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Expression of the Ets transcription factor Erm is regulated through a conventional PKC signaling pathway in the Molt4 lymphoblastic cell line

France T'Sas[a], Carmen Brenner[a], Sébastien Mauen[a], Pascale Putmans[a], Didier Monté[b], Carine Van Lint[c], Muriel Moser[d], Jean-Luc Baert[b], Yvan de Launoit[a,b,*]

[a] *Laboratoire de Virologie Moléculaire, Faculté de Médecine, ULB, CP 614, 808 route de Lennik, 1070 Brussels, Belgium*
[b] *Régulation Transcriptionnelle au cours de la Tumorigenèse Mammaire, UMR 8117/CNRS/USTL, Institut Pasteur de Lille/Institut de Biologie de Lille, 1 rue Calmette, 59021 Lille Cedex, France*
[c] *Laboratoire de Virologie Moléculaire, Institut de Biologie Moléculaire et Médicale (IBMM), ULB, 12 Rue des Professeurs Jeneer et Brachet, 6041 Gosselies, Belgium*
[d] *Laboratoire de Physiologie Animale, Faculté des Sciences, Institut de Biologie Moléculaire et Médicale (IBMM), ULB, 12 Rue des Professeurs Jeneer et Brachet, 6041 Gosselies, Belgium*

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Abstract **Erm, a member of the PEA3 group within the Ets family of transcription factors, is expressed in murine and human lymphocytes. Here, we show that in the human Molt4 lymphoblastic cell line, the *erm* gene expression is regulated by the conventional PKC (cPKC) pathway. To better characterize the molecular mechanism by which cPKC regulates Erm transcription in Molt4 cells, we tested proximal promoter deletions of the human gene, and identified a specific cPKC-regulated region between positions −420 and −115 upstream of the first exon.**

Keywords: Ets; Erm; PKC; Transcriptional regulation

1. Introduction

The Ets family transcription factors have in common the ETS-domain, an 85 amino-acid domain organized in a winged helix-turn-helix structure and responsible for DNA-binding to the specific core sequence GGA(A/T) [1]. These factors can be sub-classified in 13 groups according to the amino-acid conservation not only in the ETS-domain but also in other domains, such as transactivation or protein interaction domains. The PEA3 group is made up of three members: Pea3 (E1af, Etv4), Er81 (Etv1) and Erm (Etv5). These three factors are more than 95% identical in the ETS-domain, and highly conserved in the N-terminal acidic and C-terminal transactivation domains [2,3].

These transcription factors are involved in developmental processes and oncogenesis [2]. For example, in the mammary gland, their overexpression is observed in certain human breast cancers as well as in oncogene-induced mammary tumors [4,5]. They are also expressed upon different stages of normal mammary gland development, from embryonic emergence to postnatal evolution [6–8], with a high level of expression during extensive ductal outgrowth and branching.

Erm also plays a regulating role in the murine Th1 CD4+ lymphocytes, in which its expression is activated by a Stat4-dependent interleukin-12 pathway [9]. In human beings, the *erm* gene is strongly and preferentially expressed in phorbol 12-myristate 13-acetate (PMA) activated Th1 cells, but it is also detectable in Th2 and naïve T cells. Nevertheless, the targets and precise roles of Erm in the Th1/Th2 lymphocyte differentiation remain unclear [10].

PMA is known to promote the protein kinase C (PKC) pathways. PKC is a multigene family that encodes at least 11 distinct isoforms of serine/threonine kinases [11]. Specific isoforms play important roles in various transduction pathways that regulate cell growth, transformation and differentiation. This family is classified into three groups, based on their structure and co-factor requirement: 1. conventional PKCs (cPKCs) (α, β1, β2, and γ), which are activated by phorbol esters like diacylglycerol (DAG) or PMA, and calcium, 2. novel PKCs (δ, ε, η, θ, and μ), which are activated by DAG (or PMA) but not by calcium, and 3. novel PKCs (ζ, ι), which are insensitive to both phorbol esters and calcium. Each of these isoforms contains an amino-terminal regulatory domain and a carboxy-terminal catalytic kinase domain [11].

