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Differentiation-associated genes regulated by TPA-induced c-Jun expression via a PKC/JNK pathway in KYSE450 cells

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Abstract

A group of potential differentiation-associated genes had been identified by microarray analysis as c-Jun/AP-1 target genes essential for epithelial differentiation program. Our previous study showed that c-Jun/AP-1 could bind and activate these gene promoters in vivo using chromatin immunoprecipitation. To further understand how the mitogen-activated protein kinase signaling pathways regulate AP-1 activity and expression of c-Jun target genes, our strategy was based on the use of 12-*o*-tetradecanoylophorbol-13-acetate (TPA) and pharmacological reagents to induce or block c-Jun expression. The mRNA and protein expression of these genes increased in response to TPA-induced c-Jun/AP-1 expression. Inhibitors of JNK (SP600125) and PKC (GF109203X) mainly blocked expression and phosphorylation of c-Jun, while inhibition of MEK–ERK activity with PD98059 (an inhibitor of MEK) had little effect. Expression of involucrin and keratin 4 in response to TPA was attenuated by pretreatments with GF109203X and SP600125, but not PD98059, suggesting involvement of PKC and JNK in this response. Taken together, these results suggested that differentiation-associated genes were regulated by TPA-induced c-Jun/AP-1 mainly via a PKC/JNK pathway in esophageal cancer cell line KYSE450.
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Keywords: c-Jun/AP-1; Differentiation-associated genes; MAPK; Esophageal cancer

Esophageal squamous epithelium can be divided into two zones: basal zone and differentiated zone [1]. Cell proliferation is frequent in the epibasal layers, and migration from this area eventually triggers differentiation [2]. Our previous study indicated that a group of differentiation-associated genes were coordinately down-regulated in human esophageal squamous cell carcinoma (ESCC) [3]. A common feature of the promoter regions of these genes, including SPRRs (small proline-rich proteins), keratins, cystatin A, and involucrin, is the presence of AP-1 DNA-binding sites [4–9].

Transcription factor AP-1 is mainly composed of Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra-1, and Fra-2) [10–12]. c-Jun is the critical component of transcription factor AP-1 that composed of homo- or heterodimers of basic region-leucine zipper (bZIP) proteins that belong to the Jun, Fos, ATF, and Maf subfamilies [13–16]. The AP-1 transcription complex is associated with diverse cellular processes such as differentiation, proliferation, transformation and apoptosis. Dimers recognize the 12-*o*-tetradecanoylophorbol-13-acetate response elements (TRE, 5′-TGAG/CTCA-3′) in the gene promoter based on their ability to mediate transcriptional induction in response to phorbol esters (TPA). c-Jun/AP-1 activity is regulated by post-translational modifications, such as phosphorylation, acetylation, and ubiquitination [17–23]. The mechanism of post-translational control is most extensively documented in the case of mitogen- and cellular stress-induced hyperphosphorylation, in particular, the activation of c-Jun through the c-Jun N-terminal kinase (JNK).

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Mitogen-activated protein kinase (MAPK) pathways consist of extracellular signal-regulated kinase (ERK1/2), JNK, and p38 [24]. JNKs comprise three isoforms: JNK1, JNK2, and JNK3, and are activated by various extracellular stimuli including growth factor, heat shock, phorbol esters, and UV light [25–28]. c-Jun becomes transcriptionally active upon phosphorylation at Ser63 and Ser73 within its N-terminal transactivation domain by JNK, thereby enhanced its transactivation potential [29,30]. Upon activation, c-Jun/AP-1 regulated the expression of a large number of AP-1 target genes in both a positive and negative manner and had a role in a variety of cellular processes, including growth, differentiation, apoptosis, and survival [31,32].

In this study, we investigated whether the induction of AP-1 activity by TPA might contribute to the expression of differentiation-associated genes in KYSE450 cells. TPA, a potent activator of PKC, can activate expression of differentiation-associated genes. Activation of MAPK pathways was followed by acquisition of c-Jun/AP-1 DNA-binding and transactivation capacities. Based on this finding, we further evaluated how the mitogen-activated protein kinase (MAPK) signaling pathways regulate AP-1 activity. Our strategy was based on the use of TPA and pharmacological reagents to induce or block c-jun expression. The results demonstrate that JNK is essential for a basal level of c-Jun expression and for c-Jun phosphorylation in response to TPA. Overall, these data provide important insights into the mechanisms that ultimately determine the function of c-Jun as a regulator of cell differentiation in esophageal cancer.

