Original Paper

Journal of Biomedical Science

J Biomed Sci 2002;9:156–165

Received: July 17, 2001
Accepted: September 29, 2001

Functional Role of Extracellular Signal-Regulated Kinase Activation and c-Jun Induction in Phorbol Ester-Induced Promoter Activation of Human 12(*S*)-Lipoxygenase Gene

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Key Words
Phorbol 12-myristate 13-acetate · Extracellular signal-regulated kinase · c-Jun · Sp1 · 12(*S*)-Lipoxygenase

Abstract
The functional role of mitogen-activated protein kinase (MAPK) signaling and c-Jun induction in phorbol 12-myristate 13-acetate (PMA)-induced human 12(*S*)-lipoxygenase gene expression was studied in human epidermoid carcinoma A431 cells. Among the family of MAPK, PMA only increased the activity of extracellular signal-regulated kinase (ERK). Treatment of cells with PD98059, which is an inhibitor of mitogen-activated protein kinase kinase (MEK), decreased the PMA-induced expression of 12(*S*)-lipoxygenase. Transfection of cells with Ras, Raf and ERK2 dominant negative mutants inhibited the PMA-induced promoter activation of the 12(*S*)-lipoxygenase gene in all cases. PMA-induced expression of c-Jun was inhibited by pretreatment with PD98059. Following treatment with PMA, the interaction between c-Jun and simian virus 40 promoter factor 1 (Sp1) in cells increased with time. Enhancement of binding between the c-Jun-Sp1 complex and the Sp1 oligonucleotide was observed in cells treated with PMA, suggesting the possible interaction of c-Jun-Sp1 with GC-rich binding sites in the gene promoter. These results indicate that PMA treatment induced ERK activation mainly through the Raf-MEK-ERK signaling pathway following induction of c-Jun expression, and the formation of the c-Jun-Sp1 complex. Finally, PMA activated the promoter activity of the 12(*S*)-lipoxygenase gene in cells overexpressing protein kinase C (PKC)δ but not PKCα, indicating that PKCδ played the functional role in mediating the gene activation of 12(*S*)-lipoxygenase induced by PMA.

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Introduction

Arachidonate 12(*S*)-lipoxygenase (arachidonate: oxygen 12-oxidoreductase; EC 1.13.11.31) in a platelet was the first mammalian lipoxygenase discovered [19]. It catalyzes the transformation of arachidonic acid into 12(*S*)-hydroperoxyeicosatetraenoic acid. It is subsequently converted to 12(*S*)-hydroxyeicosatetraenoic acid by gluta-

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1021–7770/02/0092–0156$18.50/0
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thione-dependent peroxidase [4]. Human 12(*S*)-lipoxygenase has also been found in human erythroleukemia cells [16, 22], epidermal cells [34] and epidermoid carcinoma cells [3]. Overexpression of human 12(*S*)-lipoxygenase in germinal layer keratinocytes in psoriatic lesions has also been found [21]. Some findings have indicated that epidermal growth factor (EGF) induces microsomal 12-lipoxygenase activity in some types of cultured human epithelial cells [5, 33]. In addition, researchers have found that the growth-promoting effects of EGF may be modulated by 12-lipoxygenase activity in other cell types [18]. Overexpression of transforming growth factor (TGF)-α, which is an EGF homologue, has been found in inflamed psoriatic epidermis [14], further suggesting that this may be one of the factors responsible for the augmentation of 12-lipoxygenase expression in vivo. Application of phorbol ester to mouse skin has been shown to induce the formation of psoriasiform hyperplasia [20] and the expression of 8-lipoxygenase activity in an animal model [17]. Since 12(*S*)-lipoxygenase is a commonly overexpressed enzyme in human psoriatic epidermis, we used human epidermoid carcinoma A431 cells as a model to study the regulation of this enzyme. We previously found that phorbol 12-myristate 13-acetate (PMA) induced the expression of 12(*S*)-lipoxygenase mRNA in cells [28]. While studying the promoter regulation of the 12(*S*)-lipoxygenase gene, PMA [26], EGF [27] and TGF-α [9] all stimulated the promoter activation of the 12(*S*)-lipoxygenase gene in the same fashion in terms of the kinetic effect, the responsive region of the promoter and the interaction between nuclear simian virus 40 promoter factor 1 (Sp1) and promoter DNA as assayed by electrophoretic mobility shift analysis. Two Sp1 binding sites residing from −158 to −150 bp and −123 to −114 bp in the promoter were essential for the responses to PMA, EGF and TGF-α. No changes in the binding between nuclear Sp1 protein and Sp1 consensus sites of the promoter were observed in the control cells or the cells treated with PMA, EGF and TGF-α.