In the present study, we identified a cPKC pathway involved in the expression of the *erm* gene in lymphoblastic Molt4 cells.

2. Materials and methods

2.1. Materials and chemicals

Phorbol 12-myristate 13-acetate (PMA), Bis-indolylmaleimide III (BimIII), Apigenin, Staurosporine, Bryostatin 1, Thymeleatoxin, Cycloheximide and Emodin were purchased from Sigma and BAPTA/AM, Actinomycin D and H89 from Calbiochem.

2.2. Cell lines

Lymphoblastic Molt4 and SupT1 cell lines were purchased from ATCC, and maintained at 37 °C in a humidified atmosphere of 5% CO_2. The cell culture medium, RPMI-1640 medium (Gibco), was supplemented with 10% fetal bovine serum (Gibco) and non-essential amino-acids (Gibco).

*Corresponding author. Fax: +322 555 62 57.
E-mail address: ylaunoit@ulb.ac.be (Y. de Launoit).

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2.3. RNA isolation and RT-PCR

Total RNA from about 2×10^6 cells was purified by the TriPure Isolation Reagent (Roche). 1.5 μg of total RNA was reverse-transcribed with random hexamers by the use of SuperScript II reverse transcriptase (Invitrogen). PCRs were performed using the Taq DNA polymerase (Promega) with the following specific primers for amplification of the *erm* gene: 5′-CATGGACGGGTTTTATGATCAGCA-3′ and 5′-GGCACAATAGTTGTAGAGGC-3′. Briefly, 200 ng of cDNAs was submitted to 28 cycles of PCR: denaturation for 45 s at 95 °C, annealing for 45 s at 57 °C, and elongation for 45 s at 72 °C. The reaction was made with a final volume of 50 μl containing 3 mM MgCl2, 0.25 mM of each dNTP and 30 pmoles of each primer. Expression of the *actin* gene was used as control and was also studied by means of the same RT-PCR procedure using 28 PCR cycles with the following specific primers: 5′-GGGTCAGAATTCCTATG-3′ and 5′-GGTCTCAAACATGATCTGGG-3′. *Actin* control RT-PCRs were performed on the same RNA products than the *erm* RT-PCRs. PCR products were analyzed by electrophoresis in a 2% agarose gel.

2.4. Western blot

Polyclonal anti-Erm antiserum was obtained by immunizing mice with purified recombinant human Erm protein overexpressed in the bacculovirus system. Mouse anti-β-actin antibody was purchased from Amersham. Cellular proteins were separated for 1 h on gradient 10% SDS–PAGE at 20 mA and electroblotted onto nitrocellulose filters for 60 min at 100 V. The blots were treated with washing solution (5% fat-free milk, 0.1% Tween 20 in phosphate-buffered saline) and incubated overnight at 4 °C, with a 1:2500 dilution of mouse anti-Erm polyclonal antibody, or 1:5000 dilution of mouse anti-β-actin antibody. The blots were washed and incubated for 1 h at room temperature, in a 1:10000 dilution of anti-mouse immunoglobulin G coupled to horseradish peroxidase (Amersham). After three washes, detection was done by enhanced chemiluminescence (Rennaissance^R, NEN) according to the manufacturer's instructions.

2.5. Transient transfections and luciferase assays

The *erm* promoter fragments were amplified by PCR (GC-rich PCR kit, Roche) on human U2Os cell genomic DNA using the following upstream oligonucleotides: "erm$_{-420}$" 5′-GGTACGCGTTCGGGAGAGCGGCGAGAAGGGAAGTAACGG-3′ (pERM-0.5-Luc), "erm$_{-115}$" 5′-AAAACGCGTCCTCTGGCCAATGGGAACAAG-3′ (pERM-0.2-Luc) or "erm$_{+6}$" 5′-AAAACGCGTCAGCCGCTGGTGCGCGGAGCG-3′ (pERM-0.1-Luc), and the downstream oligonucleotide "erm$_{+95}$" 5′-GGCCTCGAGACGCACGCGCGCAGCG-GGCCTGGGCGCCTG-3′. PCR products were cloned into the pGL3basic vector (Promega) using the *Mlu*I and *Xho*I restriction sites present in the PCR primers, see schematic representation of these vectors in Fig. 4A. The constructs were then sequenced as described [12].

