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Repression of 5-aminolevulinate synthase gene by the potent tumor promoter, TPA, involves multiple signal transduction pathways

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Abstract

The potent tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induces activator protein-1 (AP-1) transcription factors, early response genes involved in a diverse set of transcriptional regulatory processes, and protein kinase C (PKC) activity. This work was designed to explore the signal transduction pathways involved in TPA regulation of 5-aminolevulinate synthase (ALAS) gene expression, the mitochondrial matrix enzyme that catalyzes the first and rate-limiting step of heme biosynthesis. We have previously reported that TPA causes repression of ALAS gene, but the signaling pathways mediating this effect remain elusive. The present study investigates the role of different cascades often implicated in the propagation of phorbol ester signaling. To explore this, we combined the transient overexpression of regulatory proteins involved in these pathways and the use of small cell permeant inhibitors in human hepatoma HepG2 cells. In these experimental conditions, we analyzed TPA action upon endogenous ALAS mRNA levels, as well as the promoter activity of a fusion reporter construct, harboring the TPA-responsive region of ALAS gene driving chloramphenicol acetyl transferase gene expression. We demonstrated that the participation of α isoform of PKC, phosphatidylinositol 3-kinase (PI3K), extracellular-signal regulated kinase (ERK1/2), and c-Jun N-terminal kinase (JNK) is crucial for the end point response. Remarkably, in this case, ERK activation is achieved in a Ras/Raf/MEK-independent manner. We also propose that $p90^{RSK}$ would be a convergent point between PI3K and ERK pathways. Furthermore, we elucidated the crosstalk among the components of the cascades taking part in TPA-mediated ALAS repression. Finally, by overexpression of a constitutively active $p90^{RSK}$ and the coactivator, cAMP-response element protein (CREB)-binding protein (CBP), we reinforced our previous model, that implies competition between AP-1 and CREB for CBP.

Keywords: Regulation of gene expression; Signal transduction; Tumor promoter; Protein phosphorylation; 12-*O*-Tetradecanoylphorbol-13-acetate; 5-Aminolevulinate synthase; Extracellular-signal regulated kinase; c-Jun N-terminal kinase; Phosphatidyl inositol 3-kinase; Protein kinase C

12-*O*-Tetradecanoylphorbol-13-acetate (TPA),[2] a potent tumor promoter phorbol ester, affects a number of cellular functions including gene expression and protein and DNA synthesis. At the cellular level, TPA has been demonstrated to modulate the growth, differentiation, survival, function, and metabolism of a variety of primary cell cultures and cell lines. The broad range of these phorbol ester-mediated biological effects suggests a

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[2] *Abbreviations used:* TPA, 12-*O*-tetradecanoylphorbol-13-acetate ALAS, 5-aminolevulinate synthase; AP-1, activator protein-1; CAT, chloramphenicol acetyltransferase; CREB, cAMP-responsive element protein; CBP, CREB-binding protein; CRE, cyclic AMP-responsive element; ERK, extracellular-signal regulated kinase; JIP-1, JNK-interacting protein-1; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MEK, MAPK/ERK kinase; PI3K, phosphatidyl inositol 3-kinase; PIP_3, phosphatidylinositol-3,4,5-triphosphate; PDK-1, 3-phosphoinositide-dependent kinase-1; PKA, PKB, and PKC, protein kinase A, B, and C, respectively; SEK, SAPK/ERK kinase-1; TRE, TPA-responsive element.

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role for this drug in the modulation of a variety of cellular processes, including those that affect the development, progression, and therapy of human malignancies [1].

Since TPA shares some structural similarities with diacylglycerol, the protein kinase C (PKC) physiological activator, it is commonly used to activate classical and novel PKC isoenzymes. Most of TPA-induced effects are mediated by the temporal activation, translocation, and suppression of selected PKC isoforms. These kinases play central roles in signaling pathways, participating in a variety of protein phosphorylation cascades that regulate gene expression.

It has been reported that the biological effects of the activation of a given subset of PKC isoforms in any cell will be variable, dependent upon the status of several interwoven signaling and regulatory pathways controlled by a variety of extracellular and intracellular stimuli [2]. Although cellular targets of phorbol esters are the aforementioned PKC isoforms, TPA signaling also triggers activation of other kinases, including those of mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) families [3]. There are several PKC effects mediated by activation of MAPKs, ERK, and c-Jun aminoterminal kinase (JNK), and subsequent activation of activator protein-1 (AP-1) [4].

Diacylglycerol and phorbol ester-dependent regulation of gene expression is often mediated by AP-1 transcription factors. AP-1, dimeric complexes comprising members of Fos and Jun family proteins, are early response genes involved in a diverse set of transcriptional regulatory processes [5]. They bind to and direct transcription from TPA-response elements (TREs) that are therefore known as AP-1-binding sites. Mitogens, oncoproteins, cytokines, and stress agents such as ultraviolet light activate AP-1. Frequently, AP-1 plays a role as a positive regulator of gene expression [6]. Recently, we have described a distinct mechanism of negative regulation by AP-1 on 5-aminolevulinate synthase (ALAS) gene promoter. We found that an AP-1-binding site, located at −261 bp, was crucial for the repression of ALAS promoter activity. This event involves competition between cAMP-response element (CRE) protein (CREB) and AP-1 for the coactivator CREB-binding protein (CBP) [7].

The ALAS gene has provided a useful model system for studying the integration of multiple hormonal signals at the level of a single gene [8–10]. ALAS is a mitochondrial matrix enzyme that catalyzes the first and rate-limiting step of heme biosynthesis [11]. In eukaryotes, there are two related ALAS isozymes that are encoded by two separate genes. The erythroid form of ALAS is expressed in hematopoietic tissue and is essential for the generation of functional hemoglobin in erythrocytes. The second enzyme, ubiquitous or liver type ALAS, is probably expressed in all tissues, providing heme for cytochromes and other hemeproteins [11]. Defects in genes encoding enzymes in the heme biosynthesis pathway are associated with a family of serious disorders known as porphyrias. Additionally, heme synthesis decline could explain the loss of iron homeostasis, because insufficient levels of heme would compromise iron regulation [12].

Acute hormonal regulation of ALAS is often exerted by regulating transcription. We have already demonstrated in rat hepatocytes and in human hepatoma cells that cAMP induces ALAS gene through protein kinase A (PKA) [13], and insulin represses it through extracellular-signal regulated kinase (ERK) and protein kinase B (PKB) activation [9]. These regulatory events are mediated through a region comprising 450 bp of ALAS promoter. The cAMP response is elicited through the binding of CREB to the two CRE sites located at −149 and −45 bp, respectively [13]. Moreover, insulin exerts its end point response by modulating hepatic nuclear factor 3 (HNF3) transactivation ability and through a *cis*-acting insulin-response element (IRE), present at −389 bp [10].

Here, we explored and characterized the pathways and effector targets involved in the transduction of TPA signal on ALAS gene regulation. We analyze the participation of TPA-responsive PKC isoforms, and the involvement of PI3K, ERK, and JNK pathways. To achieve this, we combined the transient overexpression of mutant versions of effector proteins involved in these pathways, and the use of small cell permeant protein inhibitors in HepG2 cells. Our findings indicate that among the TPA-responsive PKC isoforms, activation of PKCα is required for the transduction of the signal. Moreover, PI3K is involved, needless the participation of its downstream effector, PKB. The ERK, activated in a MAPK/ERK kinase (MEK)-independent manner, also contributes to the transduction of this signal. Finally, the Jun component of AP-1 complex needs to be induced and activated by JNK. This complexity indicates that activation of multiple pathways is jointly required for TPA-mediated repression of ALAS gene.