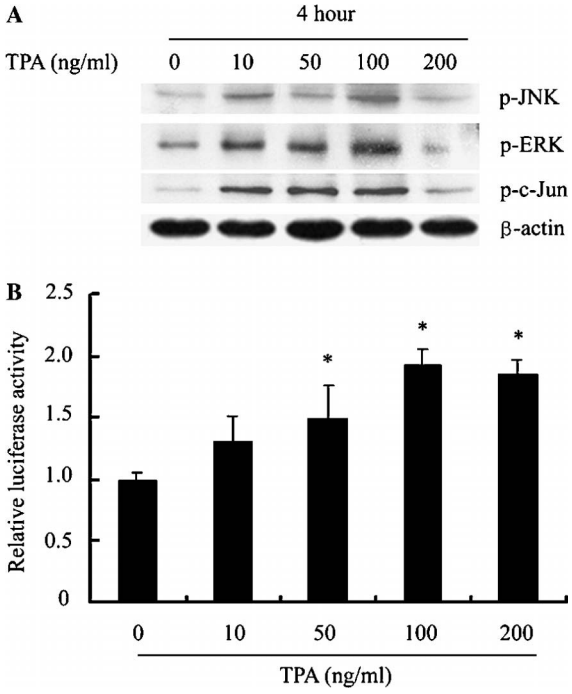

Fig. 1. TPA induces phosphorylation and activation of c-Jun/AP-1, ERK, and JNK. (A) Lysates from KYSE450 cells treated with TPA (10, 50, 100, and 200 ng/ml) for 4 h were probed with antibodies against p-JNK, p-ERK, and p-c-Jun, respectively. (B) AP-1 transactivation activity was analyzed by dual luciferase reporter assay system. KYSE450 cells were transiently co-transfected with 200 ng pAP-1-luc and 20 ng pRL-CMV. After 24 h, cells were treated with different concentrations of TPA (10, 50, 100, and 200 ng/ml) for 4 h. CMV-driven *Renilla* luciferase vector was used as an internal control. AP-1 activity was quantified in terms of the firefly luciferase activity. The data are representative of three independent experiments done in triplicate (mean values ± standard error, $^{*}p < 0.05$).

Materials and methods

Cell culture and materials. The human esophageal squamous cell carcinoma cell line KYSE450 was generous gift from Dr. Shimada Y (Kyoto University, Japan). The cells were maintained in a RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO_2 incubator. 12-*o*-Tetradecanoylophorbol-13-acetate (TPA) was purchased from Sigma (Sigma–Aldrich, Inc. St. Louis, USA). PKC inhibitor (GF109203X), MEK inhibitor (PD98059), and JNK inhibitor (SP600125) were obtained from Calbiochem (EMD Biosciences, Inc. San Diego, CA, US).

Preparation of protein extracts and immunoblotting. Cell lysates were prepared using the lysis buffer (50 mM Tris, pH 8, 150 mM NaCl, 1% NP-40, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml each of aprotinin, leupeptin, and pepstatin A (Sigma–Aldrich)). Protein concentration was determined using BCA Protein Assay Kit (Pierce Biotechnology, Inc. Rockford, USA). For immunoblotting analysis, equal amounts of proteins were loaded onto SDS–PAGE gels for electrophoresis and then transferred to PVDF membranes (Amersham Biosciences, Inc. Buckinghamshire, UK). The membranes were incubated at 4 °C with primary antibodies against JNK (sc-7345), p-JNK (sc-6254), c-Jun (sc-1694), p-c-Jun (sc-822), p-ERK (sc-7383) (Santa Cruz Biotechnology, Santa Cruz, CA), keratin 4 (ab11215) (Abcam Ltd., Cambridge, UK) involucrin (MS-126) (Lab Vision Corporation, Fremont, CA, USA), and β-actin (A5441) (Sigma–Aldrich), respectively. The blots were probed with the ECL Western blot detection system according to the manufacturer's instructions.

