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PKC Isoenzyme Expression and Cellular Responses to Phorbol Ester in JEG-3 Choriocarcinoma Cells

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Protein kinase C (PKC) is a key regulatory enzyme involved in the transduction of extracellular growth signals to the cell nucleus. It occurs in several isoforms, the exact functional roles of which have not been established as yet. The tumor-promoting agent 12-O-tetradecanoyl-phorbol acetate (TPA) is the classic activator of PKC and modulates the activity of the activating protein-1 (AP-1) transcription factor complex via this pathway. AP-1, in turn, induces cell proliferation in many tissues. In the present study, the PKC isoenzyme expression pattern in JEG-3 choriocarcinoma cells was analyzed. The results were compared with those obtained in HEC-1B endometrium adenocarcinoma cells, which had previously been characterized in this respect. To gain insight into the possible functional consequences of different PKC expression patterns, cell proliferation rates and AP-1 activity in response to TPA in both cell lines was studied. Western blot analysis of the PKC isoenzyme expression pattern revealed that JEG-3 cells are deficient in the PKC α, δ, and ε isoforms. These isoenzymes are strongly expressed in HEC-1B cells, with the α and δ being constitutively active. As opposed to HEC-1B cells, JEG-3 cells did not show an enhanced proliferation rate in response to TPA. Furthermore, TPA-treated JEG-3 cells did not exhibit any change in cell shape and refractility as observed in HEC-1B cells. AP-1 activity, as determined by a transfected AP-1-luciferase reporter plasmid, was induced 10-fold by TPA in JEG-3 cells, yet only threefold in HEC-1B cells. It is concluded from these data that differential expression of a subset of PKCs, e.g., the α, δ, and ε isoforms, may serve as an indicator of the proliferative potential in response to growth factors and mitogens. Furthermore, our data indicate that the inducibility of AP-1 activity does not necessarily reflect the proliferative capacity of a given cell type in response to classical tumor promoters such as phorbol ester.

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Introduction

Protein kinase C (PKC) is a serine/threonine kinase which mediates the effects of a large number of hormones, growth factors, and cytokines. PKC is, thus, considered a key factor in the regulation of cellular proliferation and differentiation *(1–3)*. PKC is generally thought to be activated by signal transduction systems that produce diacylglycerol (DAG), such as certain tyrosine kinase and G protein-coupled receptors *(1,4–6)*. PKC is also the major target for the well-known tumor-promoting agent 12-O-tetradecanoyl-phorbol acetate (TPA), a phorbol ester, which acts in a fashion very similar to DAG *(7–10)*. Over the last few years, a growing number of PKC isoforms has been identified *(1,5,6,11)*. Their specific expression patterns and functional properties are still largely unknown.

The downstream targets of activated PKC are only partially identified and include other protein kinases *(6,12)* and the transcription factor AP-1 *(13–15)*. The composite transcription factor AP-1 is the prototype of a mitogen-activated transactivator, and its transcriptional activity is believed to reflect cell proliferation in many tissues *(14,16–18)*. AP-1 is a homo- or heterodimeric DNA-binding protein composed of either two Jun family proteins or one Jun and one Fos family protein *(14,19)*. The activity of this transcription factor complex is modulated by growth factors, cytokines, and tumor promoters that activate PKC, such as TPA *(14,19)*. The activated AP-1 dimer binds to specific DNA sequences in the regulatory regions of mitogen-responsive genes, so-called TPA-response elements (TREs) *(20,21)*. Reporter gene constructs driven by a minimal promoter and several TREs provide a simple tool to integrate the various signal transduction pathways and protein subunits contributing to AP-1 activation. The activity of such a reporter gene is, therefore, commonly referred to as AP-1 activity.