Molt4 and SupT1 cells were transiently transfected by the DEAE-Dextran method as previously described [13]. To evaluate the transcriptional activity, cells were harvested and luciferase activity was determined using the Luciferase Assay System (Promega). Results were normalized with respect to the concentration in protein obtained by means of the Bradford method (Bradford DC method, Biorad).

3. Results

3.1. PMA treatment upregulates erm mRNA in Molt4 cells

We examined the level of *erm* mRNA in human lymphoblastic Molt4 and SupT1 cells by means of the RT-PCR method. Expression of *actin* mRNA was provided as an internal control. The two cell lines were treated with various doses of PMA, a potent PKC activator. The level of *erm* expression is very low in Molt-4 non-treated cells, when compared to its level in SupT1 non-treated cells. In Molt4 cells treated for 6 h, PMA upregulated the expression of *erm* mRNA in a dose-dependent manner and the maximal effect was obtained from the concentration of 2 ng/ml PMA. In contrast, no effect was observed in SupT1 cells (Fig. 1A). In Molt4 cells, this PMA-induced *erm* upregulation occurred as early as 2 h after PMA addition and the maximal effect was detected from 4 to 6 h after (Fig. 1B). Again, 2–48 h of PMA treatment had no effect on *erm* expression in SupT1 cells. These results strongly suggest the involvement of a PKC pathway in the transcriptional regulation of the *erm* gene in Molt4 cells.

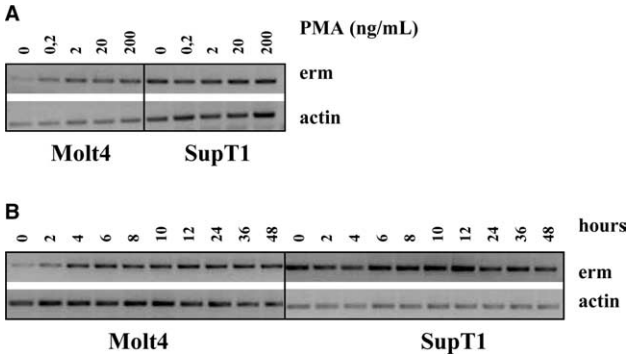

Fig. 1. PMA upregulates Erm mRNA expression in Molt4 cells. (A) Dose-dependent regulation of Erm. Lymphoblastic Molt4 or SupT1 cells (2×10^6) were treated for 6 h with the indicated concentrations of PMA. Total cellular mRNAs were extracted and RT-PCR analysis was performed to examine the expression of *erm*. The actin mRNA expression level was used as an internal experimentation control. (B) Time-dependent regulation of Erm. Cells were treated with 20 ng/ml PMA for the indicated times. RT-PCR analyses were performed as in (A).

3.2. Erm mRNA is specifically upregulated through a conventional PKC pathway

To test whether the upregulation of *erm* mRNA by PMA involves the activation of a PKC signaling pathway, expression of *erm* mRNA was analyzed in Molt4 cells treated with PMA and specific inhibitors of different pathways. We used three different PKC inhibitors – BimIII, Staurosporine and Apigenin, as well as the PKA inhibitor H89, and the Casein Kinase II (CK2) inhibitor Emodin. All three PKC inhibitors (BimIII, Staurosporine and Apigenin) blocked PMA-induced *erm* mRNA expression in the Molt4 cells. PKA and CK2 inhibitors did not affect the level of *erm* messenger. The inhibitors tested did not have any apparent effect in the SupT1 cells (data not shown). In order to more precisely identify the PKC involved in *erm* regulation, Molt4 cells were treated with the PKC pathway activators Bryostatin 1 (Bryo) and Thymeleatoxin (Ttox). These latter also activated *erm* mRNA expression in Molt4 cells. We evaluated by densitometry analysis on agarose gels of RT-PCR products that the levels of PMA-, Bryo- and Ttox-induced Erm expression are, respectively, 2.4-, 2.3- and 2.8-fold higher than that of the control one. As observed for PMA, the PKC inhibitor BimIII inhibited the Bryo- and the Ttox-induced effect. The level of Erm expression in cells treated with BimIII in the presence or the absence of one of the three PKC activators varied between 10% and 50% under the level obtained on untreated cells (Fig. 2B). The fact that Bryo and Ttox are able to activate *erm* expression suggests that the classical PKC pathway (α, β1, β2 and γ isoforms) is involved in this regulation. More particularly, Ttox is specific of α, β1 and γ isoforms [14]. In order to confirm the involvement of the cPKC sub-family in *erm* expression, we also tested the BAPTA/AM, which is an intracellular Ca^{2+} chelator. In fact, only the cPKCs require Ca^{2+} [15]. We