Plasmid constructions and luciferase assays. pAP-1-luc was a generous gift from Dr. Zhang L (Beijing Institute of Radiation Medicine, China). Cells (10^4) were seeded in 96-well plate and transfected with promoter

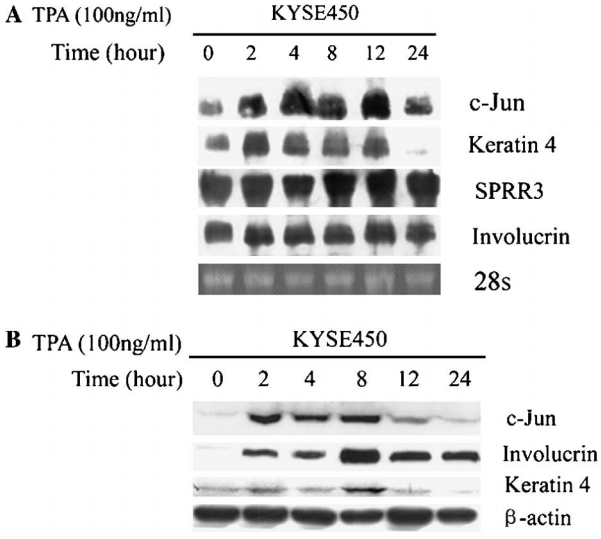

Fig. 2. c-Jun/AP-1 regulates transcription and expression of differentiation-associated genes. (A) Northern blot analyzed the time-dependent induction of c-Jun/AP-1 and differentiation-associated genes in response to TPA in KYSE450 cells. 28s rRNA was used as a loading control. (B) Cytoplasmic and nuclear proteins were extracted from KYSE450 cells treated with 100 ng/ml TPA at the indicated time and probed with antibodies against c-Jun, c-Fos, involucrin, and keratin 4. β-actin served as a loading control.

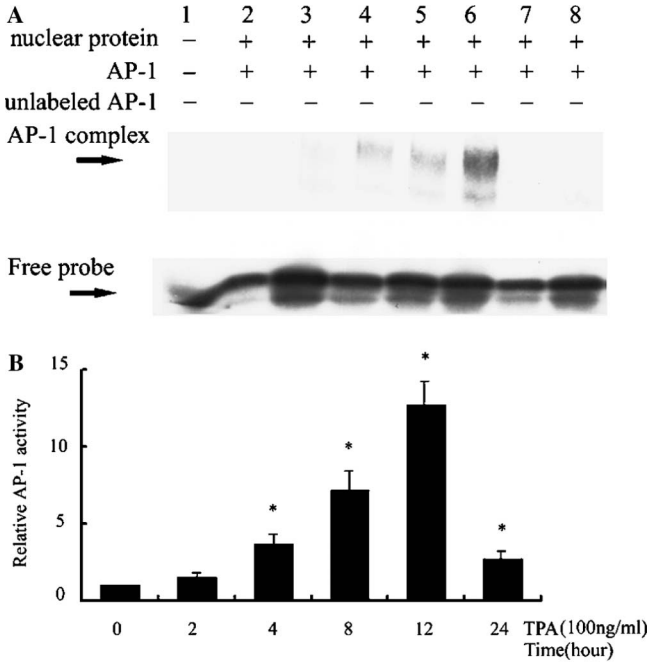

Fig. 3. c-Jun/AP-1 DNA-binding activity and transactivation activity are induced by TPA. (A) Five micrograms of nuclear extracts prepared from TPA-treated KYSE450 cells was used for electrophoretic mobility shift assays, as indicated. Lane 1, the negative control; lane 2, nuclear extracts from cells treated with TPA for 8 h were incubated with a biotin-labeled AP-1 oligonucleotide and a 100-fold excess of unlabeled AP-1 oligonucleotide; lane 3, nuclear extracts from cells treated with vehicle were incubated with a biotin-labeled AP-1 oligonucleotide; lanes 4–8, nuclear extracts from cells treated with TPA for different times (2h, 4, 8, 12, and 24 h) were incubated with a biotin-labeled AP-1 oligonucleotide. (B) AP-1 transactivation activity was analyzed by dual luciferase reporter assay system. KYSE450 cells were transiently co-transfected with 200 ng pAP-1-luc and 20 ng pRL-CMV. After 24 h, cells were treated with 100 ng/ml TPA for different times (2–24 h). $^{*}p < 0.05$.

constructs using lipofectamine™2000 (Invitrogen, Inc., Carlsbad, CA, USA) according to the manufacturer's recommendation. The activities of both firefly and *Renilla* luciferases were determined 48 h after transfection with the dual luciferase reporter assay system (Promega, Inc. San Luis Obispo CA, US). The luciferase activities were normalized to the *Renilla* luciferase activity of the internal control. The data were representative of three independent experiments done in triplicate (mean values ± standard error, $^{*}p < 0.05$).