In the present study, PKC isoenzyme expression, cell proliferation rates, and AP-1 activity in JEG-3 choriocarcinoma cells treated with TPA were analyzed. The results

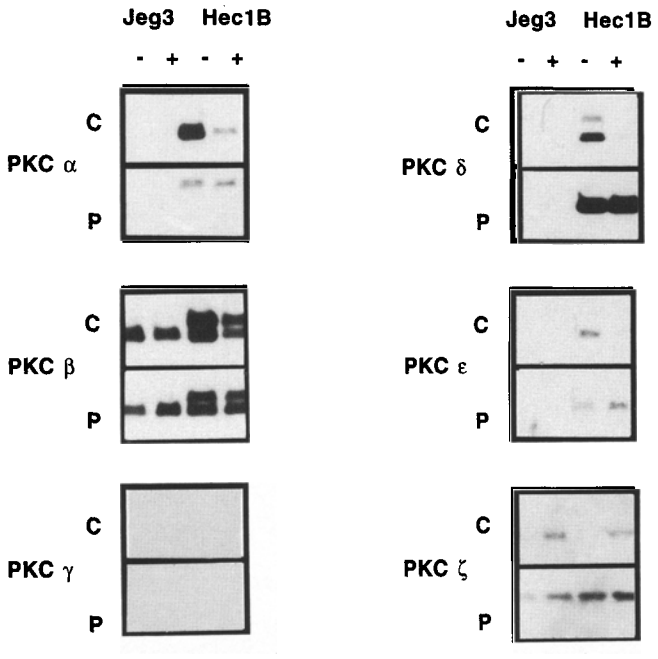

Fig. 1. Western blot analysis of the subcellular localization of PKC isoenzymes in the absence and presence of TPA. Localization of PKC isoenzymes in the cytosolic (C) and the particulate (P) fraction of JEG-3 and HEC-1B cells, either in the absence (–) or presence (+) of TPA ($10^{-7}M$, 12 h).

were compared with those obtained in HEC-1B endometrium adenocarcinoma cells, which had previously been characterized in this respect *(22)*.

Results

Western Blot Analysis of the Subcellular Localization of PKC Isoenzymes in the Absence and Presence of TPA

To investigate the differences between HEC-1B and JEG-3 cells in terms of the PKC isoform expression pattern, the subcellular localization of the PKC isoenzymes in the cytosolic (C) and the particulate (P) cell fraction were analyzed, both in the absence and in the presence of TPA. The results of these experiments are shown in Fig. 1.

As had been previously shown for HEC-1B cells *(22)*, the α isoform was found mostly in the cytosolic fraction, although an important quantity of this isoenzyme was also present in the particulate fraction in the absence of TPA, indicating constitutional activation of this isoenzyme. After TPA treatment (12 h), the expression levels were reduced in both fractions. No expression of PKC α could be detected in JEG-3 cells.

The β isoform was detected as a band doublet in HEC-1B cells, as well as in SKUT-1-B cells, another uterine tumor cell line analyzed *(22)*, and in COS-7 monkey kidney cells (unpublished results). In JEG-3 cells, a single band was observed with the same antibody. The β isoform was present both in the cytosolic and in the particulate fraction in both JEG-3 and HEC-1B cells, and was downregulated in the C fraction and upregulated in the P fraction in TPA-treated JEG-3 cells. This can be interpreted as a translocation to the cellular membrane(s), which is considered an equivalent of PKC activation. The significance of the single band versus the band-doublet is presently unknown. The γ isoform was not expressed in either cell line.

The δ isoform was not present in JEG-3 cells, whereas it was strongly expressed and had a predominant particulate localization in HEC-1B cells, indicating constitutive activation of this isoform.

The ε isoform was also absent from both the C and P fraction of JEG-3 cells, whereas it translocated from the C fraction to the P fraction in TPA-treated HEC-1B cells. Unexpectedly, an induction of PKC ζ was observed both in the cytosolic and particulate fraction of TPA-treated JEG-3 and HEC-1B cells. The expression level and intracellular localization of this isoenzyme were previously reported to be unchanged by TPA treatment in several cell lines *(23)*. In another uterine cell line (SKUT-1-B), expression of PKC ζ was below the detection both in the absence and presence of TPA *(22)*.

TPA does not Stimulate Proliferation in JEG-3 Cells

As previously shown *(22)*, HEC-1B endometrial adenocarcinoma cells responded with a marked increase in cell number to TPA treatment (Fig. 2A). In JEG-3 cells, no stimulation of proliferation was observed when TPA was added to the culture medium (Fig. 2B). It had also been shown that HEC-1B cells responded to TPA treatment with a characteristic change in cell shape and refractility, which became apparent approx 1 h after the addition of TPA to the cell culture medium. No such change in cell morphology was observed with the JEG-3 cell line after TPA treatment. All experiments were repeated at least three times in triplicates.