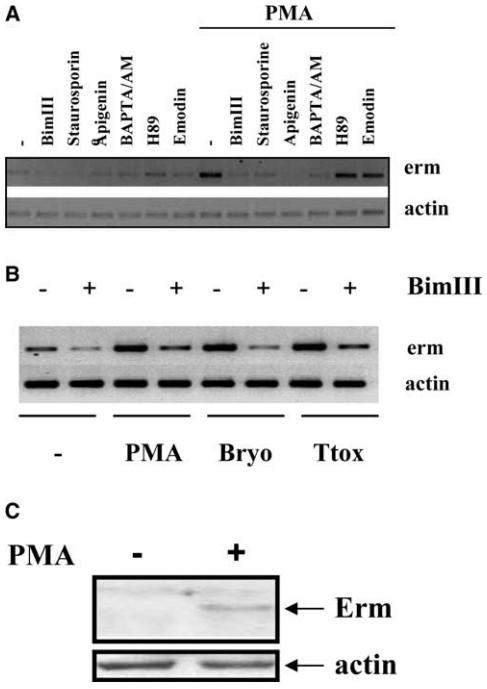

Fig. 2. Erm mRNA expression is dependent on the PKC pathway in Molt4 cells. (A) Effect of PKC inhibitors. 2×10^6 Molt4 cells were pre-treated for 1 h with the PKC inhibitors Bis-indolylmaleimide III (BimIII) (10 μM), Staurosporine (250 nM) and Apigenin (50 μM), or with the intracellular Ca^{2+} chelator BAPTA/AM (25 μM), PKA inhibitor H89 (25 μM), and CK2 inhibitor Emodin (100 μM). 0 or 20 ng/ml PMA was then added for 4 h. (B) Effect of the PKC pathway activators. Molt4 cells were pre-treated with or without the PKC inhibitor BimIII (10 μM) for 1 h, and then treated with PMA (20 ng/ml), Bryostatin 1 (Bryo: 100 nM) or Thymeleatoxin (Ttox: 100 nM) alone or in combination with BimIII, for 4 h. Erm and actin mRNAs were determined by RT-PCR analysis as described in Fig. 1. (C) Western Blot analysis. Fifty μg of total proteins from untreated and PMA-treated Molt4 cells was separated on SDS–PAGE, and the blot was incubated with either the mouse anti-Erm polyclonal antibody, or the mouse anti-β-actin antibody, followed by an anti-mouse immunoglobulin G (Amersham).

showed that *erm* mRNA upregulation depends on intracellular Ca^{2+} accessibilities, since BAPTA/AM can reverse PMA-induced *erm* expression in Molt4 cells (Fig. 2A). The PKC signaling pathway involved is thus Ca^{2+}-dependent and PMA-responsive, thus fitting with the conventional sub-family of PKC properties. Western blot analysis with an anti-Erm antibody showed that Erm protein is, as *erm* mRNA, upregulated by the PKC pathway, whereas β-actin protein is not affected by this treatment (Fig. 2C).