Electrophoretic mobility shift assays. Nuclear extracts were obtained from KYSE450 cells using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology) according to the protocol. After determining protein concentrations using BCA Protein Assay Kit (Pierce Biotechnology), electrophoretic mobility shift assays (EMSA) was performed according to the protocol (Pierce Biotechnology). Briefly, 5 μg of equal amounts of nuclear extracts was incubated with 20 μl reaction buffer (50 ng/μl poly([dI–dC]), 5 mM $MgCl_2$, 2.5% glycerol, 0.05% NP-40, and 1× binding buffer) at room temperature for 20 min. 3′-End biotin-labeled double-stranded oligonucleotide 5′-CGCTTGATGAGTCAGCC GGAA-3′ was synthesized (Takara Biotechnology, Inc. Dalian, China). For specific competition assay, a 100-fold excess of unlabeled oligonucleotide was added to the binding reaction mixture. The samples were electrophoresed on a 6% nondenaturing polyacrylamide gel. The membranes were subjected to chemiluminescent nucleic acid detection module.

Northern blot. Total RNA was extracted with Trizol reagent (Invitrogen) according to the manufacturer's recommendation. Fifteen micrograms of total cellular RNA each lane was separated electrophoretically on a 1% denaturing formaldehyde–agarose gel, transferred to Hybond N^{+} nylon membrane (Amersham Biosciences). The membranes were hybridized with biotin-labeled cDNA probe synthesized using Biotin DecaLabel™DNA Labeling kit (Fermentas, Inc. Hanover, MD, USA) and subjected to chemiluminescent nucleic acid detection module.

JNK kinase assay. This was performed with the JNK activity assay kit (EMD Biosciences, Inc. San Diego, CA, USA) according to the manufacturer's instructions. In brief, KYSE450 cells were treated with 100 ng/ml TPA for 2–24 h and lysed with 200 μl of ice-cold JNK extraction buffer on ice for 5 min. Two hundred micrograms of cellular extracts was incubated with JNK antibody at 4 °C. The immunoprecipitates (IP) were then subjected to SDS–PAGE and probed with antibody against p-c-Jun.

Results

c-Jun/AP-1 regulates transcription and expression of the differentiation-associated genes

It is known that transcription factor AP-1 translocates to the nucleus following exposure of cells to a variety of extracellular stimuli such as TPA, leading to activation and transcription of its target genes. To determine the effective concentration of TPA on the activation of c-Jun/AP-1, we analyzed the expression of p-ERK, p-JNK, and p-c-Jun in KYSE450 cells treated with 10, 50, 100, and 200 ng/ml TPA for 4 h. As it is shown in Fig. 1, AP-1 activity was induced in a dose-dependent manner and reached maximal levels with 100 ng/ml TPA addition.

To further elucidate whether c-Jun/AP-1 as transcriptional activator regulates expression of the differentiation-associated genes, we treated KYSE450 cells with 100 ng/ml TPA for different times (2–24 h) and determined expression of these genes by Northern blot and Western blot. The increase in the transcription of c-Jun was sustained for a long period of time (2–12 h), peaking between 4 and 12 h

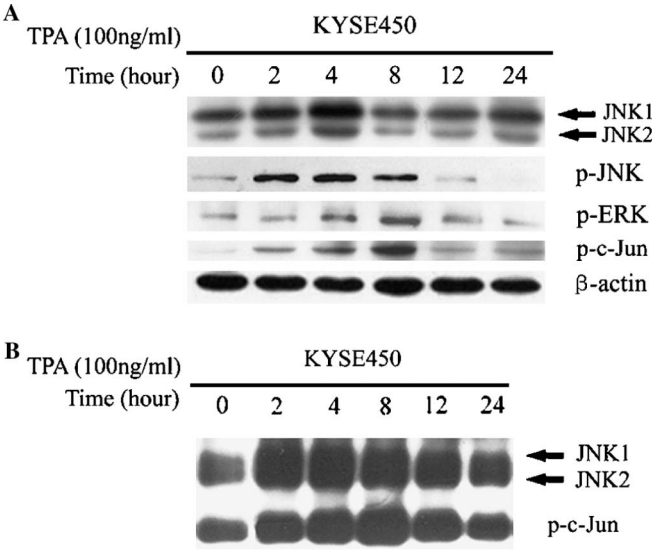

Fig. 4. TPA-triggered activation of c-Jun/AP-1 via MAPK pathways. (A) Lysates from KYSE450 cells treated with 100 ng/ml TPA for different times (2–24 h) were probed with antibodies against JNK, p-JNK, p-ERK, p-c-Jun, and β-actin, respectively. (B) JNK activity was measured by protein kinase assay. Cellular lysates were immunoprecipitated with antibody against JNK, as indicated. The immunoprecipitates (IP) were then subjected to SDS–PAGE analysis and probed with antibody against p-c-Jun.