One of the early events in the EGF signaling pathway involves the coupling of EGF to receptor tyrosine kinase, which causes Ras activation by binding to adapter protein Grb2 and the exchange protein Sos. Ras subsequently leads to the activation of Raf-1. Raf-1 phosphorylates and activates mitogen-activated protein kinase kinase (MEK), which, in turn, phosphorylates and activates extracellular signal-regulated kinases 1 and 2 (ERK1; ERK2) [29]. Ras can also activate Rac and Rho, members of the Rho family of small GTPases [31]. Rac in turn activates a protein kinase cascade that leads to the activation of c-Jun amino-terminal kinase (JNK) [13]. We previously reported that the EGF-induced expression of 12(*S*)-lipoxygenase in A431 cells was mediated through the Ras-Raf-MEK-ERK and Ras-Rac-JNK signal pathways [7]. Subsequent induction of c-Jun led by ERK and JNK activation was essential for this EGF response [7]. We recently reported that the interaction between c-Jun and Sp1 induced by EGF cooperatively activated the expression of the 12(*S*)-lipoxygenase gene [6]. The aim of this study was to determine whether PMA treatment induces ERK activation through the Raf-MEK-ERK signaling pathway followed by c-Jun induction.

Materials and Methods

Materials

[α-^{32}P]dCTP (3,000 Ci/mmol), [γ-^{32}P]ATP (5,000 Ci/mmol), [1-^{14}C]arachidonic acid (56.3 mCi/mmol), multiprime DNA labeling system and Nylon membrane (Hybond-N) were purchased from Amersham (Buckinghamshire, UK). PD98059 and GF109203X were obtained from Calbiochem (La Jolla, Calif., USA). *o*-Nitrophenyl-β-galactopyranoside and PMA were from Sigma (St. Louis, Mo., USA). Qiagen-tip 100 was from Qiagen (Hilden, Germany). β-Galactosidase plasmid driven by cytomegalovirus was from Clontech (Palo Alto, Calif., USA). Monoclonal antibodies against c-Jun and ERK2 and polyclonal antibodies against protein kinase C (PKC)α and PKCδ were obtained from Transduction Laboratories (Lexington, Ky., USA). Rabbit polyclonal antibodies directed against the phosphorylated forms of ERK1/2, JNK and p38 were purchased from New England Biolabs (Beverly, Mass., USA). Antibodies against JNK1, p38 and Sp1 agarose conjugated to Sp1 or c-Jun antibodies were from Santa Cruz Biotechnology (Santa Cruz, Calif., USA). Expression vectors of PKCα and PKCδ were kindly provided by Dr. Yoshitaka Ono of Kobe University, Kobe, Japan. Lipofectamine, Dulbecco's modified Eagle's medium and Opti-MEM medium were obtained from Life Technologies (Grand Island, N.Y., USA). Fetal bovine serum was from HyClone Laboratories (Logan, Utah, USA). All other reagents used were of the highest purity obtainable.

Cell Culture and PMA Treatment

Human epidermoid carcinoma A431 cells were grown at 37°C under 5% CO_2 in 10-cm plastic dishes containing 10 ml of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 units/ml penicillin. The confluent cells were treated with 5 n*M* PMA. After 30 min of PMA treatment, the medium was removed and the cells were then further cultured in fresh medium. Cells were treated with PMA in serum-free medium unless otherwise stated.

