Ets2 and protein kinase C ϵ are important regulators of parathyroid hormone-related protein expression in MCF-7 breast cancer cells

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Parathyroid hormone-related protein (PTHrP) promotes the metastatic potential and proliferation of breast cancer cells, and acts anti-apoptotically. In invasive MDA-MB-231 breast cancer cells, transforming growth factor β -regulated PTHrP synthesis is mediated by an Ets1/Smad3-dependent activation of the PTHrP P3 promoter. In the present study, we studied the regulation of PTHrP expression in non-invasive, Ets1-deficient and transforming growth factor β -resistant MCF-7 cells. We found PMA to be a strong stimulator of P3-dependent PTHrP expression in MCF-7 cells. Mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase (ERK) kinase 1 (MEK-1)/ERK1/2 inhibitor PD98059 interfered with this activity. Promoter studies revealed that the PMA effect depended on the Ets and stimulating protein-1 (Sp1)-binding sites. Of several Ets factors tested, Ets2, but not Ets-1, Elf-1 or Ets1, supported the PMA-dependent increase in promoter activity. PD98059 and a threonine to alanine mutation of the ERK1/2-responsive Ets2

phosphorylation site at position 72 inhibited the Ets2/PMA effect. Activated protein kinase C (PKC) ϵ could mimic PMA by stimulating the P3 promoter alone or in co-operation with Ets2 in an MEK-1/ERK1/2-dependent manner. Activated PKC α , although capable of co-operating with Ets2, failed to induce transcription from the P3 promoter on its own. The Ets2/PKC α synergistic effect was neither sensitive to PD98059 nor to Thr⁷²/Ala⁷² mutation. PMA neither increased the expression of Sp1 nor modulated the transcriptional activity of Sp1. However, it induced the displacement of a yet unknown factor from the Sp1-binding site, which may result in Sp1 recruitment to the promoter. Our results suggest an ERK1/2-dependent Ets2/PKC ϵ synergism to be involved in PTHrP expression in MCF-7 breast cancer cells.

Key words: Ets protein, protein kinase C, parathyroid hormone-related protein, phorbol ester, mitogen-activated protein kinase, stimulating protein-1.

INTRODUCTION

Parathyroid hormone (PTH)-related protein (PTHrP) has been discovered as a tumour-derived humoral factor that causes hypercalcaemia of malignancy [1,2]. Sharing with PTH the ability to bind to the PTH/PTHrP receptor in bone and kidney [3], PTHrP stimulates osteoclastic bone absorption and calcium resorption respectively. PTHrP can be cleaved into three functional fragments, the N-terminal PTH-like domain, the anti-apoptotic mid-regional domain and the C-terminal fragment or osteostatin that inhibits bone absorption [4,5]. PTHrP is expressed by a variety of tumours [6–8]. Increased PTHrP expression correlates with a more advanced tumour state and with poor prognosis [9,10]. The majority of breast tumours and most of the bony breast cancer metastases produce PTHrP [6,7,11–13]. Metastatic breast cancer cells in the bone seem to profit from the osteolytic activity of PTHrP [14]. Depending on its mid-region domain, PTHrP has also been shown to fulfil a mitogenic and anti-apoptotic function in non-invasive MCF-7 breast cancer cells [15,16]. Human PTHrP expression is regulated predominantly on the transcriptional level by three different promoters (P1–P3). Of these, the P3 promoter (formerly called P2 promoter) has been shown to be active in all breast cancer cells [17]. Within this promoter, three regulatory DNA elements, an Ets, a stimulating protein-1 (Sp1) and a Smad3/4-binding site, have

been identified [18–20], which contribute to the regulation of the promoter by transforming growth factor β (TGF β), human T-cell lymphotropic virus I-transactivator of the X region (HTLV-I-Tax), retinoic acid and adenovirus early region 1A protein [20–23].

Ets transcription factors share a unique DNA-binding domain (DBD), the Ets domain, which recognizes specifically a 5'-GGAA/T-3'-based DNA sequence [24–27]. In humans, more than 20 Ets proteins have been identified. Ets1 and Ets2 are proto-oncoproteins [28,29] that are overexpressed in a variety of tumours, including breast carcinomas [30,31]. Ets2 expression has been correlated positively with mammary tumour size in mice [32]. Ets1 and Ets2 are targets of proto-oncogenic Ras that superactivate these proteins by inducing extracellular-signal-regulated kinase (ERK) 1/2-dependent phosphorylation of a specific N-terminal threonine residue [33]. In contrast with Ets2, Ets1 is expressed only in invasive cancer cells [20,30,31].

The family of protein kinase C (PKC) is subdivided into three classes, the conventional PKC isoenzymes (α , β I, β II and γ), the novel PKCs (δ , ϵ , η , μ and θ) and the subclass of atypical PKCs (ι and ζ) [34,35]. Conventional and novel PKCs can be activated by PMA which mimics 1,2-diacylglycerol, thereby inactivating the inhibitory regulatory domain of these PKCs. Invasive tumour cells often overexpress certain forms of PKCs [36], suggesting that PKCs are involved in tumorigenesis. In support of this notion,

Abbreviations used: ChIP, chromatin immunoprecipitation; DBD, DNA-binding domain; EMSA, electrophoretic mobility-shift assay; Erg1/2, Ets-related gene 1/2; ERK, extracellular-signal-regulated kinase; HTLV-I-Tax, human T-cell lymphotropic virus I-transactivator of the X region; MAPK, mitogen-activated protein kinase; MEK-1, MAPK/ERK kinase 1; PKC, protein kinase C; PTH, parathyroid hormone; PTHrP, PTH-related protein; RT, reverse transcriptase; Sp1, stimulating protein-1; TAD, transactivating domain; TGF β , transforming growth factor β .

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PKC α has been found to stimulate invasive behaviour of MCF-7 breast cancer cells [37], whereas PKC ϵ has been shown to inhibit apoptosis of these cells [38].

Given the importance of PTHrP for breast cancer development, we decided to analyse the regulation of PTHrP expression in breast cancer cells. We have recently shown that TGF β stimulates PTHrP expression in invasive MDA-MB-231 cells by inducing an Ets1/Smad3 co-operative effect on the PTHrP P3 promoter [20]. In the present study, we examined the mechanism of PTHrP regulation in non-invasive MCF-7 cells. We show that PTHrP expression can be stimulated by PMA, involving a mechanism that is sensitive to the PD98059 inhibitor of the mitogen-activated protein kinase (MAPK)-signalling pathway. We provide evidence that PMA primarily targets the PTHrP P3 promoter by increasing its transcriptional activity by activating Ets2 and PKC ϵ .