Materials and methods

Expression vectors

The following expression vectors were used as indicated in each experiment. Deletion mutant plasmid p-354ALAS/CAT, derived from parental pALAS/CAT containing the 5′-flanking region (−833 to +42 bp) of rat ubiquitous ALAS gene cloned upstream the CAT reporter gene in vector pBLCAT6, was described previously [13]. Vectors encoding wild type, constitutively active or kinase dead mutants of α, δ or ε PKC isoforms [14] were kindly provided by Dr. Shigeo Ohno (Yokohama City University, Kanagawa, Japan).

Vectors pCMV3 myc-myrPI3K (myrPI3K) and pCMV3 myc-PI3KR947P kinase dead (kdPI3K), described by Anderson et al. [15], were a generous gift from Dr. K. Anderson (The Babraham Institute, Cambridge, UK). A pCEFL vector encoding wild type (wtPKB), or kinase inactive (substitution K179M, designated kdPKB) mutant of PKB [16]. Vectors pCEV29 and pcDNA3 encoding constitutively active (RasV12) and dominant-negative (RasN17) Ras mutants [17]. A pcDNA3 vector encoding dominant-negative mutant form of c-Raf (substitution K375W, designated dnRaf) [18]. A pcDNA3 vector encoding dominant-negative mutant (MEK A) or constitutively active mutant (MEK E) of MEK [18]. A pcDNA1 vector encoding a wild type (designated wtMAPK) or a kinase inactive (designated kdMAPK) mutant of ERK2, described by Coso et al. [19]. A vector pEBG SEKKR, encoding an inactive kinase form of SEK [20]. All these plasmids were kindly supplied by Dr. Omar Coso and Dr. J. Silvio Gutkind (NIDCR, NIH, Bethesda, USA). A pK3H vector encoding a wild type (designated wtp90) or constitutively active mutants of $p90^{RSK2}$ carrying a point mutation (substitution Y707A, designated p90Y707A) or truncation (p90Δα) of the autoinhibitory α-helix in the carboxyl-terminal tail [21] were a gift from Dr. Thomas Sturgill (University of Virginia, Virginia, USA). JIP-1 vector encoding for the JNK-interacting protein-1 [22]. A pRc/RSV vector encoding the cDNA for the wild type version of CBP was kindly provided by Dr. Paolo Sassone-Corsi (IGBMC, Strasbourg, France). The pCMV/A-Fos (designated A-Fos) is a cytomegalovirus-driven expression vector, in which the normal basic region critical for DNA binding at the N-terminus of the Fos leucine zipper was replaced for an acidic sequence (a generous gift from Dr. Charles Vinson, NCI, NIH, Bethesda, USA) [23]. Plasmid pCEFL containing the β-galactosidase gene and puroBABE vector, which conveys resistance to puromycin, were also used.

To perform transfection assays, plasmids were purified using the Wizard Plus Maxipreps (Promega). DNA concentration was estimated spectrophotometrically.

Cell culture and treatments

The human hepatoma cell line HepG2 was grown as monolayer cultures in minimum essential medium supplemented with 10%(v/v) heat-inactivated fetal calf serum and 1% penicillin–streptomycin, 100 μM non-essential aminoacids, and 2 mM glutamine (Invitrogen). Cells were cultured in a humidified atmosphere with 5% CO_2 at 37 °C. In each case, chemical inhibitors were tested in a broad range of concentrations. Effectiveness of kinase inhibitors was assessed by in vitro kinase assay as described by Scassa et al. [9].

Transient transfection experiments

HepG2 transient transfections were performed according to the standard calcium phosphate precipitation method as previously described [13]. In brief, 4 μg of p-354ALAS/CAT and 6 μg of pCEFLβgal were cotransfected into 5×10^5 cells plated on 35-mm petri dishes. The β-galactosidase plasmid was used as the internal standard to normalize transfection efficiency. The use of other cotransfected plasmids is indicated in each experiment and the amount of each expression vector used in the different experiments was previously determined through dose–response curves. The optimal quantity of each of the mentioned plasmids was cotransfected into HepG2 cells along with p-354ALAS/CAT and CAT expression was measured. Final DNA concentration was adjusted to 20 μg/35-mm dish with non-specific DNA carrier. Control transfections with carrier alone and carrier plus vector pBLCAT6 were done in parallel. Sixteen hours later, the medium was replaced for 3 ml of serum free medium, containing the reagents indicated in each experiment, and incubated for 24 h. Then, cells were harvested, and CAT activity was measured in cell extracts as described previously [13], according to the Seed and Sheen phase-extraction method [24]. The β-galactosidase activity was determined spectrophotometrically in the transfected cell extracts. CAT activity was expressed as the amount of radiolabeled chloramphenicol acetylated by 1 mg of protein in 1 min and normalized with β-galactosidase activity. β-Galactosidase activity was not modified by any of the treatments used. The protein concentration of the cell extracts was determined by the Bradford assay [25].

RNA isolation and Northern blot analysis

Total cellular RNA was isolated from transfected and cultured HepG2 cells according to Chomczinsky and Sacchi [26]. Transfections were performed with LipofectAMINE Plus reagent (Invitrogen) following the manufacturer's protocol. HepG2 cells were cotransfected with 2 μg of kdPKCα, kdPI3K, SEK KR, or kdMAPK along with 1 μg puroBABE. In parallel, cells were transfected with puroBABE alone. Puromycin (Sigma) (2.5 μg/ml) was added 24 h after transfection. Resistant clones were harvested 72 h later. Twenty-four hours before harvesting, the cells were placed in serum free medium and incubated with or without TPA for the last 8 h. As indicated in some of the experiments, 200 nM wortmannin, 25 μM SP600125, or 10 μM PD98059 were added 30 min prior to TPA addition. Wortmannin was replenished during treatment. The yield and purity of RNA samples were assessed by absorbance at 260 nm and the ratio of absorbance at 260 and 280 nm, respectively. For Northern blot analysis, 10 μg of total RNA was denatured, electrophoresed

in 1% glyoxal/agarose gels, and transferred to nylon membranes (Hybond N, Amersham Biosciences). The membranes were sequentially hybridized with ^{32}P-labeled probes to ALAS and β-tubulin. To detect ALAS mRNA, a 26-mer oligodeoxynucleotide was synthesized complementary to bases −328 to −353 of human hepatic ALAS mRNA [27]. Oligodeoxynucleotide was end-labeled using [γ-^{32}P]ATP (specific activity 222 TBq/mmol) (NEN Life Science Products) and T4 polynucleotide kinase (New England Biolabs). The resulting probe had a specific activity of about 4–6×10^3 cpm/fmol. Hybridization was carried out overnight at 70 °C in the same prehybridization solution by adding the ^{32}P-labeled oligodeoxynucleotide. To detect β-tubulin mRNA, β-tubulin cDNA was labeled by random priming using [α-^{32}P]dCTP (specific activity 111 TBq/mmol) (NEN Life Science Products) and Klenow to a specific activity of about $5–7 \times 10^8$ cpm/μg. Membranes were stripped, prehybridized, hybridized, and washed in standard conditions as described previously [9]. The membranes were scanned directly onto a Bio-Imaging Analyzer Fujifilm LAS-1000.