Preparation of Nuclear Extracts

Cells from eight dishes (8×10^7 cells) were washed twice with PBS and scraped in 6 ml of PBS. Cells were collected by centrifuging at 400 *g* for 10 min and were then resuspended in 10 volumes of buffer A (300 m*M* sucrose, 10 m*M* HEPES, pH 7.9, 10 m*M* KCl, 1.5 m*M* $MgCl_2$ and 0.1 m*M* EDTA) and homogenized by 20 strokes with a Dounce homogenizer A pestle (Wheaton, Millville, N.J.,

USA). All buffers contained phenylmethylsulfonyl fluoride (0.5 m*M*), orthovanadate (1 m*M*), pepstatin A (2 μg/ml) and leupeptin (2 μg/ml). Nuclei were pelleted by centrifugation at 400 *g* for 10 min. Pellets were resuspended in 10 volumes of buffer B [10 m*M* HEPES, pH 7.9, 400 m*M* NaCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* EGTA and 5% (v/v) glycerol] and homogenized by 20 strokes with a B pestle. The suspension was stirred for 1 h at 4°C and then centrifuged at 16,000 *g* for 60 min in a microcentrifuge. Supernatants were collected and dialyzed for 16 h against 50 volumes of buffer C [20 m*M* HEPES, pH 7.9, 0.1 m*M* EDTA, 75 m*M* NaCl and 20% (v/v) glycerol]. Dialysates were centrifuged at 7,500 *g* for 10 min and the supernatants were stored at −70°C until use.

Western Blotting

An analytical 10% sodium dodecyl sulfate (SDS)-polyacrylamide slab gel electrophoresis (PAGE) was performed. The cell nuclear extracts or lysates (30 μg of protein of each) prepared from control and PMA-treated cells were analyzed. For immunoblotting, proteins in the SDS gels were transferred to a polyvinylidene difluoride membrane using an Electroblot apparatus. Mouse monoclonal antibodies against human c-Jun or rabbit polyclonal antibodies against phospho-ERK1/2, phospho-JNK and phospho-p38 were employed as the primary antibodies. Immunoblot analysis was carried out with mouse IgG antibodies coupled to horseradish peroxidase. An enhanced chemiluminescence kit (Amersham) was used for detection.

Assay of Microsomal 12(S)-Lipoxygenase Activity

The preparation of microsomes and the analysis of 12(*S*)-lipoxygenase activity were performed as previously described [7]. The assay mixture contained 8.5 μ*M* [1-^{14}C]arachidonic acid (0.1 μCi) and an appropriate amount of microsomes in a final volume of 0.2 ml. The reaction took place at 37°C for 20 min. After extraction with 2 ml of ethyl acetate, the organic layer was evaporated. Residues were dissolved in ethanol and applied to thin-layer chromatography plates. Formation of [1-^{14}C]12(*S*)-hydroxyeicosatetraenoic acid was determined using a system 2000 Imaging Scanner (Bioscan).

Transfection of Cells with Plasmids

A luciferase reporter plasmid (pXLO-7-1) bearing a promoter region (−224 bp) of the human 12(*S*)-lipoxygenase gene was used. Two Sp1 binding sites at −158 to −150 bp and −123 to −114 bp present in the promoter were essential for the response of 12(*S*)-lipoxygenase gene activation to PMA [26]. Transient transfection of cells with plasmids was performed with Lipofectamine according to the manufacturer's instructions with a slight modification. A431 cells were replated 36 h before transfection at a density of 3×10^5 cells in 2 ml of fresh culture medium in a 3.5-cm plastic dish. For use in transfection, 12.5 μl of Lipofectamine was incubated with 0.5 μg of pXLO-7-1 plasmid, 0.2 μg of β-galactosidase plasmid or indicated plasmids as previously described in 1 ml of Opti-MEM medium for 30 min at room temperature. Cells were transfected by replacing the medium with 1 ml of Opti-MEM medium containing the plasmids and Lipofectamine. They were then incubated at 37°C in a humidified atmosphere of 5% CO_2 for 24 h. Following the replacement of Opti-MEM medium with 2 ml of fresh culture medium, cells were incubated for an additional 48 h, unless stated otherwise. The luciferase and β-galactosidase activities in cell lysate were determined as described previously [27].