and returning to basal levels 24 h after TPA treatment. Consistent with c-Jun/AP-1, transcription of differentiation-associated genes including SPRR3, keratin 4, and involucrin, was induced rapidly in response to TPA stimulation. The transcription of keratin 4 was transient and returning to basal levels 24 h after TPA addition, whereas the transcription of involucrin and SPRR3 was sustained and reached maximal levels 12–24 h after TPA addition (Fig. 2A). The kinetics of expression of c-Jun protein was analyzed by Western blot. c-Jun protein expression was induced 2–12 h after TPA stimulation. In agreement with p-c-Jun, involucrin and keratin 4 protein expression peaked at 8 h (Fig. 2B). Taken together, c-Jun/AP-1 might be involved in the regulation of differentiation-associated genes in KYSE450 cells.

c-Jun/AP-1 DNA-binding activity and transactivation activity are mediated by TPA

To investigate whether increased expression of AP-1 is followed by acquisition of AP-1 DNA-binding activity, we performed EMSA. AP-1 DNA-binding activity was prominently induced after TPA addition. Activation was observed within 2–8 h TPA treatment (Fig. 3A). Next, to test whether AP-1 DNA-binding activity had functional consequences, we performed transient transfections with pAP-1-luc into KYSE450 cells to analyze AP-1 transactivation activity. Treatment with TPA caused a significant increase in AP-1 transactivation (Fig. 3B). Thus, the results suggested TPA-induced AP-1 activity in a time-dependent manner.

TPA-triggered activation of c-Jun/AP-1 via MAPK pathways

MAPKs are critical for the transmission of stimuli to the nucleus, resulting in modification of cell growth-related transcription factors such as c-Jun and c-Fos. To gain insight into the relationship between MAPK and c-Jun activation in KYSE450 cells, we investigated the phosphorylation state over a time course following TPA addition.