TPA Stimulates AP-1 Activity in JEG-3 and HEC-1B Cells

Transient transfection experiments were performed with an AP-1-responsive reporter plasmid to characterize AP-1 activation by TPA in JEG-3 and HEC-1B cells. Experiments were performed three times in triplicates. The results are shown in Fig. 3. The AP-1 responsive vector was strongly induced by TPA in JEG-3 cells (10.05 ± 0.46-fold). It was also induced to a lesser extent (3.23 + 0.51-fold) in HEC-1B cells. These results indicate that activation of AP-1 response elements and proliferative/morphological responses to TPA treatment are not coupled in these cell lines.

Discussion

In the present study, the PKC isoenzyme expression pattern was analyzed as well as the TPA-induced changes in PKC isoenzyme expression and subcellular localization in JEG-3 cells, and compared to the HEC-1B cell line, which had previously been characterized in this respect *(22)*. A striking difference was found in PKC isoenzyme expression between JEG-3 and HEC-1B cells. Whereas HEC-1B cells expressed high levels of all isoenzymes analyzed, and

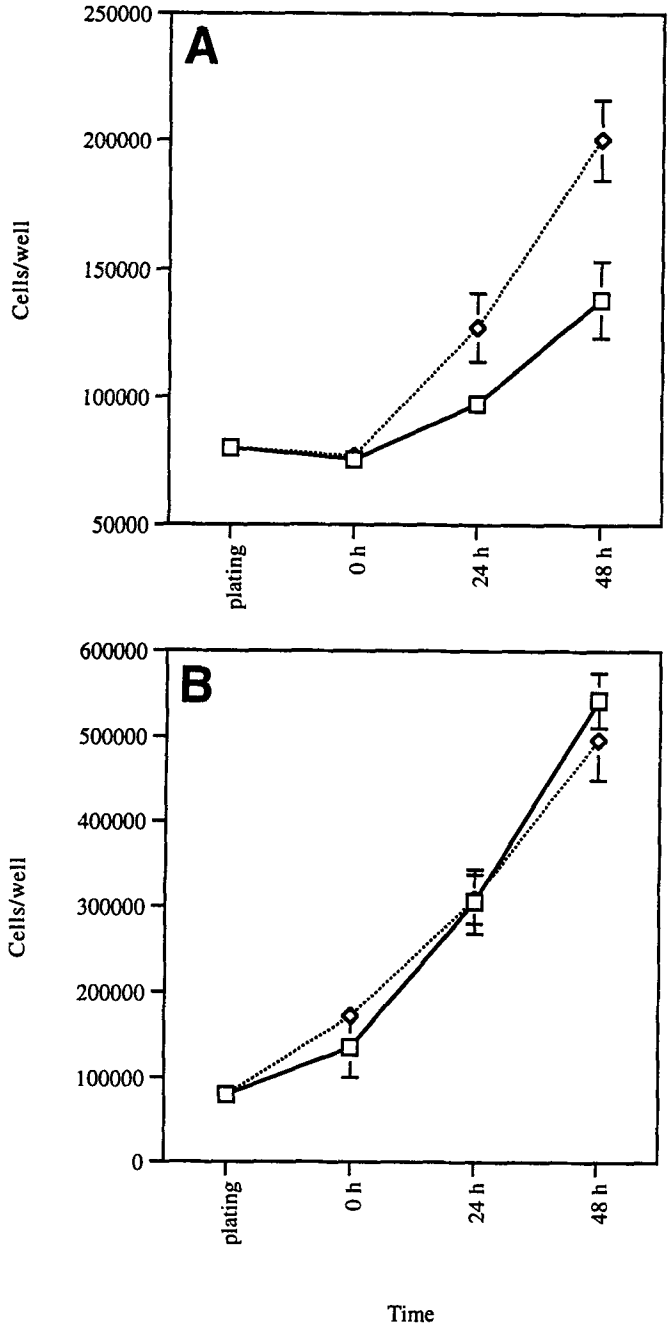

Fig. 2. Proliferative response of HEC-1B and JEG-3 cells in response to TPA. **(A)** HEC-1B endometrial adenocarcinoma cells (80,000/well) were treated for 24 h or 48 h with either $10^{-7}M$ TPA or control medium. Cell numbers were higher in TPA-treated than in control wells throughout the experiment. Squares denote –TPA (HEC), diamonds denote +TPA (HEC). **(B)** TPA treatment did not change the growth rate of JEG-3 tumor cells. Both graphs represent a typical experiment performed in triplicates. Data are expressed as mean ± SD. Squares denote –TPA (JEG), diamonds denote +TPA (JEG).