RNA Blot Analysis

Total RNA isolation and RNA blot analysis was performed as previously described [26]. Twenty micrograms of total RNA per lane were separated by electrophoresis on 1% agarose-glyoxal gel and transferred to a Nylon membrane. The cDNA probes used were the *Bam*HI fragment of c-Jun cDNA (2.3 kb) and the *Pst*I fragment of GAPDH cDNA (1.25 kb). Probes were labeled with [α-^{32}P]dCTP using a multiprime DNA labeling system, and hybridization with the ^{32}P-labeled probes was performed using a rapid hybridization system (Amersham). The Nylon membranes were washed three times at room temperature in 2 × SSPE (300 m*M* NaCl, 20 m*M* NaH_2PO_4 and 2 m*M* EDTA) containing 0.1% SDS. Each wash was carried out for 15 min. Autoradiography was then performed.

Coimmunoprecipitation

Two hundred micrograms of nuclear extracts were incubated with 10 μl of either anti-Sp1 or anti-c-Jun antibody-agarose conjugate in 300 μl of immunoprecipitation buffer [20 m*M* HEPES, pH 7.9, 420 m*M* NaCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* EDTA, 25% glycerol (v/v), 0.5 m*M* phenylmethylsulfonyl fluoride, 1 m*M* orthovanadate, 2 μg/ml pepstatin A and 2 μg/ml leupeptin] under gentle shaking at 4°C overnight. Beads were pelleted at 7,500 *g* for 2 min and washed three times with RIPA buffer [50 m*M* Tris-HCl, pH 7.5, 1% IGEPAL CA-630 (v/v), 150 m*M* NaCl and 0.5% sodium deoxycholate]. Protein was removed from the beads by boiling in sample buffer (120 m*M* Tris-HCl, pH 6.8, 10% glycerol, 3% SDS, 20 m*M* dithiothreitol and 0.4% bromophenol blue) for 5 min and subjected to SDS-PAGE on a 10% gel. Western blot analysis was carried out as described above.

Assay for Binding of c-Jun-Sp1 Complex to Consensus Sites

An immunoprecipitation method developed at our laboratories [6] was used. Sp1 oligonucleotide 5′-ATTCGATCGGGGCGGGGCGAGC-3′ was end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The binding reaction was performed in 60 μl of reaction mixture containing 0.8 μg of poly (dI-dC), 20 m*M* HEPES, pH 7.9, 0.1 m*M* KCl, 2 m*M* $MgCl_2$, 15 m*M* NaCl, 0.2 m*M* EDTA, 5 m*M* dithiothreitol, 10% (v/v) glycerol, 2% (w/v) polyvinyl alcohol, 60 μg of the cell nuclear extracts and the radiolabeled probe (2.5×10^6 cpm). The mixture was incubated at room temperature for 30 min and then incubated with 35 μl of anti-c-Jun antibody-agarose conjugate in 300 μl of immunoprecipitation buffer [20 m*M* HEPES, pH 7.9, 420 m*M* NaCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* EDTA, 25% (v/v) glycerol, 0.5 m*M* phenylmethylsulfonyl fluoride, 1 m*M* orthovanadate, 2 μg/ml pepstatin A and 2 μg/ml leupeptin] under gentle shaking at 4°C overnight. Beads were pelleted at 7,500 *g* for 2 min and washed three times with RIPA buffer. Radioactivity in the pelleted beads was determined using a scintillation counter.

Results

Activation of ERK1/2 by PMA

The effect of PMA on the activation of the MAPK family was analyzed using anti-phospho-antibodies that specifically recognized their phosphorylated and activated forms. PMA activated the phosphorylation of ERK1/2 (fig. 1), but not JNK or p38 (data not shown).