Data analysis

The experiments were carried out at least three times. Samples in each group of experiments were performed in duplicate. All data were expressed as means ± SEM. When statistical analysis was performed, data were compared by the paired Student's *t* test, and *p* values below 0.05 were considered significant.

Results

The PKCα isoform is involved in TPA inhibition of ALAS gene transcription

In a previous work, we demonstrated that TPA represses the expression of ALAS at the transcriptional level, and that this effect reflects a physiologic process rather than a general inhibitory action due to phorbol ester treatment [28]. In addition, we found that the TPA-response element (TRE), located at −261 to −255 bp of ALAS promoter, is crucial for TPA regulation [7].

Having determined that PKC mediates the inhibitory effect of TPA [7], we further investigated which isoform was involved in this regulatory process. To assess this, we first tested the effect of the overexpression of PKC mutants without kinase activity [14]. These mutants were shown to function as dominant-negative proteins [29]. We analyzed the effect of TPA responsive PKC isoforms that are expressed in hepatocytes and HepG2 cells [30]. As shown in Fig. 1A, overexpression of the PKC mutant corresponding to the α isoform completely abolished TPA inhibitory effect, indicating that PKCα activity is essential for the transduction of this signal. On the other hand, CAT activity was unaffected by the overexpression of the mutants corresponding to either, δ or ε isoforms. It should be pointed that overexpression of these mutants produced slight differences in the basal levels of promoter activity (data not shown). This effect is consistent with the involvement of the different PKC isoforms in several transduction pathways, cascades that would

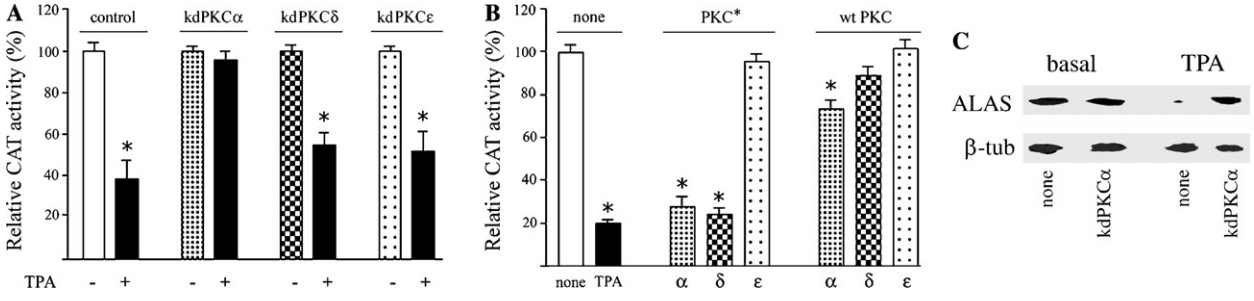

Fig. 1. PKCα is involved in TPA inhibition of ALAS gene transcription. (A) HepG2 cells were transfected with 4 μg per plate of p-354ALAS/CAT and cotransfected with 6 μg of expression vectors for kdPKCα, kdPKCδ or kdPKCε, respectively, or non-cotransfected (control). Transfected cells were incubated in serum-free medium with or without (basal) 1 μM TPA for 24 h. Results are expressed as relative CAT activity with respect to the corresponding basal values, which were set to 100. Bars represent means ± SEM of three independent experiments performed in duplicate. Student's *t* test was used to compare TPA-treated samples with non-treated (basal) samples (*$p < 0.05$). (B) HepG2 cells were transfected with 4 μg per plate of p-354ALAS/CAT and cotransfected with 6 μg of expression vectors for wtPKC or constitutively active mutants (PKC*) of α, δ or ε PKC isoforms, or non-cotransfected (control). Transfected cells were incubated in serum-free medium with or without (basal) 1 μM TPA for 24 h. Results are expressed as relative CAT activity with respect to basal value of the control group, which was set to 100. Bars represent means ± SEM of three independent experiments performed in duplicate. Student's *t* test was used to compare TPA-treated or cotransfected samples with non-treated (basal) and non-cotransfected (control) samples (*$p < 0.05$). (C) HepG2 cells were transfected with the expression vector encoding for puroBABE, which conveys resistance to puromycin or puroBABE plus kdPKCα. Twenty-four hours after transfection, cells were treated for an additional 60 h with puromycin. Resistant cells were placed in serum-free medium and incubated in the presence or absence of 1 μM TPA for the last 8 h. Total RNA (10 μg) was separated on denaturing agarose gels, blotted onto nylon membrane, and hybridized to ^{32}P-labeled probe to ALAS as described under "Materials and methods." Hybridization with a β-tubulin probe shows equal RNA loading. A representative autoradiograph of three independent experiments with similar results is shown.

be acting in concert to achieve the appropriate ALAS basal expression.

To assess if the overexpression of constitutively active mutants of PKC would mimic TPA inhibitory effect, we cotransfected HepG2 cells with p-354ALAS/CAT and constitutively active mutants of the aforementioned PKC isoforms. These PKC mutants contain single or double point mutations in the inhibitory pseudosubstrate sequence within the regulatory domain that generate constitutively active proteins with an open conformation [14]. As shown in Fig. 1B, overexpression of constitutively active mutants of both, α or δ isoforms, repressed ALAS gene as effectively as TPA treatment did. However, overexpression of wild type version of the different PKC isoforms did not have any considerable effect, with the exception of the α isoform that produced a slight decrease on the levels of reporter transcription. These data confirm that the activity of PKCα is necessary for TPA-mediated suppression of ALAS gene expression, but we cannot discard the hypothesis that PKCδ is involved, although its activity would be dispensable.

The ability of the PKCα negative mutant to abolish TPA-dependent inhibition of p-354ALAS/CAT activity prompted us to examine whether overexpression of this mutant version in HepG2 cells correspondingly suppresses TPA-dependent inhibition of the endogenous ALAS gene. As shown in Fig. 1C, the decrease in ALAS mRNA level observed after TPA treatment was prevented in cells that overexpressed kdPKCα.

PI3K activity is essential for TPA-mediated ALAS repression

Activation of conventional PKC isoforms requires three phosphorylation events; one of them is achieved by the 3-phosphoinositide-dependent kinase-1 (PDK-1) [31]. The activation of this kinase is often PI3K-dependent, although it has been reported a model in which PDK-1 phosphorylates conventional PKCs by a phosphoinositide-independent mechanism [32]. In addition, several reports provide evidences that PI3K can be activated by phorbol esters [33,34]. We wondered whether PI3K activation was necessary for TPA inhibition of ALAS gene expression. To assess this, we transfected HepG2 cells with p-354ALAS/CAT and incubated them in the presence or absence of 1 μM TPA and 200 nM wortmannin, a PI3K inhibitor that binds covalently to the p110 catalytic subunit [35]. As shown in Fig. 2A, wortmannin completely blocked TPA repression of CAT activity in a dose-dependent manner. To confirm these results, we tested the effect of the overexpression of a mutant form of PI3K catalytic subunit, p110, lacking kinase activity (kdPI3K) [15]. As shown in Fig. 2B, the mutant protein avoided TPA inhibitory effect in a dose-dependent manner, supporting the notion that PI3K activity is required for the transduction of the signal. To challenge this hypothesis, we cotransfected HepG2 cells with p-354ALAS/CAT along with myristoylated PI3K (myrPI3K), which is recruited to membrane by the addition of a N-terminal myristoylation/palmitoylation sequence from the Yes Src-Type kinase, rendering a constitutively active PI3K [15]. Overexpression of this PI3K version caused a drop in CAT activity, without reaching the levels of repression exerted by the phorbol ester (Fig. 2C). This partial inhibitory effect could be due to, at least, two reasons. First, TPA could be activating pathways other than PI3K; so, activation of PI3K would be only one of the events triggered by TPA treatment. Second, it has been reported that this post-translational modification of PI3K is not sufficient to activate all the putative intermediaries that lie downstream this kinase [36].