3.3. Erm expression is regulated by PMA at the transcriptional level

To assess whether RNA synthesis is required for the PMA-induced upregulation of *erm* mRNA, the lymphoblastic Molt4 cell line was co-treated with PMA and Actinomycin D, a DNA-primed RNA polymerase inhibitor. As shown in Fig. 3A, the *erm* mRNA upregulation by PMA was inhibited by Actinomycin D, even at concentrations as low as 1 μg/ml, suggesting the involvement of a transcriptional mechanism in the regulation process. To test whether a protein translation step is necessary for *erm* upregulation by PMA, Molt4 cells were co-treated with PMA and Cycloheximide – a protein translation inhibitor – and

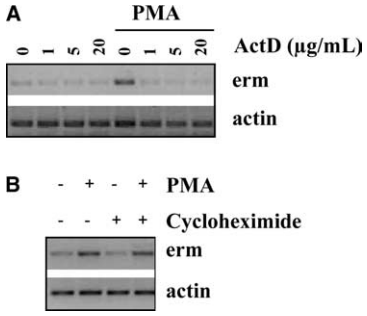

Fig. 3. PMA regulates Erm at the transcriptional level in Molt4 cells. (A) Actinomycin D treatment. Cells were treated with the indicated doses of Actinomycin D (ActD) for 30 min and then with or without PMA (20 ng/ml) for 4 h. (B) Cycloheximide treatment. Cells were treated for 30 min with or without 20 μg/ml of Cycloheximide alone and then for 4 h with or without 20 ng/ml of PMA. Erm and actin mRNAs were determined by RT-PCR analysis as described in Fig. 1.

the latter compound had no effect on PMA-induced *erm* regulation (Fig. 3B). We can thus conclude that protein translation is not required for PMA-induced *erm* mRNA. In SupT1 cells, neither Actinomycin D nor cycloheximide treatment influenced *erm* mRNA expression (data not shown).

3.4. Identification of the PMA-dependent erm promoter region

To more precisely identify the mechanism involved in the PMA-induced *erm* transcription, we analyzed the proximal promoter region of the human *erm* gene [16]. Two non-coding alternatively spliced exons (1a and 1b) were previously identified upstream of the ATG-containing exon, but exon 1a appeared to be used in most of the cases [16]. Thus, two promoter constructs upstream of exon 1a (0.5 kbp: −420 to +95, 0.2 kbp: −115 to +95) and one region, taken as control, in exon 1a (0.1 kbp: +6 to +95) were cloned into the pGL3basic vector. The pGL3basic, pERM-0.5-Luc, pERM-0.2-Luc and pERM-0.1-Luc plasmids were transiently transfected into Molt4 and SupT1 for 24 h. As expected, the pERM-0.1-Luc plasmid displayed a rather null transcriptional activity. In contrast, both pERM-0.5-Luc and pERM-0.2-Luc were transcriptionally active in Molt4 and SupT1 cells (Fig. 4A). The cells were then treated for 4 h with or without PMA. In Molt4 cells, although the pGL3basic, the pERM-0.2-Luc and the pERM-0.1-Luc reporter plasmids were not regulated by PMA, the pERM-0.5-Luc plasmid was 2.5-fold induced following PMA treatment. These data suggest the presence of a responsive element for a cPKC targeted transcription factor within the −420 to −115 *erm* promoter region. As illustrated in Fig. 4C, the PMA-induced effect on the transcriptional activity of the pERM-0.5-Luc reporter plasmid is also observed in Molt4 cells with the cPKC pathway activator Ttox; whereas on the pERM-0.2-Luc reporter plasmid this latter compound had no effect.

4. Discussion

In the present study, we have analyzed the transcriptional mechanisms involved in specific upregulation of the Ets transcription factor Erm after PMA activation in cultured human lymphoblastic Molt4 cells. Although *erm* is weakly expressed in human naïve T cells [10] and peripheral blood lymphocytes