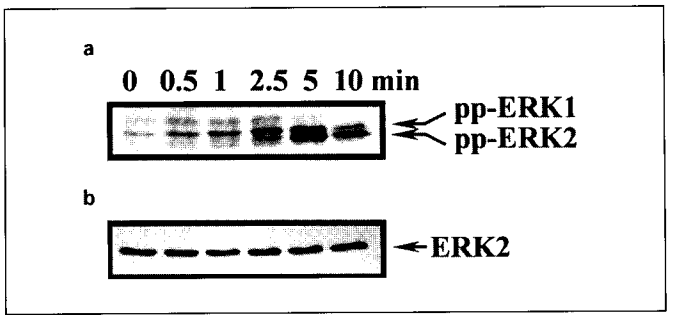

Fig. 1. Time-dependent effects of PMA on ERK activity. Confluent cells were starved for 24 h in serum-free culture medium before PMA treatment and then treated with PMA in culture medium without serum. Whole-cell lysates were prepared and subjected to Western blotting using antibodies specific for the phosphorylated form of ERK1/2 (**a**) and those against ERK2 (**b**). pp = Phosphorylated protein.

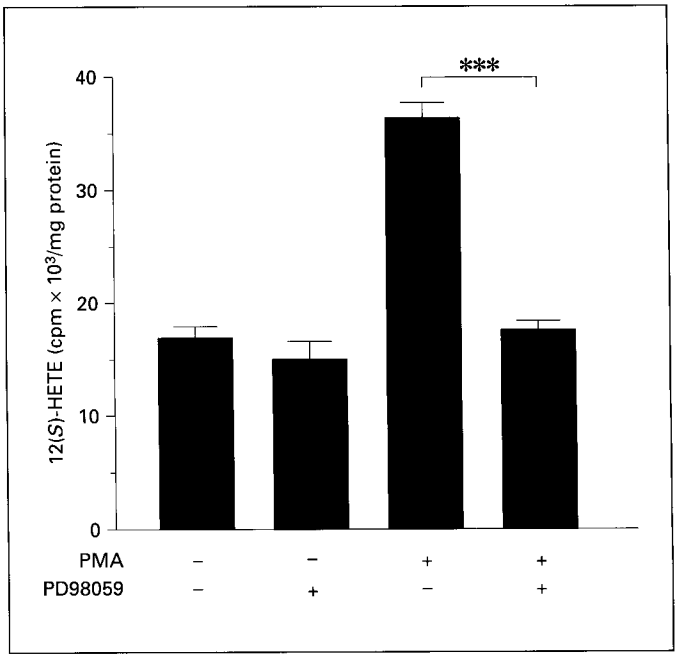

Fig. 2. Effect of PD98059 on PMA-induced 12(*S*)-lipoxygenase activity. Confluent cells were treated with 30 μ*M* PD98059 for 30 min followed by PMA treatment for 30 min. The medium was then switched to PMA-free culture medium containing 30 μ*M* PD98059 for up to 18 h for the assay of 12(*S*)-lipoxygenase activity. Values are means ± SEM of three determinations. Statistical significance was analyzed using Student's t test (*** $p < 0.005$). 12(*S*)-HETE = 12(*S*)-Hydroxyeicosatetraenoic acid.

The maximum activation of ERK phosphorylation was observed in cells treated with PMA for 5 min, but the response subsequently declined (fig. 1). Pretreatment of cells with either 5 μ*M* GF109203X, a PKC inhibitor, or 30 μ*M* PD98059, an inhibitor of MEK1/2 activation, completely inhibited the activation of ERK1/2 phosphorylation induced by PMA (data not shown).

Effect of PD98059 on PMA-Induced 12(S)-Lipoxygenase Activity

Our previous results indicated that a significant stimulation of 12(*S*)-lipoxygenase mRNA expression and enzyme activity was observed in cells treated with PMA for 14–18 h [26]. The enzyme activity assay was therefore performed with cells treated with PMA for 18 h. To determine whether the PMA-induced 12(*S*)-lipoxygenase activity was mediated by ERK1/2 activation, the effects of PD98059 were studied. Pretreatment of cells with 30 μ*M* PD98059 completely inhibited the PMA-induced enhancement of 12(*S*)-lipoxygenase activity (fig. 2), indicating that ERK activation mediated the stimulation of 12(*S*)-lipoxygenase activity induced by PMA.