EXPERIMENTAL

Cell line, plasmids and inhibitors

MDA-MB-231 and MCF-7 breast cancer cell lines were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Karlsruhe, Germany), supplemented with 10 % (v/v) foetal calf serum (PAA, Laboratories, Wiener Strasse, Austria) in the absence of antibiotics. For PTHrP P3 promoter analysis by transient transfection assay, a $-328/+20$ promoter fragment cloned upstream of a luciferase gene was used [20]. Ets-binding site, CCCACC element and AGAC box mutants of the P3 promoter were as described previously [18–20]. pcDNA3-based expression plasmids were used for the expression of Ets1, Ets2, Elf-1 and Ets-1 [20]. pCIN4-based expression plasmids, E2full, E2DBD or E2TAD [39], were introduced into cells to express Flag-Ets2, Flag-Ets2-DBD or Flag-Ets2-TAD respectively. Plasmids pHANE-PKC- α -CAT and pHANE-PKC- ϵ -CAT for the expression of PKC α -CAT and PKC ϵ -CAT respectively, were kindly provided by Jae-Won Soh (Herbert Irving Comprehensive Cancer Center, Columbia University, New York, NY, U.S.A.). Gal-Sp1 and Gal-DBD plasmids were gifts from Gunnar Westin (Department of Medical Center, Biomedical Center, University of Uppsala, Uppsala, Sweden). Actinomycin D and PD98059 (Calbiochem, Bad Soden, Germany) were used at final concentrations of 1 and 5 μ g/ml or 25 μ M respectively. Control cells were incubated with the equivalent concentration of DMSO.

Transient transfection and luciferase assay

PTHrP P3 promoter assays by transient transfections using lipofectAMINETM reagent (Invitrogen) were performed as described previously [20], except that 2 μ g of reporter plasmid and 0.7 μ g of expression plasmid were used. Cells were incubated with 10 nM PMA or mock (DMSO)-treated for 7 h, and lysed for the measurement of luciferase activity, 16 h after transfection.

Nuclear extraction and Western-blot analysis

Preparation of nuclear extracts and Western-blot analysis were performed as described previously [40]. Polyclonal antibodies anti-Ets1 (C-20), anti-Elf1 (C-20), anti-Erg1/2 (Ets-related gene 1/2; C-20), anti-PKC α (C-20), anti-PKC ϵ (C-15), anti-Sp1 (PEP2) and anti-Sp3 (D-20) were purchased from Santa Cruz Biotechnology. Each antibody, except anti-Ets1 (1:5000) and anti-Elf1 (1:4000), was diluted to 1:2000 before use. Monoclonal antibodies anti-Flag M2 (Upstate Biotechnology, Virginia, VA,

U.S.A.) and anti-HA (12CA5; Roche, Mannheim, Germany) were diluted to 1:1000 or 1:1500 respectively.

Electrophoretic mobility-shift assay (EMSA)

EMSAs were performed essentially as described previously [20]. For the detection of factors binding to Ets-binding site or the CCCACC element of the PTHrP P3 promoter, the following oligonucleotide ($-79/+49$ WT) was radiolabelled by fill-in reaction using Klenow enzyme (New England Biolabs, Schwalbach, Germany): 5'-CAACTTTCCGGAAGCAACCAGC-3' (sense strand) and 5'-CTCTGGTGGGCTGGTTGCTTCCGGAAAG-TTG-3' (antisense strand). The $-79/+49$ Sp1M competitor oligonucleotide contained a triple mutation (indicated by lower-case letters) in the CCCACC motif: 5'-CAACTTTCCGGAAG-CAACCAGgCtAgCAGAG-3' (sense strand). The $-51/+28$ WT and $-51/+28$ M PTHrP-specific oligonucleotides were as described previously [20]. For supershift experiments, 0.5 μ l of anti-Elf1 or anti-Erg1/2, or 0.1 μ l of anti-Ets1 antibody was added to the reaction mixture.

Reverse transcriptase (RT)-PCR

RNA isolation, conventional and real-time RT-PCR were performed as described by Lindemann et al. [20]. PTHrP-specific primers were described in [20]. Primers for human Ets2-specific RNA amplification by real-time PCR were as follows: 5'-TTTCTCATGACTCCGCCAACT-3' (forward) and 5'-GGCTTGACTCATCACAGCCTT-3' (reverse).

Chromatin immunoprecipitation (ChIP) assay

MCF-7 cells from four confluent cultures (225 cm² flask) were trypsinized and transfected with a total of 80 μ g of either Flag-Ets2-expressing plasmid E2full or with the control vector pcDNA3 by electroporation. After 3 h of incubation in fresh medium allowing cells to re-attach to a 225 cm² flask, the supernatant was removed and PMA-containing medium was added. After incubation for another 3 h, cells were washed with 20 ml of PBS and then treated with 20 ml of PBS containing 1 % (v/v) formaldehyde for 10 min at room temperature (22 °C). The reaction was stopped by incubating the cells with 20 ml of 0.125 M glycine/PBS solution for 15 min at room temperature. Each cell layer was washed three times with 20 ml of PBS and then treated with 2 ml of trypsin solution for 15 min at room temperature. After the addition of 8 ml of medium, supplemented with 10 % foetal calf serum to inactivate trypsin, cells were scraped off and collected in 50 ml tubes by centrifugation at 150 g for 5 min at room temperature. Cells were resuspended in 1 ml of PBS and transferred on to a microtube. After centrifugation at maximum speed in a microfuge, cells were lysed in lysis buffer [50 mM Hepes (pH 7.9)/1 mM EDTA/140 mM NaCl/10 % (v/v) glycerol/0.5 % Nonidet P40/0.25 % Triton X-100]. The subsequent steps were performed at 4 °C. Cells were rotated for 10 min, centrifuged at 2500 g for 10 min and resuspended in high-salt buffer [20 mM Tris/HCl (pH 8.1)/2 mM EDTA/0.5 M NaCl/0.1 % SDS/1 % (v/v) Triton X-100] containing a mixture of protease inhibitors (Roche), and incubated for 10 min. Following another centrifugation step, cells were suspended in 700 μ l of sonication buffer [10 mM Tris/HCl (pH 8.0)/1 mM EDTA/0.5 mM EGTA] and sonicated 20 times for 15 s each, by using a Branson Sonifier 250 (duty cycle, 50 %; output control, 3.5). Between subsequent sonication steps, cells were kept on ice for 15 s. The homogenate was cleared by