PKB/Akt has been shown to be a downstream effector of PI3K when stimulated by the action of different signaling molecules [37]. To determine whether PKB/Akt activation is required for TPA repression of ALAS gene, we cotransfected HepG2 cells with an expression vector encoding for a mutant form of PKB without kinase activity or for wild type PKB, and p-354ALAS/CAT reporter vector. Cotransfection of either kinase dead or wild type PKB/Akt did not modify the activity of ALAS promoter, neither in the presence, nor in the absence of TPA (data not shown).

The physiological relevance of the above results was challenged by testing the effect of wortmannin or by the overexpression of kdPI3K on ALAS mRNA. As assessed by Northern blot analysis the addition of 200 nM wortmannin or the overexpression of kdPI3K markedly impaired TPA-dependent inhibition of ALAS transcription. Moreover, neither PI3K inhibitor nor the overexpressed mutant altered ALAS mRNA levels under basal conditions (Fig. 2D). As a whole, these data indicate that PI3K activity is necessary for TPA-mediated suppression of ALAS gene expression, but PKB/Akt would not be a crucial mediator in this process.

ERK1/2 mediates TPA inhibition of ALAS gene transcription

A common transduction pathway driving ERK activation often involves Ras, Raf, and MEK proteins. The participation of this transduction pathway in TPA repression of ALAS gene was analyzed by preventing the activation of different components of this circuit. First, we blocked Ras farnesylation with two structurally unrelated farnesyltransferase inhibitors, lovastatin and PD152440. Pretreatment with any of these two inhibitors lowers the pool of intracellular Ras available for subsequent activation by growth factors and abolished the stimulation of MAPK activity [38]. The results summarized in Table 1 show that none of these

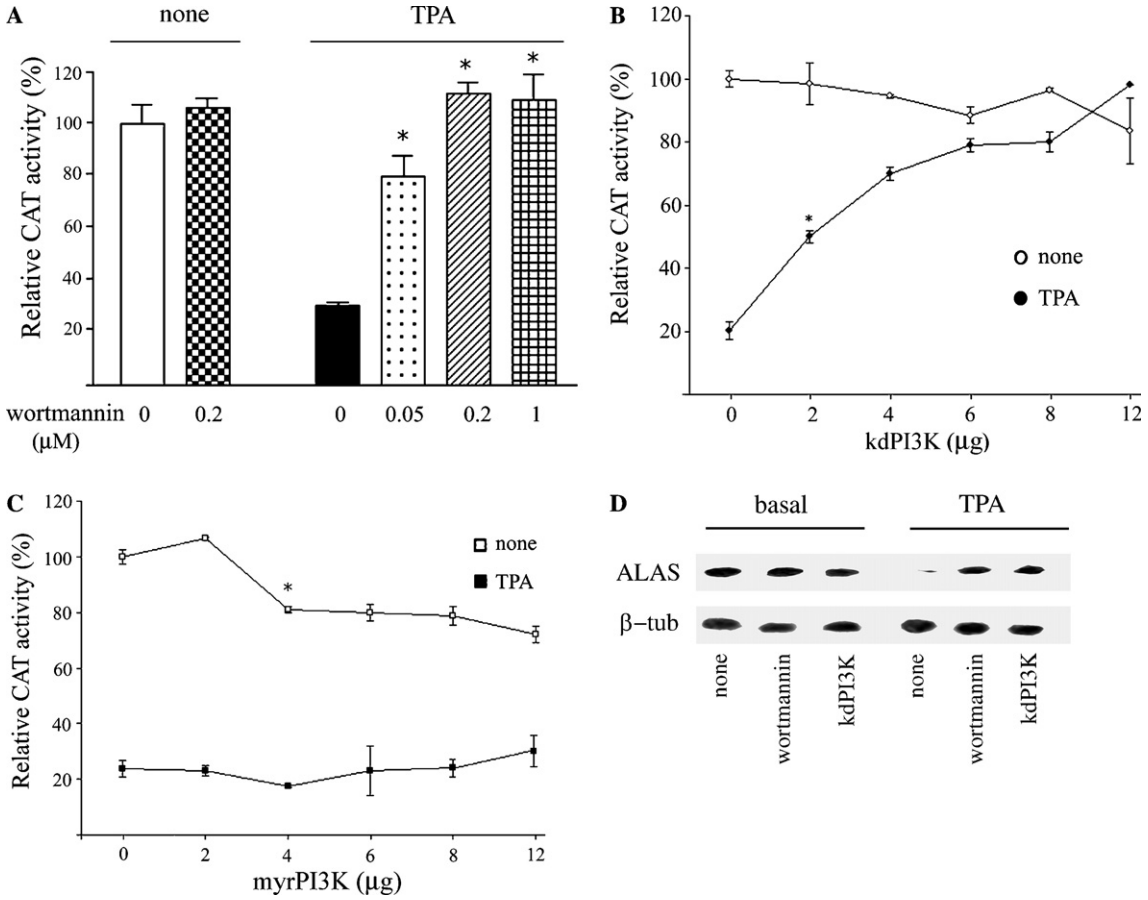

Fig. 2. PI3K is required for TPA inhibition of ALAS promoter transcriptional activity. (A) HepG2 cells were transfected with 4 μg per plate of p-354ALAS/CAT. Transfected cells were incubated in serum-free medium with or without 1 μM TPA, and 0, 0.05, 0.2 or 1 μM wortmannin for 24 h. Since this compound has been shown to lose effectiveness after incubation at physiological pH [54], it was replenished during treatment. Results are expressed as relative CAT activity with respect to the control basal value, which was set to 100. Bars represent means ± SEM of three independent experiments performed in duplicate. Student's *t* test was used to compare wortmannin-treated with non-treated samples (*$p < 0.05$). (B) HepG2 cells were transfected with 4 μg per plate of p-354ALAS/CAT and cotransfected with different amounts of kdPI3K. Transfected cells were incubated in serum-free medium with (●) or without (○) 1 μM TPA for 24 h. Results are expressed as relative CAT activity with respect to the control basal value, which was set to 100. Each point represents mean ± SEM of three independent experiments performed in duplicate. Student's *t* test was used to compare samples cotransfected with kdPI3K with non-cotransfected samples (*$p < 0.05$, indicates the minimum kdPI3K amount that causes a significant blockage of TPA repression). (C) HepG2 cells were transfected with 4 μg/plate of p-354ALAS/CAT and cotransfected with different amounts of myrPI3K. Transfected cells were incubated in serum-free medium with (■) or without (□) 1 μM TPA for 24 h. Results are expressed as relative CAT activity with respect to the control basal value, which was set to 100. Each point represents mean ± SEM of three independent experiments performed in duplicate. Student's *t* test was used to compare samples cotransfected with myrPI3K with non-cotransfected samples (*$p < 0.05$, indicates the minimum myrPI3K amount that causes a significant repression). (D) HepG2 cells were transfected with the expression vector encoding for puroBABE, which conveys resistance to puromycin or puroBABE plus kdPI3K. Twenty-four hours after transfection, cells were treated for an additional 60 h with puromycin. Resistant cells were placed in serum-free medium and incubated in the presence or absence of 1 μM TPA for the last 8 h. Total RNA (10 μg) was separated on denaturing agarose gels, blotted onto nylon membrane, and hybridized to ^{32}P-labeled probe to ALAS as described under "Materials and methods." Hybridization with a β-tubulin probe shows equal RNA loading. A representative autoradiograph of three independent experiments with similar results is shown.

inhibitors modified basal or TPA-repressed ALAS promoter activity. In addition, experiments carried out using a specific inhibitor of MEK, PD98059, demonstrated that MEK would not be an essential component of this signal because treatment of transfected cells with this agent did not modify either basal or TPA-repressed CAT activity (Table 1).