Effect of Dominant Negative Mutants of Ras, Raf and ERK on PMA Response

The expression vector of the Ras dominant negative mutant pMMrasDN [15] was used in order to determine whether PMA-induced expression of 12(*S*)-lipoxygenase was mediated through Ras activation. Cells were transfected with a luciferase reporter gene pXLO-7-1 (–224 bp) and a dominant negative vector pMMrasDN for 68 h, followed by PMA stimulation. As shown in figure 3, transfection of pMMrasDN in cells dose-dependently inhibited the PMA-induced promoter activation of 12(*S*)-lipoxygenase. However, the inhibition was only partial. Transfection with 4 μg of pMMrasDN only induced a 50% inhibition. By comparison, an 80% inhibition induced by pMMrasDN was observed in EGF-induced promoter activation of 12(*S*)-lipoxygenase under the same experimental conditions [7]. Transfection of cells with the expression vectors of dominant negative mutants C4B for Raf [2] and K52R ERK2 for ERK [32] also inhibited the PMA-induced promoter activation of 12(*S*)-lipoxygenase in a dose-dependent manner. The inhibitory effect of Raf and ERK dominant negative mutants was much more significant than that of Ras dominant negative mutants. Transfection with 4 μg of either vector C4B or vector K52R ERK2 almost completely inhibited the PMA response (fig. 3).

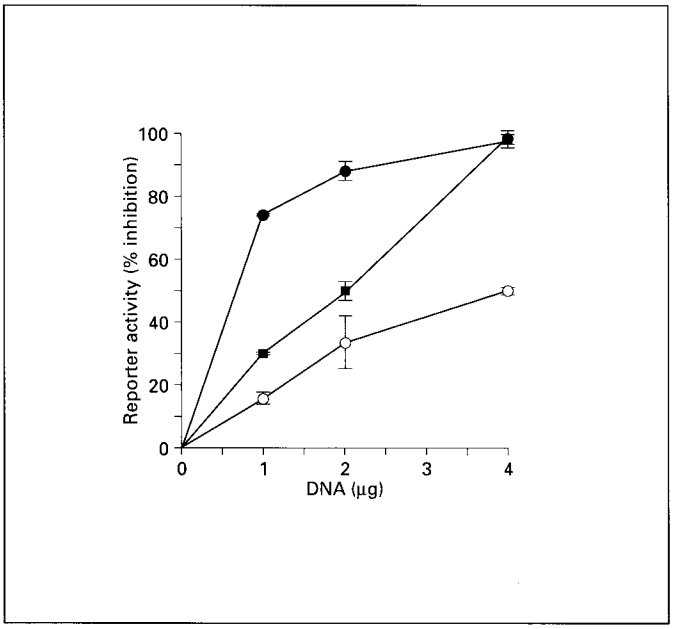

Fig. 3. Effects of dominant negative mutants of Ras, Raf and ERK2 on PMA-induced promoter activation of the 12(*S*)-lipoxygenase gene. Cells were cotransfected with pXLO-7-1 (–224 bp) luciferase plasmid (0.5 μg), β-galactosidase plasmid (0.2 μg) and dominant negative vectors pMMrasDN (○), C4B (■) and K52R ERK2 (●) by the lipofection method. After replacing the Opti-MEM medium with 2 ml of fresh culture medium, cells were incubated for an additional 24 h and then treated with 5 n*M* PMA. After 30 min of PMA treatment, the medium was removed and then the cells were further cultured in fresh medium for up to 18 h. Medium containing 10% fetal bovine serum was used throughout the experiment. The expression of luciferase activity and β-galactosidase activity was determined. Values are means ± SEM from three separate experiments. In each experiment, assays for the promoter activity in control and dominant negative mutant-treated cells were performed in triplicate.