centrifugation at 16 000 *g* for 10 min. A portion of the solution (50 μ l) was removed and incubated at 65 °C overnight to be used as input control. The rest was diluted with ChIP dilution buffer [17 mM Tris/HCl (pH 8.1)/1.2 mM EDTA/170 mM NaCl/0.01 % SDS/1.1 % Triton X-100] containing protease inhibitors to a final volume of 2 ml. Pretreated Sepharose G (Amersham Biosciences, Buckinghamshires, U.K.) slurry (80 μ l) was then added. Pretreatment of Sepharose G was performed as follows. Sepharose G (750 μ l) was washed four times with TE buffer [10 mM Tris/HCl (pH 8.0)/1 mM EDTA]. Each washing step was followed by centrifugation at 50 *g* for 1 min in a microfuge. Sepharose G was then resuspended in 1.5 ml of TE buffer containing 1.5 % (w/v) BSA (fraction V; Applichem, Germany) and 600 μ g of sheared salmon sperm DNA (Invitrogen), and incubated under rotation for 1 h. The Sepharose G/cell extract mixture was then rotated for 30 min and centrifuged at 50 *g* for 1 min. The supernatant was divided into 500 μ l aliquots. After addition of 2 μ g of anti-Flag or anti-HA antibody, or no antibody, aliquots were incubated overnight under rotation. Pretreated Sepharose slurry (60 μ l) was added and the mixture incubated for another 60 min. After centrifugation for 1 min at 50 *g*, the Sepharose beads were washed once with low-salt buffer [20 mM Tris/HCl (pH 8.1)/2 mM EDTA/150 mM NaCl/0.1 % SDS/1 % Triton X-100], once with high-salt buffer, once with LiCl buffer [10 mM Tris/HCl (pH 8.1)/1 mM EDTA/0.25 M LiCl/1 % (v/v) Nonidet P40] and twice with TE buffer. Each washing step was performed for 5 min under rotation. Chromatin was eluted from the beads in two subsequent elution steps. In each step, the beads were resuspended with 250 μ l of elution buffer [1 % (w/v) SDS/0.1 M NaHCO₃] containing protease inhibitors, vortex-mixed and incubated for 15 min at room temperature under rotation. The resulting 500 μ l of immunoprecipitated chromatin solution was mixed with 20 μ l of 5 M NaCl and incubated overnight at 65 °C. After the addition of 10 μ l of 0.5 M EDTA, 20 μ l of Tris/HCl (pH 6.5) and 1.3 μ l of Proteinase K (15 mg/ml; Roche), the mixture was incubated at 45 °C for 1 h. Proteins were removed by phenol-chloroform extraction and DNA recovered by ethanol precipitation in the presence of glycogen. DNA pellets were dissolved in 10 μ l of water. For conventional PCR, 0.1 μ l of DNA solution was mixed with 2.5 μ l of JumpStart RedTaq polymerase (Sigma), 5 μ l of 10 $\times$ PCR buffer (Sigma), 1 μ l of 10 mM dNTPs and 1 μ g of each primer (forward, 5'-TGCCTCGAGCGTGTGAACA-3'; reverse, 5'-TCCCATAGCAATGTCTAATTAATCTGG) in a final volume of 50 μ l. After 45 cycles, PCRs were subjected to DNA electrophoresis in a 6 % (w/v) polyacrylamide gel and the DNA was visualized by ethidium bromide staining.

RESULTS

MCF-7 cells regulate PTHrP expression primarily through the P3 promoter

First, we examined the importance of the PTHrP P3 promoter for PTHrP gene regulation in MCF-7 cells. Conventional RT-PCR, using primers designed specifically to amplify the PTHrP P3 transcript (Figure 1A), revealed that the P3 promoter was involved in maintaining basal PTHrP-RNA levels in MCF-7 (Figure 1B). Next, we attempted to determine the contribution of P3-dependent transcription to PTHrP expression under stimulating conditions. Since, in contrast with MDA-MB-231 cells, MCF-7 cells are not TGF β -responsive [20], we had to search for an alternative stimulator of PTHrP expression. By using quantitative real-

Figure 1 PMA up-regulates promoter P3-specific PTHrP expression in MCF-7 cells in a PD98059-sensitive manner

(A) Organization of the human PTHrP gene. Boxes represent exons. Shaded areas indicate the translated sequences. Splicing events are denoted below the map. Arrows show the positions of the primers used for conventional RT-PCR (black arrows) or real-time quantitative RT-PCR (grey arrows). Primer pairs P1/2, P3 or A were used to detect promoter P1/2-specific, P3-specific or all PTHrP transcripts respectively. (B) Detection of the P3 transcript and all PTHrP transcripts in MCF-7- and MDA-MB-231-specific RNA by conventional RT-PCR. (C, D) Real-time RT-PCR of RNA isolated from MCF-7 or MDA-MB-231 cells. (C) Time course of the PMA effect on the level of the P3 and P1/2 transcripts, and on the expression of all PTHrP-RNA species in MCF-7 or MDA-MB-231 cells. (D) Inhibitory effect of 1 or 5 μ g/ml actinomycin D (Act D) and 25 μ M PD98059 on the PMA-induced P3-dependent PTHrP-RNA expression in MCF-7 cells. Cells were incubated with PMA for 4 h before harvest.

time RT-PCR, we found that the PKC activator PMA could markedly up-regulate the overall level of the PTHrP-RNA by 25-fold, whereas it had no effect on PTHrP expression in MDA-MB-231 cells (Figure 1C). PMA up-regulated the level of the P3-specific transcript by up to 38-fold, whereas it increased the combined level of the RNA species derived from P1/P2-dependent transcription 17-fold (Figure 1C). We calculated that, on treatment with PMA, >50 % of the PTHrP-RNA species in MCF-7 cells were generated from the P3 promoter. In the

presence of actinomycin D, the effect of PMA on the overall and P3-dependent PTHrP expression was found to be reduced significantly (Figure 1D), suggesting that PMA increased the transcriptional activity of the P3 promoter. In the presence of the MAPK/ERK kinase (MEK)-1 inhibitor PD98059, PMA failed to induce PTHrP expression (Figure 1D), suggesting that, in MCF-7 cells, PMA mediates its effect on PTHrP, at least partially, by activating the Raf/MEK-1/ERK1/2 pathway [41]. We were able to demonstrate that PMA increased ERK1/2 phosphorylation and that this effect was inhibited by PD98059 (results not shown). Collectively, these results suggest that activation of PKC by PMA stimulates PTHrP expression in MCF-7 cells by a mechanism that involves transcriptional stimulation of the P3 promoter via activation of the Raf/MEK-1/ERK1/2 pathway.