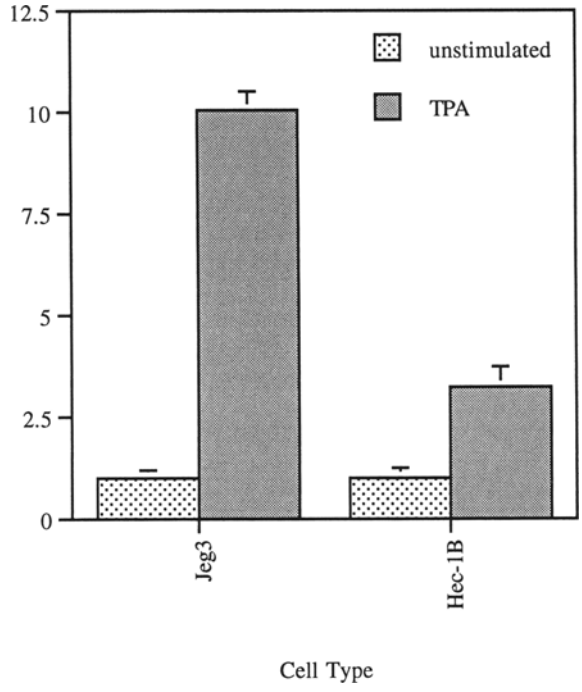

Fig. 3. TPA stimulates AP-1 activity in JEG-3 and HEC-1B cells. Cells were transiently transfected with AP-1-tk81-Luc and either left unstimulated or stimulated with TPA for 12 h. Results are presented from a typical experiment performed in triplicates. Data are expressed as mean fold induction ± SD. Induction in JEG-3 cells was 10.05 ± 0.46-fold, and in HEC-1B cells 3.23 ± 0.51-fold.

showed constitutive activation of PKC α and δ and translocation of PKC ε in response to TPA, JEG-3 did not express any detectable levels of these isoenzymes. Previous studies in other cell types and tissues indicate that PKC α, β, δ, ε, and ζ are widely distributed throughout the body *(5,11,23,24)*, whereas expression of PKC γ is restricted to CNS tissues *(5,11,23,24)*. This is consistent with the present data, showing no expression of this isoform in either cell line. The notion that each cell type expresses a particular subset of PKC isoforms *(5,11)* is also supported by this data.

An important functional consequence of PKC activation is a change in cell proliferation rates. However, cellular proliferation and differentiation may not only depend on overall PKC activity, but also on the expression of certain PKC isoforms. Over the last few years, a growing number of PKC isoforms exhibiting specific expression patterns and functional properties have been identified, thus providing a possible molecular explanation for the observed differences in mitogen and tumor promoter responsiveness in different cell types *(1,5,6,11)*. The PKC family now comprises at least 12 members, which can be divided into two groups *(5)*, the Ca^{2+}-dependent or conventional PKCs (cPKCs) α, βI, βII, and γ, and the Ca^{2+}- independent or novel PKCs (nPKCs) δ, ε, ζ, η, θ, ι, λ, and μ. cPKCs do not require the presence of Ca^{2+} for activation by phorbol esters; however, Ca^{2+} lowers the concentration of phorbol ester necessary to obtain full activity *(25)*. The members of the nPKC group do not require Ca^{2+} for activation, but need phosphatidylserine as a cofactor, except for PKC ζ, which exhibits low but constitutive activator-independent kinase activity *(26,27)*.

