated a stable clone (LΔ-555 clone 5), constitutively expressing LΔ-555 construct, with TPA.

Downregulation of PKC (by TPA treatment for 24 h at a concentration of 200 ng/ml) led to a decrease of CAT transcriptional activity in LΔ-555 clone 5 compared with that of TPA-untreated cells (22.1% versus 60.7% in Fig. 5). Conversely, PKC stimulation (by TPA treatment for 1 h at a concentration of 10 ng/ml) caused a relevant induction of CAT activity in LΔ-555 clone 5 (108.1% versus 60.7% in Fig. 5). TPA treatment was performed also with a stable mutant clone (LΔ-555 clone MN1), constitutively expressing the LΔ-555MutN1 construct. Previously, we found that the substitution of the binding motif (AGAGA) of AldA-NRE with the CCGCG sequence in the LΔ-555MutN1 construct reduces

the binding activity and leads to an increased CAT transcriptional activity [11]. Here, we demonstrate that the transcriptional activity of the stable mutant LΔ-555 clone MN1 was not affected by modulation of PKC activity. In fact, the relative CAT activity was similar in TPA-untreated and TPA-treated cells (99.3% and 102.5% versus 100% in Fig. 5), thus demonstrating that effects exerted by TPA upon the interaction between p97 and AldA-NRE require the wild-type AldA-NRE motif.

These results indicate that the PKC-dependent phosphorylation of p97, leading to the displacement of p97 from AldA-NRE, is the basic event that in vivo regulates the aldolase A L-type promoter activity.

4. Discussion

Gene expression is actively repressed through silencers and negative regulatory elements; the former, defined 'position-independent regulatory elements', direct an active repression mechanism by interfering, usually, with general transcription factors. The latter, defined 'non-classical, position-dependent elements', drive a passive repression mechanism by interacting with auxiliary transcription factors [19]. Within the human aldolase A pL promoter we have identified a negative regulatory element, AldA-NRE, that represses promoter activity in an orientation-independent fashion, in the context of a native and a heterologous promoter [8]. In this respect, AldA-NRE belongs to the classical silencer family.

We previously showed that mouse AldA-NRE binds a 97-kDa nuclear repressor protein and that this DNA-protein interaction modulates the expression of aldolase A L-type mRNA in a cell cycle-dependent fashion in NIH 3T3 cells [11]. Here, we demonstrate that during the differentiation of human Caco-2 cells the increased expression of aldolase A L-type mRNA is caused by a decreased binding of a 97-kDa protein (p97) to AldA-NRE. Therefore, we have investigated whether phosphorylation of the p97 repressor affects its DNA binding activity. We found that p97 is phosphorylated in differentiated cells and that the phosphorylation leads to a decrease in binding activity (see Fig. 2) and to an increase in the transcription of pL promoter (see Fig. 5). Post-translational modification of transcription factors, often phosphorylation

	Relative CAT Activity %	
	LΔ-555 clone 5	LΔ-555 clone MN1
TPA-untreated cells	60.7 ± 8.7	100
TPA-treated cells (200 ng / ml) / 24 hrs	22.1 ± 2.8	99.3 ± 14.9
TPA-treated cells (10 ng / ml) / 1 hrs	108.1 ± 17.4	102.5 ± 18.6
serum-deprived cells	116.8 ± 21.1	N.D.

Fig. 5. Transcriptional regulation of aldolase A-CAT constructs by p97/AldA-NRE interaction in NIH 3T3 cells. Values represent relative CAT activities of the wild-type LΔ-555 clone 5 compared with that of the mutant LΔ-555 clone MN1 arbitrarily taken as 100%. The same results were obtained with at least two different stable clones. Each value is mean ± S.D. from 3–5 independent experiments. N.D.: not detected.

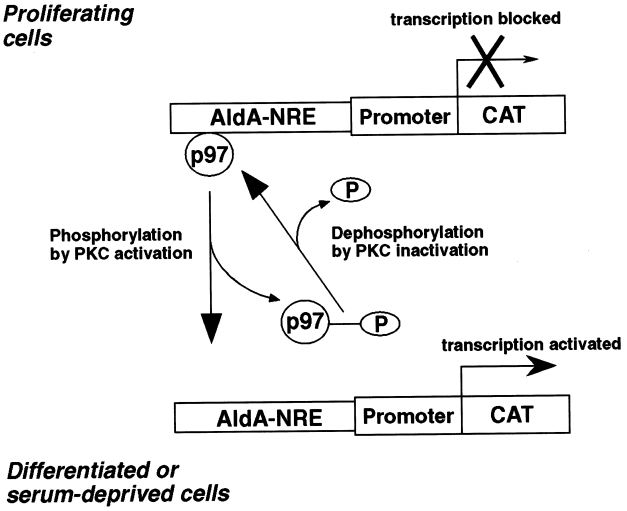

Fig. 6. Proposed scheme of the transcriptional regulation mediated by the p97/AldA-NRE interaction.

events, is a rapid system with which to control gene induction and repression in eukaryotes during proliferation/differentiation stages [20]. Data presented in this work confirm and enlarge the notion that the affinity of the protein/DNA interaction is generally decreased by phosphorylation.

There are several well-documented examples of transcriptional activators whose function is modulated by phosphorylation [21]. To our knowledge, p97 is one of the few transcriptional repressors whose binding activity is modulated by phosphorylation. The findings that p97 is cell cycle-regulated [11] and that its binding activity is modulated by phosphorylation (this work) suggest that p97 is a phosphorylation-dependent transcriptional repressor regulated during the cell cycle. Protein kinase C is one of the major regulators of cell growth and cell proliferation [22,23]. Compelling evidence points to a role for PKC isoenzymes as regulators linking signal transduction pathways to the cell cycle machinery [24,25]. Our data suggest that phosphorylation of p97 repressor is mediated by members of the PKC family. Indeed, PKC downregulation caused an increase of p97/AldA-NRE binding, whereas stimulation of PKC activity by TPA provoked a severe decrease of p97 binding activity (see Fig. 3). The reduced binding activity observed under conditions of PKC activation was reversed in the presence of a PKC inhibitor (H7) (see Fig. 3). Finally, we demonstrate that the in vivo PKC-dependent phosphorylation of p97 repressor was the event responsible for transcriptional regulation of the promoter region harboring AldA-NRE in its 5′ sequences. In fact, clones stably expressing CAT mRNA driven by the aldolase A pL promoter showed decreased CAT activity when PKC was downregulated, and increased CAT activity under conditions of PKC stimulation (see Fig. 5). Our results, summarised in a schematic model in Fig. 6, suggest that transcriptional regulation of the AldA L-type promoter is mediated by p97/AldA-NRE interaction and is modulated by PKC-dependent phosphorylation of p97. However, at the moment we do not know whether in vivo phosphorylation of p97 is mediated directly by PKC, or other proteins are involved in this pathway.

The isolation and cloning of p97 repressor protein will provide insights into the molecular mechanisms that regulate p97

activity during the cell cycle and into the relationship between cell cycle control and the PKC signal transduction pathway.

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