PMA up-regulates the PTHrP P3 promoter in MCF-7 cells

By using transient transfection assays, we next tested a $-328/+20$ PTHrP P3 promoter fragment for its ability to respond to PMA in MCF-7 cells (Figures 2B, 3A, 3B, 4C, and 5A, 5C). Fold induction of this promoter by PMA depended on the batch of serum in which MCF-7 cells were grown. The serum batch that we initially used (Figures 2B, 3A, 3B and 5A) allowed PMA to stimulate moderately P3 promoter activity 2–4-fold. Using a different batch, PMA activated the promoter 7–8-fold (Figures 4C and 5C). The higher PMA response, as observed with the latter batch, could be attributed partly to a lower basal P3 activity. Similar to its effect on P3-dependent PTHrP expression, PD98059 completely inhibited stimulation of the P3 promoter by PMA, while only slightly affecting basal promoter activity (Figure 2B).

Next, we studied the effect of mutations in the Ets-binding site, the Sp1 site or in the Smad recognition element on P3 promoter activity in MCF-7 cells (Figure 2A). A single mutation in the GGAA core motif of the Ets-binding site significantly down-regulated basal and PMA-induced promoter activity (Figure 2B). When the Ets-binding site was mutated, addition of PD98059 did not reduce the promoter activity further, suggesting that the PD98059-sensitive PMA effect on the P3 promoter relied entirely on the Ets-binding site. A triple mutation in the Sp1-binding element, already shown [19] to abrogate Sp1 binding to this site, also decreased PMA-dependent promoter activity, but did not affect basal activity. However, complete replacement of the Sp1-binding motif by a Gal4 recognition element [21] decreased both PMA-dependent and basal P3 promoter activity. A mutation in the AGAC box, which eliminates Smad3/4 binding to this site [20], had a positive effect on basal promoter activity and activated the promoter more than 2-fold. In contrast, the same mutation had no effect on promoter activity in the presence of PMA. In MDA-MB-231 cells, PMA failed to activate the PTHrP P3 promoter (Figure 2B). This is consistent with the observation that these cells do not support PMA-dependent stimulation of PTHrP expression from the endogenous P3 promoter (Figure 1C). Collectively, these results show that PMA can activate transcription from the PTHrP P3 promoter in MCF-7 cells in a PD98059-dependent manner and that this effect requires the Ets-binding site and, to a lesser extent, also the Sp1 recognition motif to be intact. Basal P3 promoter activity in MCF-7 cells, even though dependent on the Ets-binding motif, did not rely on the Sp1 recognition element by itself, but on a sequence that overlaps with the Sp1-binding site.

Ets2 supports PMA-induced activation of the PTHrP P3 promoter

The importance of the Ets-binding site suggests that an Ets factor may be involved in mediating the PMA effect on the PTHrP P3

Figure 2 The Ets-binding site of the P3 promoter is required for PMA-dependent promoter activation

(A) Schematic drawing of the proximal PTHrP P3 promoter showing the Ets-binding site, the CCCACC element and the AGAC box. EM, SM or AM represent P3 mutant promoter harbouring mutations that prevent binding of Ets, Sp1 or Smad3/4 protein respectively to their corresponding site. Gal denotes a mutant PTHrP P3 promoter where the sequence between -63 and -47 , which includes the Sp1-binding site, was replaced by a Gal4 recognition site (CGGN₁CGG) [21]. (B) Transient transfection analysis of the EM, SM, Gal or the AM mutation on basal or PMA-induced P3 activity by using a $-328/+20$ P3 promoter–luciferase construct. Experiments that were performed in the presence of PD98059 are indicated by 'PD'. Relative promoter activity was calculated by dividing the activity of luciferase by that of galactosidase, generated by a co-transfected control plasmid. Error bars represent the average value of 2–4 independent experiments.

promoter. We tested the Ets proteins Ets1, Ets2, Elf-1 and Ets-1 for their ability to activate the P3 promoter in MCF-7 cells. Ets2, Elf-1 and Ets-1 proteins are expressed in MCF-7 cells (Figure 6A and results not shown). Ets1 was found not to be expressed in MCF-7 cells, even when these cells were treated with PMA (Figure 6A), a treatment known to induce Ets1 expression in vascular-smooth-muscle and endothelial cells [42,43]. Of the Ets proteins tested, Ets2 proved to be the most potent activator. Under non-stimulating conditions, Ets2 increased basal activity 4.5-fold, whereas in the presence of PMA, Ets2 activated the promoter 11-fold (Figure 3A). In contrast, Ets1 induced the P3 promoter only 2-fold in the absence and 4.5-fold in the presence of PMA. Similar to Ets2, Ets-1 significantly affected the promoter activity under basal conditions, but failed significantly to enhance further promoter activity in response to PMA. Elf-1 did not have any effect on the P3 promoter activity. These results suggest that Ets2 and Ets-1 may play a role in maintaining basal P3-dependent transcription in MCF-7 cells, whereas Ets2 may be involved in the regulation of P3 transcription by PMA.

To analyse further the Ets2-dependent regulation of the P3 promoter, we first used the different mutant versions of the P3 promoter depicted in Figure 2(A). Under non-stimulated conditions, the mutation in the Ets-binding site significantly decreased P3 promoter activity 2.2-fold, whereas mutations in the Smad- or Sp1-binding site had no effect (Figure 3A). In the presence of PMA, the effect of the Ets mutation on Ets2-dependent transcription was even more pronounced, leading to a 4.6-fold reduction in promoter activity. Under PMA conditions,

Figure 3 Ets2 supports PMA-dependent activation of the PTHrP promoter in MCF-7 cells

(A) Effect of various Ets factors on basal and PMA-dependent P3 promoter activity as measured by transient transfection assays using lipofectAMINETM. Wild-type (2 μ g) – 328/+ 20 P3 promoter–luciferase construct was transfected alone or together with 0.7 μ g of pCDNA3-based expression plasmid containing the cDNA of Ets1, Ets2, Elf-1 or Ese-1 [20] in the presence or absence of PMA. (B) Effect of P3 promoter mutations in the Ets, Sp1 and Smad3/4-binding site (promoter constructs EM, SM and AM respectively as shown in Figure 2A) on WT-Ets2-dependent activation of the PTHrP P3 promoter. Effect of two transdominant-negative versions of the Ets2 protein (DBD and TAD) [39] on basal and PMA-dependent P3 activity were also tested. DBD-Ets2 is a Ets2 protein fragment that contains the C-terminal part of the Ets2 protein from amino acids 332–468, which includes the DBD. TAD-Ets2 comprises the first 331 N-terminal amino acids of the Ets2 protein, which contain the TAD. Ets2 proteins used in the experiments presented in (B) were Flag-tagged.

the mutation in the Sp1 recognition sequence also significantly decreased P3 promoter activity. The mutation in the Smad-binding site had no effect. These results resemble those obtained with the different P3 mutant promoters in the absence of Ets2 in that PMA-induced P3 activation depends on the Ets- and Sp1-binding sites, whereas only the Ets-binding motif is required for basal promoter activity.