To gain insight into the possible physiological consequences of different PKC isoenzyme expression patterns, the JEG-3 and HEC-1B cell lines were compared with respect to cell proliferation rates and AP-1 activity. The classical PKC-activating agent TPA was used to stimulate PKC in these experiments. The responses of different cell types to TPA treatment fall into three categories: stimulation of cell growth and proliferation, inhibition of proliferation, or no change in proliferation rates. The proliferative activity of NIH 3T3 fibroblasts and resting T lymphocytes, for instance, is stimulated by TPA treatment *(28–30)*. It has also been suggested that some cell types that overexpress PKC proliferate more vigorously and are more susceptible to the mitogenic effects of growth factors and/or TPA, and some also showed morphologic alterations *(1,31–33)*. In other cell types, TPA was reported to inhibit proliferation and to induce differentiation *(34–36)*. It was found that TPA treatment (24 and 48 h) did not stimulate the proliferation rate of JEG-3 choriocarcinoma cells. In contrast, HEC-1B endometrial adenocarcinoma cells had shown a marked proliferative response when treated with TPA *(22)*. As opposed to HEC-1B cells, JEG-3 cells did also not show any change in cell morphology in response to TPA. AP-1 activity was strongly induced by TPA in JEG-3 cells. This induction was stronger in JEG-3 than in HEC-1B cells, indicating that stimulation of cell proliferation and induction of AP-1 activity are dissociated in these cell lines.

In summary, JEG-3 cells did not respond to TPA with enhanced proliferation or a change in cellular morphology. They did not express PKC α and δ, which are strongly expressed and constitutively active in HEC-1B cells. In addition, JEG-3 cells were deficient in PKC ε, which was activated in response to TPA in HEC-1B cells. It had been previously shown that another cell line (SKUT-1-B uterine tumor cells), which did not show an increase in proliferation rates in response to TPA either, was deficient in PKC α, δ, and ε *(22)*. The fact that these isoenzymes are now found to be absent in JEG-3 as well, further supports the notion that they might be implicated in proliferative responses to TPA in tumor cells. Thus, the absence of expression of these isoenzymes can be interpreted as a possible indicator of proliferative potential of tumor cells in response to mitogens. On the other hand, these isoforms are not for the activation of AP-1-responsive promoters, since JEG-3 cells showed an induction of such a promoter which was even stronger than in HEC-1B cells. The only two PKC isoforms expressed in JEG-3 cells were PKC β, which was strongly expressed, and PKC ζ. One or both of these isoforms should, thus, be involved in the transmission of the signal resulting in AP-1 activation in these cells.

In conclusion, the molecular and functional heterogeneity of the PKC family make them attractive targets for anticancer drug development. As isoform-specific PKC activators and inhibitors will become available as therapeutic agents, analysis of the PKC isoenzyme expression profile of tumors will be essential to establish tumor-specific treatment protocols.

Materials and Methods

Cell Culture

JEG-3 human choriocarcinoma cells and HEC-1B human endometrial adenocarcinoma cells and were obtained from ATCC (Rockville, MD) and maintained in low glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 m*M* L-glutamine, and penicillin/streptomycin. Cells were passaged twice weekly and plated for cell proliferation experiments when 60–80% confluent.

Preparation of Cytosolic and Membrane Fractions for Western Blot Analysis

Cytosolic and membrane fraction preparation was performed as previously described *(2,3,37)*. Briefly, approx 5×10^6 cells were washed with 5 mL of buffer A (25 m*M* Tris/HCl, pH 7.4, 250 m*M* sucrose, 2.5 magnesium acetate, and 1 m*M* dithithreitol [DTT]). Cells were harvested in 1.5 mL ice-cold buffer B (buffer A plus 2.5 m*M* EGTA, 20 μ*M* Leupetin, 100 μ*M* phenylmethylsulphonyl fluoride, PMSF). The cell lysate was sonicated for 30 s at 4°C and then centrifuged at 100,000*g* at 4°C for 40 min. The supernatant (=the cytosolic fraction) and the pellet (=the particulate fraction) were processed as follows.

Cytosolic proteins were precipitated with 10% trichloroacetic acid (final concentration=10%). The samples were then centrifuged for 5 min at 14,000 rpm. The precipitate was washed two times with 1 mL ether and then vacuum-dried for 15–30 min. Then 200 μL of modified sample buffer was added (50 m*M* Tris, pH 6.8, 1% SDS, and 12% sucrose), and the proteins were resolubilized by sonication (5 × 1 s at room temperature). The samples were again centrifuged for 5 min, and the supernatants were stored as frozen aliquots. Protein concentration was determined with a detergent-compatible protein assay system (Biorad, München, Germany) and with bovine serum albumine (BSA) protein standards diluted in modified sample buffer.