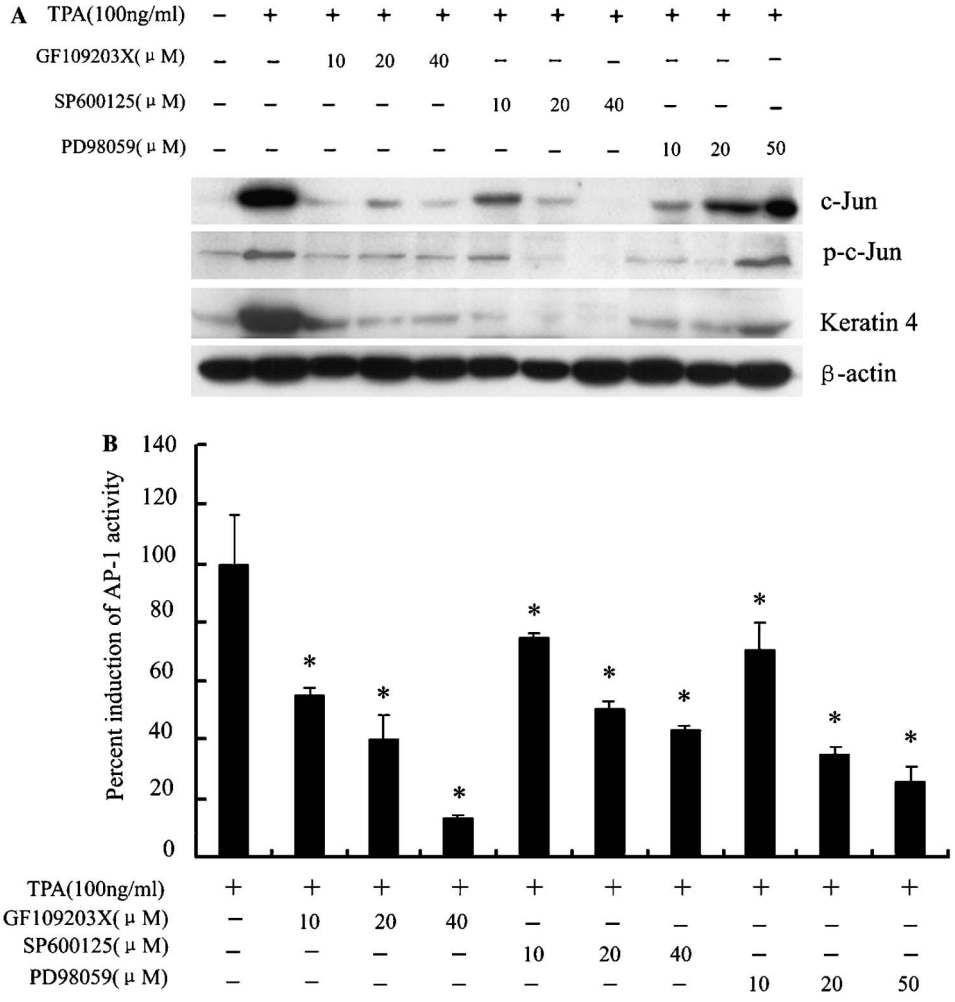

Fig. 5. MAPK inhibitors suppressed TPA-triggered activation of c-Jun/AP-1. (A) KYSE 450 cells were pretreated with PKC inhibitor GF109203X (10, 20, and 40 μM), JNK inhibitor SP600125 (10, 20, and 40 μM) and MEK inhibitor PD98059 (10, 20, and 50 μM), respectively, for 1 h and then treated with TPA for 4 h. Cellular lysate protein was extracted and probed with antibodies against c-Jun, p-c-Jun, keratin 4, and β-actin. (B) AP-1-driven luciferase reporter activity was assayed in KYSE450 cells transiently co-transfected with pAP-1-luc and pRL-CMV. After 24 h, cells were pretreated with various concentrations of inhibitors as indicated for 1 h and then treated with 100 ng/ml TPA for 4 h. $^{*}p < 0.05$.

The phosphorylation of endogenous JNK, ERK, and c-Jun proteins was coordinately increased and reached maximal levels from 4 to 8 h after TPA treatment of KYSE450 cells (Figs. 1A and 4). Consistently, JNK activity was elevated upon exposure of cells to TPA using protein kinase assay (Fig. 4B). Although the kinetics and amplitude of JNK and ERK activity were compatible with the phosphorylation of c-Jun upon TPA treatment, to seek the direct evidence of MAPK pathways involved in the TPA-stimulated expression of c-Jun/AP-1, we utilized PKC inhibitor (GF109203X), MEK inhibitor (PD98059), and JNK inhibitor (SP600125), respectively, to prevent MAPKs in response to TPA stimulation. SP600125 dose dependently inhibited phosphorylation of c-Jun. The different doses of GF109203X similarly inhibited phosphorylation of c-Jun, whereas partial inhibition of phosphorylation of c-Jun was observed at 10, 20 μM, at a concentration of 50 μM, PD98059 weakly blocked phosphorylation of c-Jun. The inhibition of keratin 4 was consistent with the change of p-c-Jun (Fig. 5A). We observed that 20 μM PD98059, SP600125, and GF109203X blocked the activity of c-Jun/AP-1 (~50%) (Fig. 5B).

To further investigate whether PKC and MAPK inhibitors can block expression of differentiation-associated genes, we pretreated KYSE450 cells with 20 μM PD98059, SP600125, and GF109203X, respectively. The inhibitors resulted in a strong though not complete inhibition of TPA-stimulated induction of c-Jun/AP-1 at 4 h after TPA addition (Fig. 6A). c-Jun/AP-1 DNA-binding activity was obviously blocked by GF109203X and SP600125, whereas weakly inhibited by PD98059 at 4–8 h after TPA addition. Moreover, AP-1 DNA-binding activity was weakly inhibited by GF109203X at 2 h after TPA addition (Fig. 6B). Inhibition of JNK and PKC mainly prevented expression and phosphorylation of c-Jun, while