Next, we tested transdominant-negative versions of the Ets2 proteins, the N-terminal deletion mutant DBD-Ets2, containing amino acids 332–348 and the DBD or the C-terminal deletion mutant TAD-Ets2, harbouring amino acids 1–331 including the transactivating domain (TAD) [39], for their ability to interfere with PTHrP P3 promoter activity in MCF-7 cells. We found that both Ets2 mutants significantly reduced basal as well as PMA-induced transcription from the PTHrP P3 promoter (Figure 3B). This supports the notion that an Ets factor, such as Ets2, is important for PMA-dependent regulation of the P3 promoter.

Ets2 binds to the PTHrP P3 promoter *in vivo*

We next performed ChIP assay to study whether Ets2 binds to the PTHrP P3 promoter *in vivo*. We transfected MCF-7 cells with Flag-Ets2 and treated them with PMA. After chromatin isolation, we compared PTHrP P3-specific PCR product formation, resulting from precipitating chromatin with an anti-Flag antibody, with those that arose from treatment with a control antibody (anti-HA) or with no antibody. As shown in Figure 4(A), the P3-specific PCR product was only visible when the anti-Flag antibody was used for immunoprecipitation. This suggests that, under PMA conditions, Ets2 is binding to the endogenous PTHrP P3 promoter in MCF-7 cells.

PMA modulates Ets2 activity in MCF-7 cells

So far, our results suggest that PMA may mediate its effect on the PTHrP P3 promoter, at least partially, by increasing the transcriptional activity of Ets2. PMA could affect Ets2 activity in different ways. It could induce Ets2 expression, increase the activating potential of the Ets2 protein and/or recruit an Ets2-co-operating factor to the P3 promoter. By using real-time RT-PCR, we found that PMA increased the Ets2 RNA level in MCF-7 cells by up to 6-fold (Figure 4B). Maximum induction was observed 1 h after addition of PMA, which is 1 h before the peak level of PTHrP P3-specific transcript in response to PMA was reached (Figure 1C). To activate the Ets2 protein, PMA could trigger phosphorylation at Thr⁷² [33] by stimulating either the Raf/MEK-1/ERK1/2 or the phosphoinositide 3-kinase pathway [33,44]. As shown in Figures 1(D) and 2(B), we have already demonstrated that MEK-1 inhibitor PD98059 inhibits PMA-induced P3-dependent PTHrP expression and P3-dependent transcription. When we used this inhibitor in the presence of PMA and Ets2, we similarly found a dramatic effect on promoter activity (Figure 4C). Again, PD98059 failed to inhibit promoter activity when the Ets site was mutated. We next explored the possibility that the PD98059-sensitive action of PMA on the PTHrP P3 promoter involves phosphorylation of Ets2 at Thr⁷². To block phosphorylation of Ets2 at this site, we used a version of the Ets2 protein (A72-Ets2) that contains alanine at position 72 instead of threonine. This mutation did not affect the ability of Ets2 to activate the PTHrP P3 promoter in the absence of PMA, but resulted in approx. 2-fold lower activation of this promoter in the presence of PMA (Figure 4C). Since the expression level of A72-Ets2 was similar to wild-type Ets2 (Figure 4D), it can be concluded that blocking phosphorylation at Ets2-specific amino acid 72 affects the ability of Ets2 to support PMA-induced transcription from the P3 promoter. However, the effect of the threonine to alanine mutation on promoter activity was much less pronounced when compared with PD98059. This suggests that phosphorylation of Thr⁷² can only partially explain the PD98059-sensitive effect of PMA on the PTHrP P3 promoter.

A constitutively active form of PKC ϵ mimics PMA in its ability to activate the PTHrP P3 promoter in an ERK1/2-dependent manner

Of the many PKC subtypes, PKC α and PKC ϵ have been shown to activate the Raf/MEK-1/ERK1/2 pathway. Both PKC forms are expressed in MCF-7 cells [45]. To analyse the effect of these particular PKCs on P3 promoter activity, we used constitutively active forms of these kinases (PKC α -CAT and PKC ϵ -CAT). PKC α -CAT and PKC ϵ -CAT are truncated versions of PKC α and PKC ϵ respectively [41]. They contain the C-terminal catalytic domain, but are lacking the N-terminal regulatory domain. Therefore the activities of PKC α -CAT and PKC ϵ -CAT are independent of the presence of PKC activators, such as 1,2-diacylglycerol or PMA. When we tested PKC ϵ -CAT for its ability to modify the activity of the PTHrP P3 promoter, we found that PKC ϵ -CAT was able to activate the P3 promoter 9.3-fold (Figure 5A). Together with WT-Ets2, PKC ϵ -CAT stimulated the promoter 45-fold. As was found with the PMA-induced P3 promoter activation (Figure 4C), the effect of PKC ϵ -CAT on the P3 promoter was reduced by the MEK-1/ERK1/2 inhibitor PD98059 (Figure 5C). Also, using the ERK1/2-resistant A72-Ets2 mutant resulted in a 2-fold decrease in the Ets2/PKC ϵ -dependent activation of the P3 promoter (results not shown). The same mutation reduced similarly the Ets2/PMA effect on the promoter (Figure 4C). Therefore PKC ϵ -CAT and PMA share

Figure 4 Mutating the Ets2 protein at the MAPK-responsive phosphorylation site reduces the Ets2/PMA effect on the PTHrP P3 promoter