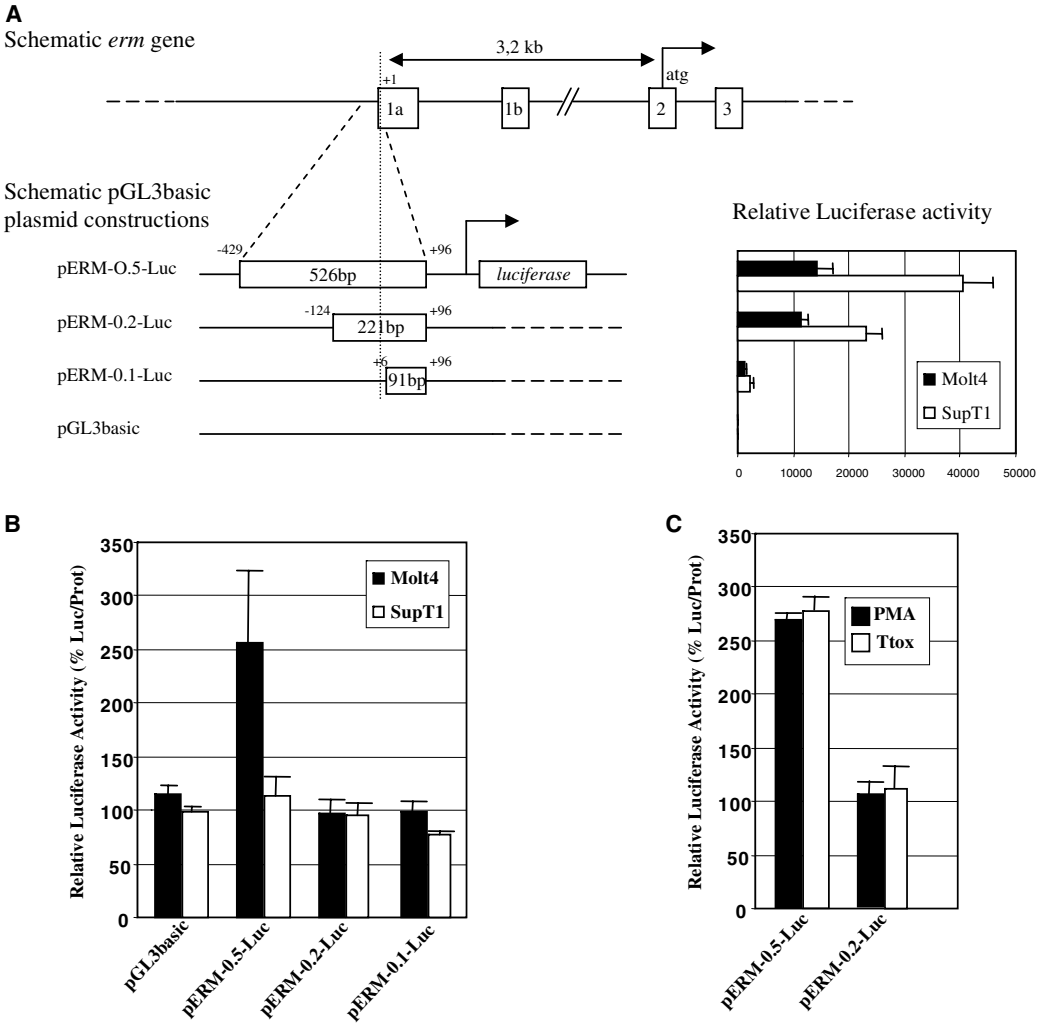

Fig. 4. The −500 bp promoter of the human Erm gene is regulated by the PKC pathway in Molt4 cells. (A) Schematic representation of the human *erm* promoter regions studied. These promoter regions were cloned upstream of the luciferase reporter gene into the pGL3basic plasmid. Molt4 or SupT1 cells (3×10^6) were transfected with 250 ng of pERM-0.5-Luc, pERM-0.2-Luc, or pERM-0.1-Luc. Twenty-four hours after transfection, cells were harvested for luciferase activity and protein measurements. Results for each construct were normalized using protein concentration and are expressed as luciferase activity. The data represent means ± S.E.M. of three independent experiments. (B) Molt4 or SupT1 cells were transfected with 250 ng of pERM-0.5-Luc, pERM-0.2-Luc, or pERM-0.1-Luc. Twenty-four hours after transfection, cells were incubated with or without PMA (20 ng/ml) for 4 h. The luciferase activities were obtained as in (A) and are presented relative to the corresponding promoter construct control without PMA (=100%). (C) Molt4 cells were transfected with 250 ng of pERM-0.5-Luc or pERM-0.2-Luc reporter plasmid. Twenty-four hours after transfection, cells were treated for 4 h with PMA (20 ng/ml) or Thymeleatoxin (Ttox: 100 nM). The luciferase activities were obtained as in (A); 100% corresponding to the non-treated cells.