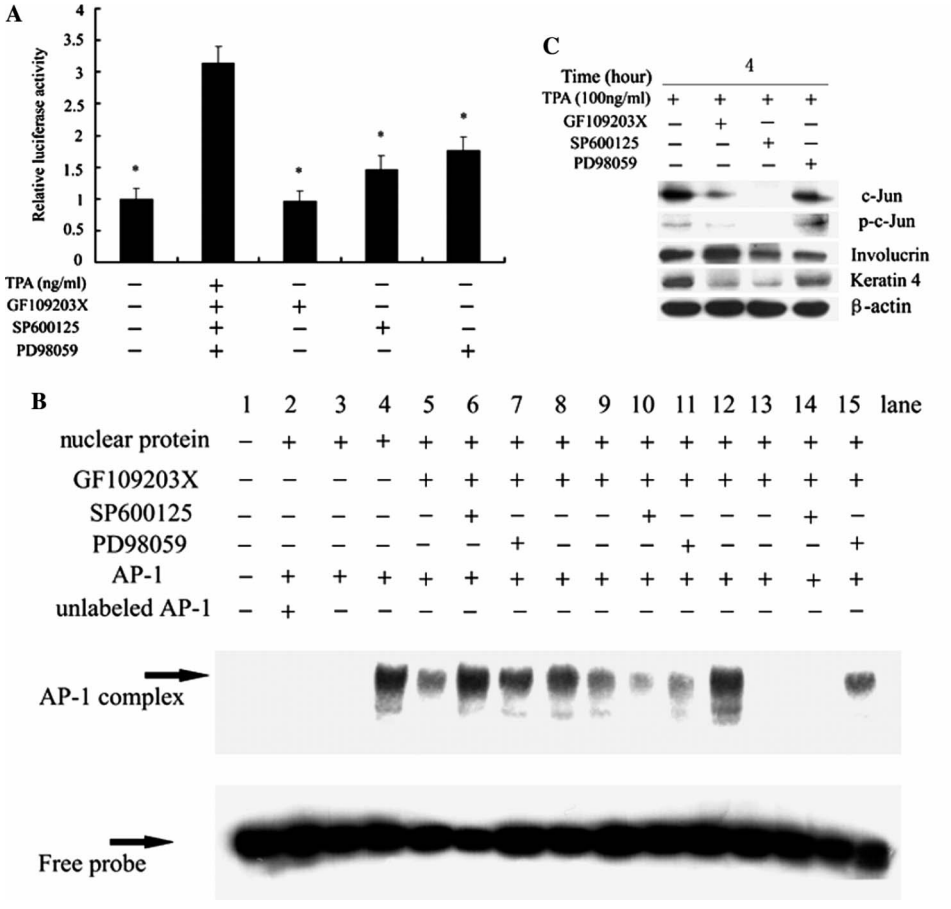

Fig. 6. MAPK inhibitors block TPA-mediated induction of differentiation-associated gene expression. (A) Transient co-transfection was made with pAP-1-luc and pRL-CMV in KYSE450 cells. After 24 h, cells were pretreated with GF109203X (20 μM), SP600125 (20 μM), and PD98059 (20 μM), respectively, for 1 h and then treated with 100 ng/ml TPA for 4 h. The AP-1 activity was detected using the dual luciferase reporter assay system. $^{*}p < 0.05$. (B) Nuclear extracts (5 μg) were used for electrophoretic mobility shift assays. Lane 1, the negative control. Lane 2, nuclear extracts from cells treated with TPA for 8 h were incubated with a biotin-labeled AP-1 oligonucleotide and a 100-fold excess of unlabeled AP-1 oligonucleotide. Lane 3, nuclear extracts from cells treated with vehicle were incubated with a biotin-labeled AP-1 oligonucleotide. Lanes 4–7, lanes 8–11, and lanes 12–15, nuclear extracts from cells pretreated with PKC inhibitor GF109203X (20 μM), JNK inhibitor SP600125 (20 μM), and MEK inhibitor PD98059 (20 μM) for 1 h before treated with 100 ng/ml TPA for 2, 4, and 8 h, respectively, were incubated with a biotin-labeled AP-1 oligonucleotide. (C) KYSE450 cells were pretreated with PKC, JNK, and MEK inhibitors, respectively, for 1 h and then treated with TPA for 4 h. Nuclear or cytoplasmic extracts were subjected to immunoblotting for detection of the expression of c-Jun, p-c-Jun, and target genes such as involucrin and keratin 4.