(A) ChIP assay of chromatin derived from either Flag-Ets2 or mock-transfected MCF-7 cells. Chromatin from MCF-7 cells was isolated and pretreated as described in the Experimental section. For immunoprecipitation, an anti-Flag, a control antibody (anti-HA) or no antibody was used. The purified immunoprecipitated DNA was then subjected to a PCR analysis by using PTHrP gene-specific primers whose sequences are shown in the Experimental section; input denotes the PTHrP-specific PCR product generated from chromatin that was not subjected to immunoprecipitation. (B) PMA up-regulates *ets2*-RNA expression. The *ets2*-RNA level was measured by real-time RT-PCR using Ets2-specific primers after treatment of MCF-7 cells with PMA for 0.25, 0.5, 0.75, 1, 2 or 4 h. (C) Blockage of ERK1/2-inducible phosphorylation of Ets2 by replacing threonine at position 72 by alanine (A72) reduces the ability of Ets2 to transactivate the P3 promoter in the presence of PMA. Transfection experiments with WT-Ets2 or A72-Ets2 in the absence or presence of PMA and PD98059 (PD) were performed as described in the legend to Figure 3(A). P3-EM denotes the Ets2-mutant P3 promoter as shown in Figure 2(A). (D) Western-blot analysis of nuclear extracts derived from MCF-7 cells overexpressing Flag-tagged WT-Ets2, DBD-Ets2, TAD-Ets2 or A72-Ets2 by using an anti-Flag antibody.

the ability to activate the promoter alone or in co-operation with Ets2 in an ERK1/2-dependent manner.

Although expressed at a similar level as PKC ϵ -CAT (Figure 5B), PKC α -CAT failed to activate the PTHrP P3 promoter (Figure 5A). However, PKC α -CAT was capable of synergizing with Ets2 and both, together, stimulated the promoter 12-fold (Figure 5A). In contrast with the Ets2/PKC ϵ co-operative effect, the Ets2/PKC α -CAT synergism was affected neither by PD98059 nor by the Thr⁷²/Ala⁷² mutation (Figure 5C). This suggests that PKC ϵ , but not PKC α , may, at least partially, mediate the effect of PMA on the PTHrP P3 promoter in MCF-7 cells.

PMA does not increase expression or transcriptional activity of Sp1 in MCF-7 cells

Mutation in the Sp1-binding site or replacement of this site by a Gal4 recognition sequence decreased PMA- and PMA/Ets2-regulated P3 promoter activity. This suggests that Sp1 or a related factor is important for the PMA effect on the P3 promoter. PMA has been shown to up-regulate expression and transcriptional activity of Sp1 previously [46,47]. To test whether PMA also affects Sp1 activity in MCF-7 cells, we first determined the Sp1

protein level in the absence and in the presence of PMA. As shown in Figure 6(A), PMA did not modulate Sp1 protein expression in these cells. Also, we did not observe the effect of PMA on the expression of the Sp1-like protein, Sp3. It was noticed that the expression of Sp1 was found to be lower in MDA-MB-231 cells.

To test possible changes of Sp1 transcriptional activity in response to PMA, we used a Gal4-PTHrP P3 promoter construct (Figure 2A) and a Gal4-Sp1 fusion protein. The Gal4-Sp1 fusion protein contained the first 612 amino acids of the Sp1 protein and a Gal4-DBD at the C-terminus [48]. The Sp1-specific sequence within this fusion protein includes the major activation domains A, B and most of C, but lacks the activation domain D and the DBD [49]. Gal4-DBD contains the first 147 amino acids of the yeast transcriptional activator Gal4 [50]. In the Gal4 mutant of the PTHrP P3 promoter the sequence between -63 and -47, which includes the Sp1-binding site (Figure 2A), has been replaced by a Gal4-binding site (CGGN₁₁CGG) [21]. Using these constructs we found that, in the absence of PMA, Gal4-Sp1 activated the Gal4-P3 promoter approx. 3-fold, whereas it had no effect on the wild-type P3 promoter (Figure 6B). As a positive control we also used Gal4-Tax (Figure 6B), a strong activator of the Gal-mutant version of the PTHrP P3 promoter [21]. In the

Figure 5 A constitutively active form of PKC ϵ can substitute for PMA to activate the PTHrP promoter and is capable of co-operating with Ets2

(A) PKC ϵ activates the PTHrP P3 promoter and enhances Ets2 transcriptional activity in MCF-7 cells. A C-terminal fragment of PKC α (HA-PKC α -CAT) or PKC ϵ (HA-PKC ϵ -CAT) that contains the catalytic, but not the regulatory domain [41] was expressed alone or together with Ets2 to test the effect of these proteins on the activity of the PTHrP P3 promoter in transient transfection experiments. Capabilities of these PKC subtypes to activate the P3 promoter were compared with that of PMA in the presence of the empty vector (pCDNA3). (B) Ectopic expressions of HA-PKC α -CAT and PKC ϵ HA-PKC ϵ -CAT as determined by the Western-blot technique using the indicated antibodies are shown. (C) The Raf/MEK-1/ERK1/2 pathway is involved in PKC ϵ -, but not in PKC α -dependent stimulation of the PTHrP P3 promoter. The MEK-1 inhibitor, PD98059, and a Thr⁷²/Ala⁷² mutation in the Ets2 protein were tested for their abilities to interfere with Ets2-, PKC ϵ -CAT- or Ets2/PKC α -CAT-mediated P3 promoter activation.

Gal4-Tax fusion protein, the Gal4-DBD is fused to the entire sequence of the HTLV-I-Tax protein at its N-terminus. Gal4-Tax stimulated the Gal4-P3 promoter 12-fold, whereas it had a minor effect on the wild-type P3 promoter (Figure 6B). The isolated Gal4-DBD failed to increase the activity of the Gal4-P3 promoter. In the presence of Gal4-Sp1, addition of PMA activated the Gal4-P3 promoter 2.5-fold, but also the wild-type promoter 3.3-fold, suggesting that PMA did not specifically affect the transcriptional activity of Sp1. In conclusion, the results suggest that PMA does not directly target the Sp1 protein in MCF-7 cells.