In a different approach, we transiently cotransfected HepG2 cells with plasmids encoding dominant-negative mutant forms of factors involved in the transduction and regulation of signals through the Ras/MAPK pathway, either in the presence or absence of TPA. For the experiments, whose results are shown in Table 1, HepG2 cells were cotransfected with p-354ALAS/CAT and expression vectors for dominant-negative Ras (RasN17), Raf (dnRaf), or MEK (MEK A). The expression of any of these proteins had no effect on basal or TPA-repressed p-354ALAS/CAT expression. The aforementioned results suggest that signaling through Ras, Raf, and MEK is not essential in TPA repression of ALAS gene.

Table 1
Activation of Ras, Raf or MEK is not essential for TPA-mediated ALAS repression

Treatment	Relative CAT activity (%)						
	Treatment				Transfection		
	None (control)	Lovastatin	PD152440	PD98059	RasN17	dnRaf	MEK A
None (basal)	100 ± 4	116 ± 18	114 ± 5	99 ± 3	107 ± 3	104 ± 4	106 ± 4
TPA	32 ± 6	30 ± 8	31 ± 5	32 ± 4	28 ± 3	36 ± 3	20 ± 6

HepG2 cells were transfected with 4 μg per plate of p-354ALAS/CAT and 6 μg of expression vectors encoding for RasN17, dnRaf or MEK A, as indicated. Transfected cells were incubated in serum-free medium with or without (basal) 1 μM TPA and 500 nM lovastatin, 10 μM PD152440 or 10 μM PD98059, as indicated, for 24 h. Results are expressed as relative CAT activity with respect to the control basal value, which was set to 100. Values represent means ± SEM of three independent experiments performed in duplicate. Student's *t* test was used to compare samples cotransfected or inhibitor-treated with their respective control sample (basal or TPA-treated). No statistical significances were observed.

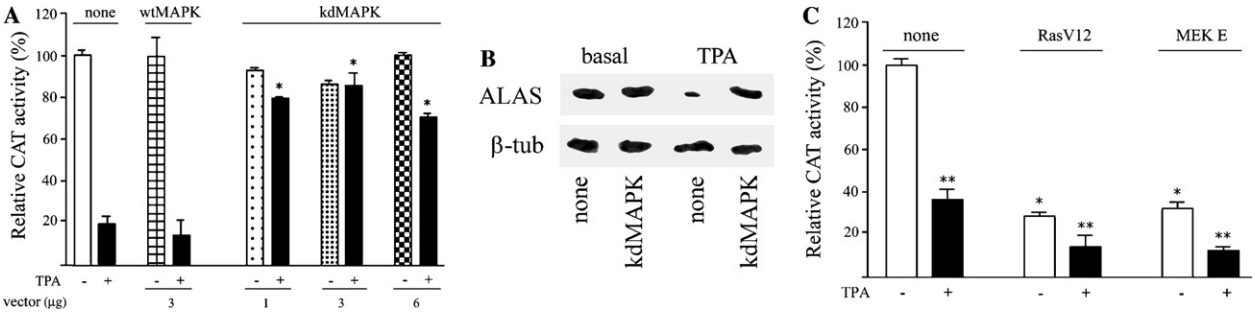

Fig. 3. ERK is required for TPA repression. (A) HepG2 cells were transfected with 4 μg per plate of p-354ALAS/CAT and cotransfected with different amounts of wtMAPK or kdMAPK. Transfected cells were incubated in serum-free medium with or without 1 μM TPA for 24 h, as indicated. Results are expressed as relative CAT activity with respect to the control basal value, which was set to 100. Bars represent means ± SEM of three independent experiments performed in duplicate. Student's *t* test was used to compare TPA-treated cotransfected samples with TPA-treated control (non-cotransfected) samples (*$p < 0.05$). (B) HepG2 cells were transfected with the expression vector encoding for puroBABE, which conveys resistance to puromycin or puroBABE plus kdMAPK. Twenty-four hours after transfection, cells were treated for an additional 60 h with puromycin. Resistant cells were placed in serum-free medium and incubated in the presence or absence of 1 μM TPA for the last 8 h. Total RNA (10 μg) was separated on denaturing agarose gels, blotted onto nylon membrane, and hybridized to ^{32}P-labeled probe to ALAS as described under "Materials and methods." Hybridization with a β-tubulin probe shows equal RNA loading. A representative autoradiograph of three independent experiments with similar results is shown. (C) HepG2 cells were transfected with 4 μg per plate of p-354ALAS/CAT and cotransfected with 6 μg of RasV12 or MEK E. Transfected cells were incubated in serum-free medium with or without 1 μM TPA for 24 h, as indicated. Results are expressed as relative CAT activity with respect to the control basal value, which was set to 100. Bars represent means ± SEM of three independent experiments performed in duplicate. Student's *t* test was used to compare non-treated cotransfected samples with basal control samples (*$p < 0.05$) or TPA-treated samples with the corresponding non-treated samples (**$p < 0.05$).

Although ERK1/2 can be activated by alternative pathways, most of them remain elusive, and many involve the activation of conventional PKC isoforms [39]. To investigate the role of ERK in this regulatory process, we cotransfected expression vectors for wild type (wtMAPK) or a kinase dead version of ERK2 (kdMAPK) along with p-354ALAS/CAT reporter vector into HepG2 cells. As shown in Fig. 3A, cotransfection with kdMAPK blocked the TPA repression of the reporter vector. In contrast, cotransfection with wtMAPK showed no significant effects. None of them modified promoter basal activity. These results strongly suggest that ERK1/2 would be crucial for the transduction of the signal. To further establish the role of ERK relevant to TPA inhibition of ALAS gene transcription, we performed Northern blot analysis from kdMAPK overexpressing HepG2 cells. These experiments revealed that the presence of the MAPK mutant hindered TPA action on ALAS gene (Fig. 3B). We next overexpressed constitutively active forms of Ras (RasV12) or MEK (MEK E), which activate downstream components independently of signaling input. Cotransfection of HepG2 cells with p-354ALAS/CAT and RasV12 or MEK E repressed reporter expression as effectively as treatment with TPA did (Fig. 3C).