Particulate proteins were extracted as follows: 200 μL ice-cold buffer C (buffer B + 0.5% Triton X 100) were added to the particulate fraction. The sample was then sonicated for 5 × 1 s at 0°C, and centrifuged for 30 min (14,000 rpm, 4°C). Protein concentration was determined as described above, except that BSA protein standards were diluted in buffer C. The cytosolic and particulate fractions were diluted with 2X sample buffer (100 m*M* Tris/HCl pH 6.8, 8% SDS, 24% glycerol, 4% 2-mercaptoethanol, and 0.02% Serva blue G) to a final volume of 100 μL and a final protein concentration of 300 μg/mL.

PAGE and Western Blotting

Electrophoresis was performed in a 10% polyacrylamide separating gel and a 3% stacking gel as previously

described *(38)*. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon P, Millipore, Eschborn, Germany) using the technique described by Towbin et al. *(39)*. Membranes were stained with Ponceau S to determine transfer efficiency and homogeneity of protein lanes. After destaining with TBS (Tris-buffered saline: 20 m*M* Tris/HCl, pH 7.6, 137 m*M* NaCl) for 5 min, membranes were incubated overnight at 4°C in blocking solution (0.1*M* maleic acid, pH 7.5, 0.15*M* NaCl, 0.005% thimerosal, and 1% blocking reagent, Boehringer Mannheim, Germany). The membranes were washed for 10 min in TBST (TBS plus 0.05% Tween 20) and incubated with affinity-purified isoenzyme-specific antibody solution. Anti-PKC α, ε, and ζ were obtained from Gibco, BRL (Eggenstein, Germany). Anti-PKC β, γ, and δ were obtained from Santa Cruz Biotechnology, Inc. The antibodies were used in the following dilutions: 1:300 for PKC α, 1:500 for PKC β, 1:1000 for PKC γ, 1:500 for PKC δ, 1:1000 for PKC ε, and 1:300 for PKC ζ. Dilution was performed with 9:1 TBST/blocking solution. Blots were incubated for 1 h at room temperature, washed 3 × 10 min in TBST, and incubated with the second antibody (goat-antirabbit IgG-peroxidase conjugate, Sigma, 1:1000) for 1 h at room temperature. The second antibody was visualized by enhanced chemiluminescence (ECL) reagents (Amersham, Braunschweig, Germany) and Fuji RX 400 films.

Analysis of Cell Proliferation

Cells were plated in 12-well plates at a density of 80,000 cells/well. After 12 h, cells were counted in three wells to obtain baseline numbers. Cells were then incubated with either normal medium or medium containing $10^{-7}M$ TPA. Cells were removed after 24 and 48 h for determination of cell numbers. Cell counting was performed in an automated cell counter (Coulter Electronics Ltd., Luton, UK).

Plasmid Construction

AP-1-tk81-luc was constructed as follows: Oligonucleotides 5'-CGTGACTCAGCGCGGTGACTCAGCGCGG-3' and 5'-GATCCCGCGCTGAGTCACCGCGCTGAGTCA CGAGCT-3', containing two TRE consensus sites (underlined), were annealed to generate a double-stranded TRE_2 oligomer. Two TRE_2 oligomers were ligated via their compatible *Bam*HI overhangs and inserted into the *Sst*I site of the plasmid tk81-Luc via their *Sst*I overhangs. The correct insertion of the double-stranded TRE_4 oligonucleotide was confirmed by dideoxynucleotide DNA sequencing. Tk81-luc contains the firefly luciferase gene under the control of a truncated herpes simplex virus thymidine kinase (tk) promoter *(40)*, and was a gift from Dr. S. K. Nordeen.

Transfections and Luciferase Assays

Cells were plated in 12-well plates (Costar, Cambridge, MA) at a density of 2×10^5 cells/well. After 24 h, cells were transfected by the lipofection method as previously described *(41)*, using 1 μg reporter plasmid/well. At 8 h after transfection, cells were either left unstimulated or treated with TPA ($10^{-7}M$, Sigma, Deisenhofen, Germany) for 12 h.

At the end of the experiment, the cells were trypsinized, washed, pelleted, and lysed with reporter lysis buffer (Promega, Madison, WI). After one freeze–thaw cycle, luciferase activity in the lysate was determined in a luminometer (Lumat LB 9501, Berthold, Wildbad, Germany).

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