PMA abrogates binding of an MCF-7 nuclear protein to a sequence that overlaps with the Sp1-binding site

We have shown that, in the absence of PMA, complete replacement of the Sp1-binding site by a Gal4 recognition sequence significantly decreased basal PTHrP P3 promoter activity in MCF-7 cells, whereas a triple mutation in the Sp1-binding site had no effect. In contrast, both mutations equally affected promoter activity in the presence of PMA. One possible explanation might be that, under non-stimulated conditions, a protein other than an Sp-like factor occupies the Sp1-binding site and contributes to basal promoter activity. In response to PMA, this putative factor might then be replaced by Sp1 or a related protein. To test this hypothesis, we performed EMSA. We incubated nuclear extracts from MCF-7 with an oligonucleotide probe that corresponded to the P3-specific sequence between -79 and -49 containing the Ets- and Sp1-binding sites (Figure 7A). We detected three major complexes C1-C3 (Figure 7B, lane 2). Complexes C1 and C3 contained Ets proteins. This is indicated by the observation that all competitors that contained an Ets-binding site, -79/-58 (Figure 7C, lanes 3 and 4; 7D, lane 4), -79/-58 Sp1M (Figure 7D, lane 5), and the Ets consensus sequence (Figure 7E, lane 5), inhibited the formation of the C1

Figure 6 Sp1 is not directly targeted by PMA to induce PTHrP transcription in MCF-7 cells

(A) Western-blot analysis of nuclear extracts from PMA- or mock-treated MCF-7 or MDA-MB-231 cells to study endogenous expression of Sp1, Sp3, Ets1, Elf-1 and PKC α . All three bands visualized by the anti-Sp3 antibody represent Sp3 proteins as described previously [57]. (B) Effect of Gal4-Sp1 or Gal4-DBD on the wild-type or Gal4-P3 promoter in the presence or absence of PMA. The Gal4-Sp1 fusion protein contains the first 612 amino acids of the Sp1 protein, whereas the Sp1-DBD at the C-terminus was replaced by the Gal4-DBD [48]. The Gal4-PTHrP P3 promoter contains a Gal4-binding site between -63 and -47, which replaces the Sp1-binding site (Figure 2A) [21]. The Gal4-Tax fusion protein combines the Gal4-DBD with the entire sequence of the HTLV-I-Tax protein. An empty vector (pCDNA3) was used under the control conditions.

and C3 complexes, whereas the AGAC box harbouring -51/-28 oligonucleotide did not (Figure 7D, lane 3). The C1 complex could specifically be supershifted by an anti-Elf-1 antibody (Figure 7E, lane 4) and formation of this complex was enhanced when nuclear extracts from MCF-7 that overexpressed exogenous Elf-1 were used (results not shown). We conclude from these results that the C1-specific complex contains Elf-1. The C3-specific protein could not be characterized. It is possible that the C3-specific protein is Ets-1, as overexpression of Ets-1 led to the formation of a complex of similar mobility (results not shown). Complexes with Ets2 could not be detected, even when Ets2 was overexpressed (results not shown). Similar to Ets1, Ets2 contains an autoinhibitory module that decreases its DNA-binding activity

Figure 7 EMSAs using a PTHrP P3-specific probe that contains the Ets-binding site and CCCACC motif

(A) Structure of the -79/-49 DNA probe used for EMSA and the -79/-49 Sp1M competitor. The -79/-49 Sp1M oligonucleotide harbours a triple mutation that interferes with Sp1 binding to the CCCACC motif. (B-E) EMSAs using nuclear extracts from PMA- or mock-treated MCF-7 cells were performed as described in the Experimental section. Ets cons. denotes an oligonucleotide that contains an Ets consensus site [18].

by 50-fold [51]. The formation of both Ets-containing C1 and C3 complexes were not altered in response to PMA (Figure 7B, cf. lanes 2 and 3).

Another complex, C2, was visible when nuclear extracts from untreated MCF cells were used (Figure 7B, lane 2). On stimulation with PMA, this complex could no longer be detected. Also, the complex was not found when extracts from MDA-MB-231 cells were used (results not shown). In MDA-MB-231 cells, basal PTHrP P3 promoter activity was sensitive to the Sp1-specific mutation (Figure 7C). Complex C2 did not contain an Ets protein, as it did not disappear in the presence of an oligonucleotide that contained an Ets consensus-binding site (Figure 7E, lane 5). Also, it did not harbour an Sp-like protein, since a PTHrP-specific -79/-49 oligonucleotide carrying a triple mutation in the Sp1 (-79/-49 Sp1M) was as effective in inhibiting the formation of the C2 complex as the -79/-49 WT oligonucleotide (Figure 7D, cf. lanes 4 and 5), and since the C2 complex disappeared in the presence of an Sp1 consensus-binding site (results not shown). A comparative analysis of the effect of a (-79/-49)-specific oligonucleotide versus the effect of a (-79/-58)-specific oligonucleotide on C2 formation revealed that the C2-specific protein required the sequence between -49 and -58 for binding to the PTHrP probe (Figure 7C, cf. lanes 3 and 4). This sequence contains exactly the CCCACC motif, which is also needed for Sp1 to bind to the PTHrP P3 promoter (Figure 7A). However, it is unlikely that the C2-specific protein relies only on this element for its interaction with the P3 promoter, as the triple mutation in this site, that specifically inhibits Sp1 binding, severely alters this sequence. Instead, we

believe that the C2 protein, by recognizing a sequence that overlaps with the CCCACC element requires some nucleotides from this motif, which were not affected by the triple mutation.

Despite the high level of Sp1 and Sp3 expression in MCF-7 cells, we could not find Sp1- or Sp3-specific complexes with the PTHrP P3 probe. Also, we were not able to detect Sp1 or Sp3 complex with an Sp1 consensus sequence (results not shown). This is quite probably due to the buffer conditions, as used in the present study for EMSA that were unfavourable for Sp1/Sp3 binding.

In conclusion, the results suggest that the CCCACC element is required for both basal and PMA-induced PTHrP P3 promoter activity in MCF-7 cells. Yet, it seems that different proteins occupy this site depending on whether PMA is present or not. It is possible that PMA actively induces the replacement of the C2-specific protein by an Sp-like protein.

In MCF-7 cells, a nuclear protein binds to the AGAC box that is not found in MDA-MB-231 cells

Under non-stimulated conditions, mutation of the AGAC box affected PTHrP P3 promoter activity in MCF-7, but not in MDA-MB-231 cells. By using EMSA, we examined MCF-7 and MDA-MB-231 nuclear extracts for proteins that could bind to this element. When MCF-7 nuclear extracts were incubated with a P3-specific oligonucleotide (-51/-28) that contained the AGAC box (Figure 7A), three different complexes, C5-C7, were observed (Figure 8B, lane 5). The formation of all these

Figure 8 EMSAs using a PTHrP P3-specific probe that contains the AGAC box

(A) Structure of the -51/-28 probe used. (B, C) EMSAs using nuclear extracts from PMA- or mock-treated MCF-7 cells or MDA-MB-231 cells were performed as described in the Experimental section.

complexes was dependent on the integrity of the AGAC box, as a mutation in the AGAC motif abrogated the ability of the unlabelled -51/-28 oligonucleotide to compete with the probe for binding of the (C5-C7)-specific proteins (cf. lanes 6 and 7). Interestingly, in MDA-MB-231 nuclear extracts, the C5- or C7-specific-binding activity was found to be completely absent or much reduced respectively (Figure 8B, lane 2; 8C, lane 3). Formation of the C5 and C7 complexes were not altered when MCF-7 cells were treated with PMA (Figure 8C, cf. lanes 1 and 2). These results show that at least one AGAC-binding protein is present in MCF-7 cells, which is missing in MDA-MB-231 cells. This may account for the difference in importance of the AGAC box for basal PTHrP P3-dependent transcription in these cells.