[17], PMA/ionomycine-activated Th1 cells overexpress it [10]. Moreover, during murine Th1/Th2 commitment, the transcription factors Erm and T-bet are selectively expressed in Th1 cells [9]. We could thus hypothesize that this transcription factor plays a key role in normal T cell differentiation. In leukemia, as indicated by our previous data, Erm is expressed in the following cell lines: the CEM, Molt4 and Jurkat lymphoblastic cells, the HEL erythroleukemia cells, the U937 promonocytic cells and the Dami megakaryocytic cells [17]. Although *erm* is expressed in both cell lines tested in the present study, i.e., SupT1 and Molt4, we showed that *erm* is transcriptionally upregulated by phorbol esters in the latter only. Absence of *erm*-transcriptional regulation by PMA in SupT1 cells is probably due to the relatively high level of *erm* transcript in these cells, when compared to that in Molt4 cells. Since the phorbol ester PMA acts through the PKC pathway [18,19], and using specific inhibitors such as emodin and H89, we were able to exclude the involvement of the CK2 and PKA pathways, respectively, in the PMA-induced *erm* expression in Molt4 cells. The data obtained on *erm* expression, with either the specific intracellular Ca^{2+} chelator BAPTA/AM [15] or the specific PKC activators Bryostatin 1 and Thymeleatoxin [14], permit us to more precisely determine which of the 11 PKCs are involved in this process, i.e., the α, β1 and γ isoforms.

Other *ets* genes are also transcriptionally regulated by the PKC pathway. This is the case for *ese*-1 and *ese*-3, which are transcriptionally upregulated by PMA in bronchial epithelial cells. Interestingly, in these cells, PMA induced *SPRR1B* gene expression by upregulating these two Ets transcription factors which directly bind to the *SPRR1B* promoter [20]. Moreover, in vascular smooth muscle cells, PMA induced a dose-

dependent *ets*-1 mRNA expression [21]. Many other genes are also transcriptionally regulated by PMA. For example, treatment of mouse primary keratinocyte cultures with PMA reduced DNA synthesis and sequentially induced an upregulation of *p21Cip1/WAF1*, *hairless* and *involucrin* mRNAs in a time-dependent fashion [22]. Moreover, PMA induces *MMP*-9 expression via NF-κB activation, and mutation of its promoter at the NF-κB site completely abolishes PMA-induced transcriptional activity [23].

In this paper, we provided evidence that the *erm* gene is regulated by the cPKC pathway in Molt4 cells at the transcriptional level. In fact, PMA-induced *erm* mRNA expression is blocked by Actinomycin D. This result led us to identify more precisely the promoter region involved in PMA-induced *erm* regulation. We took advantage of the previous work mapping the human *erm* gene and its regulatory regions [16]. We focused our interest on the region upstream of the first exon 1A and identified a functional cPKC sensitive region between positions −420 and −115. Interestingly, putative binding sites for AP1, MEF2, NFY, CAAT, CREB, Myb and EKLF transcription factors were identified in this region. For example, the AP1 binding site at position −357 could be a target of this pathway. In fact, the AP-1 family of proteins has previously been shown to be involved in PMA-gene regulation. In the myeloid leukemic U937 cell line, the expression of hMSH2 MMR protein is regulated by PKC activity [24]. In NK cells, transcription of the *2B4 (CD244)* gene is regulated by PMA and mutation of the AP1 binding site induces a loss of PMA induction [25]. However, our preliminary results indicated that mutation of the putative AP1 site of the human *erm* gene did not affect PMA-regulation (data not shown). In K562 hematopoietic cells, transcriptional regulation by PMA of the human GM3 synthase (*hST3 Gal V*) gene is dependent of the CREB responsive site within the promoter [26]. Similarly, in Jurkat cells, expression of the human *MAT2A* gene is also induced by PMA through the transcription factor c-Myb [27]. Identification of the transcription factors that are responsible for PKC-induced Erm transcription in Molt-4 cells would be of help to explain the absence of response in PMA-treated SupT1 cells. We thus focus our interest on these two latter different sites; and subsequently the identification of the targeted genes after PMA-activation in Molt-4 cells would be an important step in the understanding of the function of this transcription factor.

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