As a whole, these results indicate that ERK is necessary for TPA-mediated negative regulation of ALAS promoter activity, and that it would be activated by a Ras/Raf/MEK-independent pathway. As seen in Fig. 3C, ALAS promoter activity displayed further decrease upon TPA treatment of cotransfected cells. This additive effect suggests that an additional pathway other than ERK1/2 would be involved. Evidences for the implication of other cascades are described below

Constitutively active p90^{RSK2} mimics TPA-mediated repression of ALAS promoter

The p90^{RSK} proteins, members of one of the subfamilies of MAPK-activated protein kinases (MAPKAPKs),

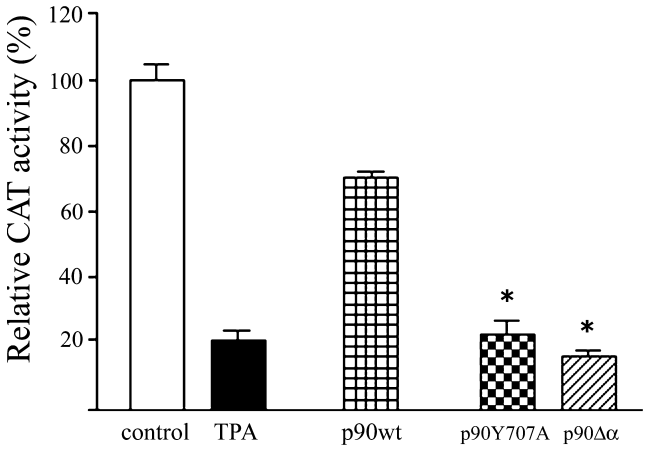

Fig. 4. Constitutively active $p90^{RSK2}$ mimics TPA-mediated repression of ALAS promoter. HepG2 cells were transfected with 4 μg per plate of p-354ALAS/CAT and cotransfected with 3 μg of expression vectors for wild type (wtp90) or constitutively active forms for $p90^{RSK2}$ (p90Y707A or p90Δα) Transfected cells were incubated in serum-free medium with or without 1 μM TPA for 24 h, as indicated. Results are expressed as relative CAT activity with respect to the control basal value, which was set to 100. Bars represent means ± SEM of three independent experiments performed in duplicate. Student's *t* test was used to compare the samples with the control (non-cotransfected) TPA-treated samples (*$p < 0.05$ indicates no significance).

are involved in regulating survival and proliferation, and are activated by a myriad of stimuli, including TPA [40]. For its complete activation two phosphorylation events are necessary, one achieved by ERK and the other by PDK-1 [41].

As we have demonstrated above that ERK and PI3K pathways are involved in TPA repression, we hypothesized that $p90^{RSK}$ would be a convergent point between these two pathways. To explore the contribution of this kinase on TPA regulation of ALAS gene, we cotransfected expression vectors for wild type $p90^{RSK2}$ or two different constitutively active mutants (p90Δα or p90Y707A) along with p-354ALAS/CAT into HepG2 cells. As shown in Fig. 4, overexpression of either $p90^{RSK2}$ constitutively active mutant produced a decrease in CAT activity, similar to that obtained with TPA treatment. On the other hand, cotransfection of $wtp90^{RSK2}$ produced only a slight decrease in ALAS promoter activity. These results support the hypothesis about $p90^{RSK2}$ mediating TPA inhibition of ALAS gene expression.

JNK activity is crucial for the transduction of the signal

As c-Jun and Jun D are transcription factors involved in this regulatory process [7], we then asked if the JNK is responsible for the induction and activation of these AP-1 components. To assess this query, we analyzed the effect of TPA treatment on HepG2 cells transfected with p-354ALAS/CAT in the presence of the JNK-specific inhibitor, SP600125 [42]. As shown in Fig. 5A, treatment with this agent prevented TPA repression in a dose-dependent manner without affecting the basal transcriptional activity. To corroborate these results, we use two different strategies in order to impede JNK activation. In one experiment, we cotransfected HepG2 cells with an expression vector encoding a kinase dead, dominant-negative version of stress-activated protein kinase (SAPK)/ERK kinase-1 (SEK 1), SEK KR [20], and in another, we overexpressed the scaffold protein JNK-interacting protein-1 (JIP-1). The presence of this scaffold protein, in non-stoichiometric amounts, leads to JNK cascade inactivation, by titration of endogenous components [43]. As depicted in Figs. 5B and C, overexpression of either the kinase inactive SEK KR, or JIP-1 blocked phorbol ester inhibitory effect in a dose-dependent way.

Finally, we asked whether the blockage of JNK would affect the regulation of endogenous ALAS exerted by TPA. To address this question we performed Northern blot analysis from HepG2 cells treated with 25 μM SP600125 or transfected with SEK KR expression vector. As depicted in Fig. 5D, under these conditions, the ability of TPA to block ALAS gene expression was impaired. These results demonstrate the requirement of JNK activity for the transduction of this signal.

How do these effectors crosstalk?

At this point, having a whole picture of the participating factors in the transduction of TPA signal driving ALAS gene repression, we wondered how are these factors related. To gain insight into this issue, we performed a group of assays where TPA repression was mimicked by overexpression of the constitutively active $p90^{RSK}$ mutant, p90Y707A, and, simultaneously, the activity of a particular effector was suppressed. In each case, to impair the activity of the selected intermediary, we used either a specific inhibitor, or overexpressed a dominant-negative mutant.

In the first set of experiments the activity of PKC, ERK or PI3K was blocked, by overexpression of kdPKCα, kdMAPK or wortmannin treatment, respectively. As seen in Fig. 6A, inactivation of each of these kinases avoided TPA repression. Importantly, no effect upon the promoter activity was observed in the presence of overexpressed p90Y707A. These results strongly suggest that PKCα, ERK, and PI3K would lie upstream of $p90^{RSK}$, in this signal transduction. In a second set of experiments, JNK activity was blocked by treatment with SP600125 or by overexpression of a c-Fos mutant version, A-Fos, that impairs functional AP-1 assembly, thus preventing its binding to DNA [44]. In this case, either blockage of AP-1 assembly or inhibition of JNK activity avoided repression produced by both, TPA treatment or p90Y707A overexpression (Fig. 6B). These results indicate that JNK and AP-1 activation are downstream or