DISCUSSION

Various stimulators, including HTLV-I-Tax, TGF β 1 and retinoic acid, have been shown to stimulate PTHrP gene expression primarily by activating transcription from human PTHrP P3 promoter or its mouse counterpart [18,20,22]. In invasive MDA-MB-231 breast cancer cells, TGF β stimulates the P3 promoter by inducing a co-operative interaction between Ets1 and Smad3 [20]. In the present study, we examined the regulation of the P3 promoter in non-invasive, Ets1-deficient and TGF β -resistant MCF-7 breast cancer cells. We found that, in these cells, endogenous expression from the PTHrP P3 promoter could be significantly up-regulated by treatment with PMA. This activity could be completely blocked by MEK-1 inhibitor PD98059, suggesting that PMA mediates its effect on P3-dependent PTHrP expression by activating the Raf/MEK-1/ERK1/2 pathway. Promoter analyses confirmed that the P3 promoter is responsive to

PMA in a PD98059-sensitive manner. Mutation analyses showed that the PMA effect depended on the Ets- and Sp1-binding sites, suggesting that Ets- and Sp-like factors are involved in this PMA action. Of the four Ets factors tested, Ets1, Ets2, Ets-1 and Elf-1, Ets2 was the strongest activator of the P3 promoter and was the only one whose stimulatory activity could be significantly increased by PMA, again in a PD98059-sensitive fashion. Other Ets proteins, such as members of the polyoma enhancer A3 ('PEA3') Ets protein subgroup that are often overexpressed in mammary carcinoma or Friend leukaemia integration 1 ('FLI-1') protein, were not studied because they are not expressed in MCF-7 cells [31,52]. The involvement of Ets2 in PMA-mediated PTHrP expression is further supported by the observation that transdominant-negative versions of Ets2, Ets2-DBD and Ets2-TAD, abolished PMA-induced P3 promoter activity. Also, ChIP analysis showed that ectopically expressed Ets2 co-localizes with the PTHrP P3 promoter in the chromatin of MCF-7 cells.

Several lines of evidence indicate that PMA increases Ets2 activity. We demonstrate that PMA increases expression of Ets2 in MCF-7 cells. It has also been reported that PMA can stabilize the Ets2 protein [53]. Furthermore, a threonine to alanine substitution at position 72 of the Ets2 protein was found to reduce the PMA/Ets2 effect on the P3 promoter. This suggests that PMA may superactivate Ets2 by inducing phosphorylation at Thr⁷² by activating ERK1/2 [33]. However, this mutation affected Ets2/PMA-dependent P3 promoter activity much less than the MEK-1/ERK1/2 inhibitor PD98059, which suggests that Ets2 phosphorylation can only partly explain this PD98059-sensitive effect of PMA. Therefore PMA may target additional P3-regulating transcription factors, such as Sp1, which can be activated by ERK1/2-dependent phosphorylation [54]. Although Sp1 supported PMA-induced P3 promoter activity, its

transcriptional activity was not increased by PMA. This suggests that Sp1 is not directly targeted by PMA in MCF-7 cells. Since Sp1 is capable of co-operating with Ets2 [22], it could play an important role as a cofactor for Ets2. Intriguingly, a protein, named C2-specific protein, was found to bind a P3 promoter sequence that overlaps with the Sp1-binding site only when PMA was absent. This may suggest that PMA may actively induce the displacement of the C2-specific protein from the promoter to allow recruitment of Sp1 or another Sp-like protein to the Sp1-binding site.

The two PKC subtypes PKC α and PKC ϵ , capable of activating the Raf/MEK-1/ERK1/2 pathway, were both found to stimulate transcription from the P3 promoter in the presence of exogenous Ets2. However, only the PKC ϵ effect was sensitive to PD98059. In addition, only PKC ϵ could activate the P3 promoter in the absence of exogenous Ets2. This suggests that PKC ϵ , but not PKC α , is involved in the PD98059-sensitive effect of PMA on the PTHrP P3 promoter. In support of this notion, we found that Gö6976, an inhibitor that blocks the activity of PKC α , but not of PKC ϵ , had no effect on PMA-induced P3-dependent PTHrP expression (results not shown). Interestingly, both PKC ϵ and PTHrP have been shown to have an anti-apoptotic effect on MCF-7 cells [15,38]. During apoptosis of MCF-7 cells, PKC ϵ is activated by caspase 7 that removes the inhibitory regulatory domain from this protein [55]. It is tempting to speculate, therefore, that the anti-apoptotic effect of PKC ϵ is, at least partially, mediated by PTHrP.

Interestingly, in MDA-MB-231 cells, PMA hardly affected PTHrP expression (Figure 1C). This is probably due to a generally anomalous PMA response that correlates with constitutive activation of PKCs, such as PKC α , in these cells [37]. These permanently activated PKCs may also contribute to the regulation of the P3 promoter, as PKC inhibitors reduced TGF β -induced P3 promoter activity in MDA-MB-231 cells [20]. However, the stimulatory effect of TGF β on the P3 promoter was found to be resistant to PD98059 (results not shown). This supports the notion that P3-dependent PTHrP expression is differently regulated in MCF-7 as opposed to MDA-MB-231 cells.

The closely related transcription factors, Ets1 and Ets2, both participate in tumour-promoting processes. For example, both proteins have been shown to activate a number of genes coding for extracellular matrix-degrading proteases and, therefore, are assumed to be involved in the acquisition of cellular invasiveness ([27] and references therein). Recently, Ets1 was found to be associated also with the regulation of the promyelocytic leukaemia protein oncogenic domains ('PODs') that seem to be deregulated in invasive tumours [56]. In the present study, combined with the results we have published previously [20], we provide evidence suggesting that these Ets factors also participate in the regulation of the expression of the (breast) tumour-promoting protein, PTHrP. This further underscores the importance of Ets1 and Ets2 for tumorigenesis.

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