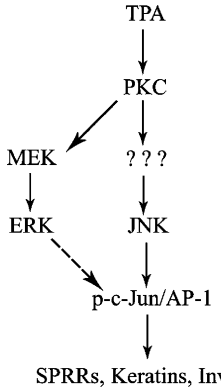

Fig. 7. Model of c-Jun/AP-1 regulating the expression of differentiation-associated genes via MAPK pathways. TPA activates ERK and JNK, leading to the subsequent increase of c-Jun/AP-1 expression and activity. Whereas only JNK pathway triggers c-Jun phosphorylation, ERK pathway shows a weak effect. Phosphorylation of c-Jun/AP-1 can bind to AP-1 DNA-binding sites on the promoter regions of differentiation-associated genes. The basal transcription machinery leads to an increase in the expression of SPRRs, keratins, and involucrin in KYSE450 cells.

inhibition of ERK pathway had a weak effect. Indeed, JNK inhibitor SP600125 suppressed expression of keratin 4 and involucrin. GF109203X can inhibit expression of keratin 4, whereas PD98059 can inhibit the expression of neither keratin 4 nor involucrin (Fig. 6C).

Taken together, the results strongly suggested that activation of JNK pathway was mainly required for the expression of c-Jun/AP-1 and differentiation-associated genes (Fig. 7).

Discussion

In our previous study, a group of potential differentiation-associated genes had been identified by microarray analysis as c-Jun/AP-1 target genes essential for epithelial differentiation program. ChIP assays showed that c-Jun/AP-1 could bind and activate these gene promoters in vivo (unpublished data). The data presented here demonstrated that TPA-induced c-Jun could regulate the expression of the differentiation-associated genes, however, the molecular signaling mechanism of TPA-induced c-Jun expression was poorly understood in esophageal cancer.

The conventional position of c-Jun/AP-1 in signal transduction cascades has been closer to the end of MAPK pathways such as ERK, JNK, and p38. Among the AP-1 subunits, c-Jun is a main component and thus plays a key role in response to a variety of extracellular stimuli. TPA is known to effectively induce AP-1 transcriptional activity through activating the JNK pathway that phosphorylates and activates c-Jun in various cell lines. We selected KYSE450 cells as a useful model to elucidate how MAPK cascades control gene expression via the transcriptional regulation of c-*jun*. It was indicated that in response to TPA, c-Jun was directly phosphorylated by PKC/JNK signaling. The inhibitors of PKC (GF109203X) and JNK (SP600125) can obviously block the DNA-binding activity and transactivation activity of AP-1 in KYSE450 cells induced by TPA. Nonetheless, inhibition of MEK–ERK activity with PD98059 had little effect. It is well documented that JNK phosphorylates c-Jun more efficiently than ERKs in vitro [33,34]. The phosphorylated c-Jun with enhanced transactivation and DNA-binding activity mediated signaling transductions, which control many different, sometimes opposing cellular processes [35]. Taken together, these results suggested that differentiation-associated genes were regulated by TPA-induced c-Jun/AP-1 mainly via a PKC/JNK pathway in esophageal cancer cell line KYSE450.

Activation of Jun N-terminal kinase leads to phosphorylation and thereby activation of transcription factor c-Jun/AP-1 and consequently alters gene expression. There are many nuclear Jun N-terminal kinase substrates including c-Jun, ATF-2, and Elk-1 proteins [36,37]. AP-1 proteins are the final target of signal-transducing kinase cascades and upon phosphorylation become transcriptionally active triggering the activity of AP-1-driven promoters and the expression of their corresponding regulated genes [38]. We demonstrated that the transcription level and protein expression of a group of differentiation-associated genes keratin 4, keratin 13, SPRR3, and involucrin increased in response to TPA stimulation. Notably, though phosphorylation of ERKs and JNKs is induced by TPA, only SP600125 pretreatment attenuates TPA-induced keratin 4 protein expression, suggesting an important role of JNK in TPA-induced keratin 4 expression in KYSE450 cells. Keratin 4 is highly expressed in the esophageal squamous suprabasal cells and its expression is associated with the switch from proliferation to differentiation [9]. Keratin 4 homozygous null mice have a phenotype largely restricted to the esophagus and cornea where there is impairment of the normal differentiation program [10]. Involucrin is a major component of the cornified envelope and is considered to be an appropriate marker for terminal differentiation. Our results suggested that c-Jun/AP-1 target genes were essential for esophageal squamous epithelium differentiation program.

In conclusion, we found that c-Jun/AP-1 orchestrated the modulation of a group of TPA-induced differentiation-associated genes in esophageal squamous cell carcinoma through PKC/JNK signaling pathway.

Acknowledgments

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