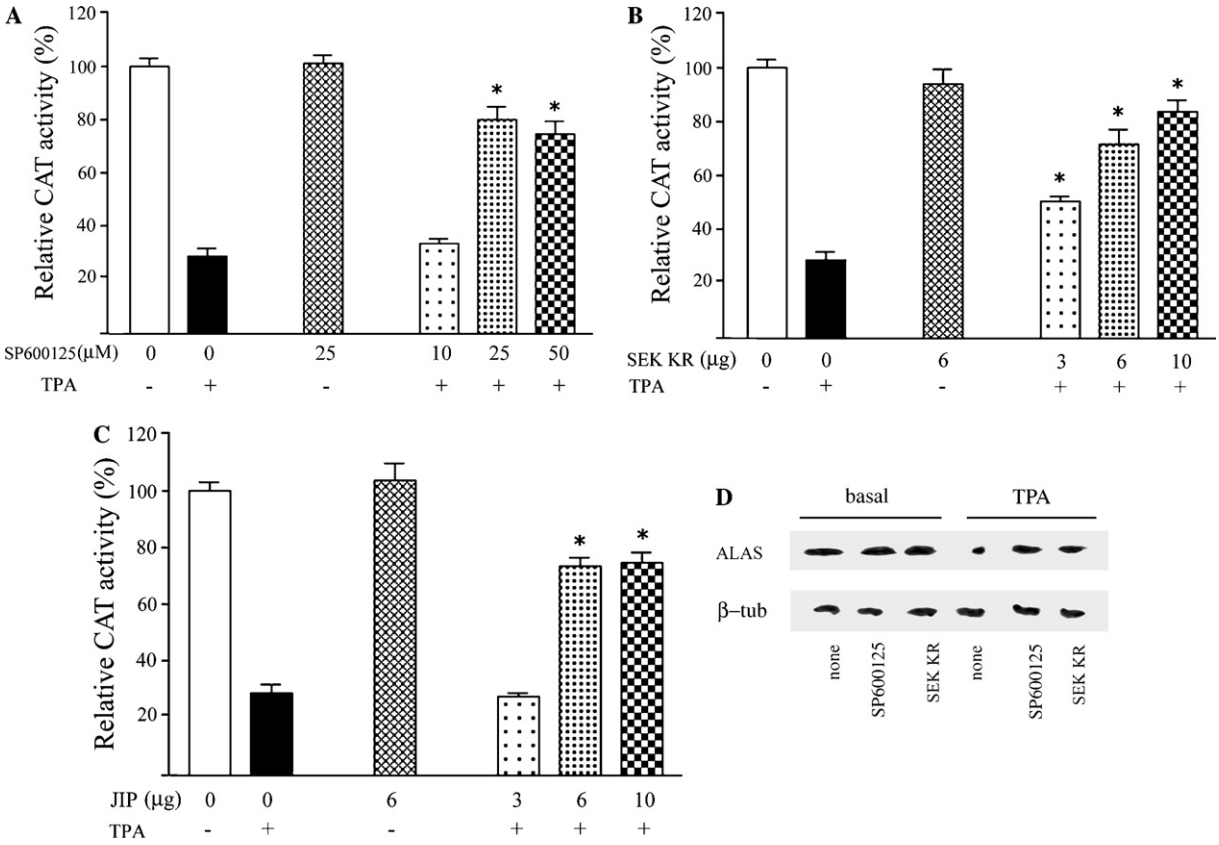

Fig. 5. JNK is required for TPA inhibition of ALAS transcription. (A) HepG2 cells were transfected with 4 μg per plate of p-354ALAS/CAT. Transfected cells were incubated in serum-free medium with or without 1 μM TPA, and the indicated amounts of the JNK inhibitor, SP600125 for 24 h. Results are expressed as relative CAT activity with respect to the control basal value, which was set to 100. Bars represent means ± SEM of three independent experiments performed in duplicate. Student's *t* test was used to compare SP600125 and TPA-treated samples with control TPA-treated samples (*$p < 0.05$). (B) HepG2 cells were transfected with 4 μg per plate of p-354ALAS/CAT and cotransfected with the indicated amounts of SEK KR. Transfected cells were incubated in serum-free medium with or without 1 μM TPA for 24 h, as indicated. Results are expressed as relative CAT activity with respect to the control basal value, which was set to 100. Bars represent means ± SEM of three independent experiments performed in duplicate. Student's *t* test was used to compare TPA-treated cotransfected samples with the TPA-treated control samples (*$p < 0.05$). (C) HepG2 cells were transfected with 4 μg per plate of p-354ALAS/CAT and cotransfected with the indicated amounts of an expression vector for the scaffold protein JIP-1. Transfected cells were incubated in serum-free medium with or without 1 μM TPA for 24 h, as indicated. Results are expressed as relative CAT activity with respect to the control basal value, which was set to 100. Bars represent means ± SEM of three independent experiments performed in duplicate. Student's *t* test was used to compare TPA-treated cotransfected samples with TPA-treated control samples (*$p < 0.05$). (D) HepG2 cells were transfected with the expression vector encoding for puroBABE, which conveys resistance to puromycin or puroBABE plus SEK KR. Twenty-four hours after transfection, cells were treated for an additional 60 h with puromycin. Resistant cells were placed in serum-free medium and incubated in the presence or absence of 1 μM TPA for the last 8 h. Total RNA (10 μg) was separated on denaturing agarose gels, blotted onto nylon membrane, and hybridized to ^{32}P-labeled probe to ALAS as described under "Materials and methods." Hybridization with a β-tubulin probe shows equal RNA loading. A representative autoradiograph of three independent experiments with similar results is shown.

are independent events with respect to $p90^{RSK}$ activation. In other words, if JNK pathway is blocked or formation of a functional AP-1 dimer is prevented, the activation of $p90^{RSK}$ is not enough to mimic TPA repression. It should be noted that none of the treatments affected the basal transcriptional activity (Figs. 6A and B).

In a previous work, we demonstrated that the decrease in ALAS basal activity observed in the presence of TPA is due to CBP sequestration by the AP-1 dimer bound to the TRE located in ALAS promoter. This event diminishes the ability of this promoter to assemble the productive pre-initiation complex that involves CBP interaction with CREB dimers bound to CRE sites present on it. TPA repression of ALAS gene was largely relieved when CBP was ectopically expressed [7]. To reinforce the whole model, we analyzed the effect of p90Y707A overexpression in the presence of non-limiting amounts of CBP. In agreement with our model, overexpression of CBP prevented repression produced by both TPA treatment or p90Y707A overexpression, without modifying basal CAT activity (Fig. 6B), supporting our proposed mechanism involving competition between CREB and AP-1 for CBP coactivator [7]. It is important to note the significant induction of ALAS promoter activity observed in the presence of p90Y707A and CBP overexpression. Under this experimental condition, the

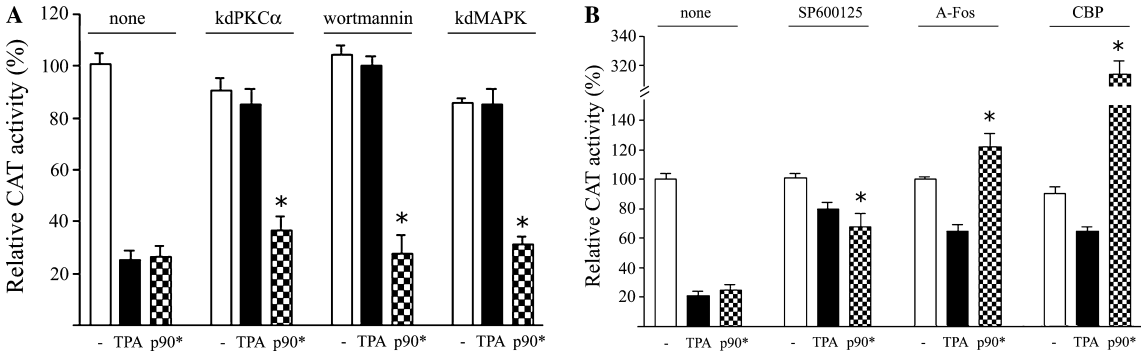

Fig. 6. p90^{RSK2} would lie downstream PKCα, PI3K, and ERK, and upstream AP-1 activation in the signaling pathway. (A) HepG2 cells were transfected with 4 μg per plate of p-354ALAS/CAT and cotransfected with 3 μg of p90Y707A (p90∗) and 6 μg of kdPKCα or 3 μg of kdMAPK, as indicated. Transfected cells were incubated in serum-free medium with or without 1 μM TPA and/or 200 nM wortmannin for 24 h, as indicated. Results are expressed as relative CAT activity with respect to the control basal value, which was set to 100. Bars represent means ± SEM of three independent experiments performed in duplicate. Student's *t* test was used to compare p90Y707A-cotransfected samples with the corresponding TPA-treated samples (*$p < 0.05$). (B) HepG2 cells were transfected with 4 μg per plate of p-354ALAS/CAT and cotransfected with 3 μg of p90Y707A (p90∗) alone or plus 6 μg of A-Fos, or 4 μg of pRc/RSV CBP, as indicated. Transfected cells were incubated in serum-free medium with or without 1 μM TPA and/or 25 μM SP600125 for 24 h, as indicated. Results are expressed as relative CAT activity with respect to the control basal value, which was set to 100. Bars represent means ± SEM of three independent experiments performed in duplicate. Student's *t* test was used to compare p90Y707A-expressing samples that were treated with SP600125, or cotransfected with A-Fos or CBP, with the control p90Y707A-cotransfected samples (*$p < 0.05$).

transcriptional activity achieved would resemble the one obtained upon signal triggered by cAMP agonists [13].

Discussion

The work reported here was designed to identify the molecular link between the TPA signaling pathway and the transcription of ALAS gene in human hepatoma HepG2 cells. Our previous results identified a proximal TRE on ALAS promoter, where AP-1 binds, as the main target for TPA action [7]. The well-documented interaction of phorbol esters with PKC [45] suggests that protein phosphorylation is involved in the mechanism by which TPA suppresses ALAS mRNA transcription. The results of the assays using mutant forms of different TPA-responsive PKC isoforms [30], indicate that the activity of PKCα is crucial for TPA repression (Fig. 1). With regard to PI3K role, our results from experiments using wortmannin or kdPI3K indicate that this protein is also involved in the transduction of this signal (Fig. 2). Ectopic expression of mutant forms or utilization of inhibitors of MAPK cascade components revealed that ERK1/2 became an essential mediator. Importantly, in this case, this kinase is activated in a Ras/Raf/MEK-independent manner (Table 1). Although several stimuli lead to ERK activation through the aforementioned cascade, there are alternative pathways that may participate; most remain elusive and many involve conventional PKC isoforms [39]. Our results suggest that p90^{RSK}, the direct target of ERK1/2, would be involved in this event, since ectopic expression of p90^{RSK2} constitutively active mutants mimicked TPA action (Fig. 4). We propose that it would be one of the kinases involved in the induction and activation of c-Fos component of AP-1 dimers. We have previously elucidated that c-Jun and Jun D integrate this AP-1 complex. Three different experimental approaches, treatment with a JNK inhibitor, ectopic expression of SEK KR, and overexpression of JIP-1 support the finding that JNK is an essential mediator of this regulatory process (Fig. 5). With regard to p90^{RSK}, we conjectured that it could be a convergent point between PI3K and ERK pathways. There are many evidences that support our premise. First, the overexpression of p90^{RSK2} constitutively active mutant forms completely mimicked the effect of TPA. In contrast, overexpression of myrPI3K only caused a partial inhibition (Fig. 3C). Moreover, ALAS repression elicited by ERK activation showed an additive effect upon TPA treatment in cotransfected cells. As a whole, these facts confirm that TPA would be activating both pathways. The sole activation of one of them, ERK or PI3K, is not enough to mimic TPA repression, but the activation of the putative convergent effector, p90^{RSK}, would be sufficient, even though if the activation of PKC, PI3K or ERK is impaired (Fig. 6A), in agreement with the fact that activation of p90^{RSK} would be a downstream event. Conversely, if JNK pathway is blocked or functional AP-1 dimers are impeded to assemble, activation of p90^{RSK} is not enough to resemble TPA action (Fig. 6B). These findings indicate that activations of both p90^{RSK} and JNK are necessarily independent events, which jointly lead to AP-1 assembly on the *cis*-element present in the promoter. On the other hand, when p90Y707A was overexpressed along with CBP, the repression exerted by this kinase was impaired, reinforcing our model involving competition between CREB and AP-1 for CBP [7]. Interestingly, in this scenario ALAS

promoter activity was markedly induced, resembling the signal triggered by cAMP [13]. One possible explanation would be that this cellular context mimics in part, the one achieved upon cAMP stimulation. As it has been reported, activated $p90^{RSK}$ phosphorylates CREB in Ser 133 [46], enabling CREB to recruit CBP [47]. Finally, CBP recruitment by phosphorylated CREB dimers bound in ALAS promoter CRE sites would be favored because, in these conditions, the coactivator is present in non-limiting amounts. So, the enhanced gene expression observed would be due to the unperturbed CBP recruitment to ALAS promoter CRE sites.

In several promoters TPA resembles insulin action. Particularly, in ALAS gene, both molecules markedly repress transcriptional promoter activity [8,28]. TPA exerts its action through a *cis*-acting element, TRE, and *trans*-acting factors, AP-1; and insulin through an IRE, and HNF3 and nuclear factor 1 (NF-1) [7,10]. Previously, we found that both pathways, PI3K and ERK/$p90^{RSK}$, are jointly required for insulin-mediated ALAS gene repression [9]. Although the activation of these pathways is critical to propagate the signal triggered by insulin and TPA, different actors elicit the end point response. Thus, we asked which were the key divergent network points that determine the difference. On the one hand, having established the involvement of PI3K pathway in TPA repression, we determined that PKB/Akt is not an essential mediator in this route, but results indispensable for insulin action. On the other hand, whereas in insulin-mediated ERK/$p90^{RSK}$ activation, Ras/Raf/MEK participation is crucial; in TPA stimulation these effectors result dispensable. Thus, in this case, signaling triggered by these two molecules bifurcates downstream PI3K and induces ERK by different pathways. However, activation of PKC is indispensable for the transduction of both signals, in insulin-mediated regulation, this kinase targets remain unclear [8].

At this point, a tentative model emerges, that points to the crosstalk among the participating cascades. Upon stimulation, this phorbol ester would trigger the activation of PKCα and PI3K. Hence, activation of PI3K increases the intracellular pools of PIP_3 leading to PDK-1 activation, contributing to PKCα stimulation. Once activated, PDK-1 phosphorylates $p90^{RSK}$ [41]. Additionally, TPA would also promote ERK1/2 phosphorylation, probably through PKCα, in a Ras/Raf/MEK-independent manner, then, activated ERK phosphorylates $p90^{RSK}$ [48]. Once, undergoing these two post-translational modifications, $p90^{RSK}$ is able to catalyze c-Fos activation [49]. Upon signaling input, PI3K would also stimulate a separate pathway that renders an activated JNK [50], that induces and transactivates Jun component of AP-1 [51]. Finally, the propagated signal would allow the assembled dimer to determine the transcriptional response upon its binding to the TRE present on ALAS promoter.

ALAS repression can have important physiologic outcomes. Its imbalanced expression results in a deficit of vital hemeproteins [11]. Reactive oxygen species can cause oxidative DNA damage and deregulated cell signaling which are involved in carcinogenesis. Various reported types of cancer manifest an imbalance in their antioxidant mechanisms [52], suggesting that this DNA damage would lead to enhanced tumor promotion susceptibility. It has been reported that apoptotic processes are associated with genetic mechanism involving this type of DNA damage [53]. Thus, it is conceivable to think that TPA-mediated ALAS repression could be one of the mechanisms leading to either tumorigenesis or apoptosis triggered by phorbol esters. The cause could be the high levels of DNA injury due to the increase of free radicals produced by TPA in conjunction with the decrease in the amount of hemeproteins crucial for reactive oxygen species neutralization. In summary, we have delineated a novel mechanism driving TPA action on ALAS promoter. Further identification of unknown TPA gene targets should help to understand the complexity of its signaling.

Acknowledgments

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