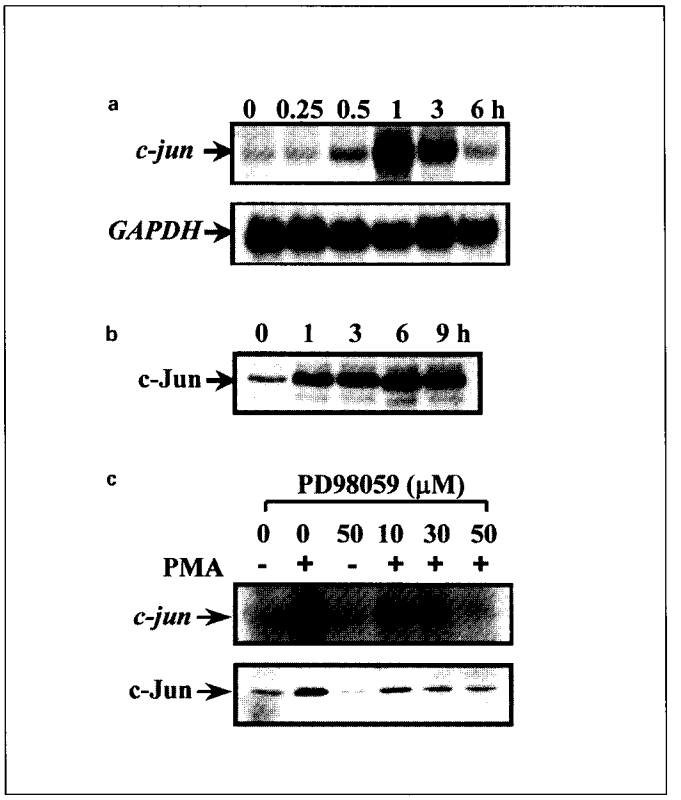

Fig. 4. Effects of PMA on the expression of c-Jun. **a**, **b** Confluent cells maintained for 24 h in serum-free culture medium were treated with 5 n*M* PMA for different time periods as indicated. Northern blot analysis of *c-jun* mRNA and GAPDH (**a**) and immunoblot analysis of c-Jun expression in nuclear extracts (**b**) were performed. **c** Confluent cells were starved for 24 h in serum-free culture medium before PMA treatment and then treated with PD98059 for 30 min followed by PMA treatment for 1 h. The expression of c-Jun was analyzed.

Induction of c-Jun Expression by PMA

When ERK is activated, it is translocated to the nucleus to activate the expression of some immediate early genes [24]. Induction of the immediate early gene c-Jun by PMA in A431 cells was thus studied. PMA induced the expression of both c-Jun mRNA and protein in a time-dependent manner (fig. 4a, b). The maximum induction of mRNA and protein was observed in cells treated with PMA for 1 and 3 h, respectively, and the maximum induction of c-Jun protein persisted for up to 9 h after PMA treatment. Pretreatment of cells with PD98059 inhibited the PMA-induced expression of c-Jun mRNA and protein in a dose-dependent manner (fig. 4c), indicating that it was mediated through the activation of MEK-ERK signaling.

Coimmunoprecipitation of c-Jun and Sp1 in PMA-Treated Cells

Expression of Sp1 in nuclear extracts prepared from cells treated with PMA was studied from 1 to 9 h using immunoblot analysis. No differences in Sp1 expression between control and PMA-treated cells were observed (fig. 5a). Interactions between c-Jun and Sp1 upon PMA treatment were then studied by coimmunoprecipitation using either Sp1 or c-Jun antibodies. When agarose-bearing Sp1 antibodies were used, no changes in the immunoprecipitated Sp1 between control and PMA-treated cells were observed; however, the coimmunoprecipitated c-Jun increased in a time-dependent manner in PMA-treated cells (fig. 5b). After PMA treatment for 1 h, a significant interaction between c-Jun and Sp1 was observed

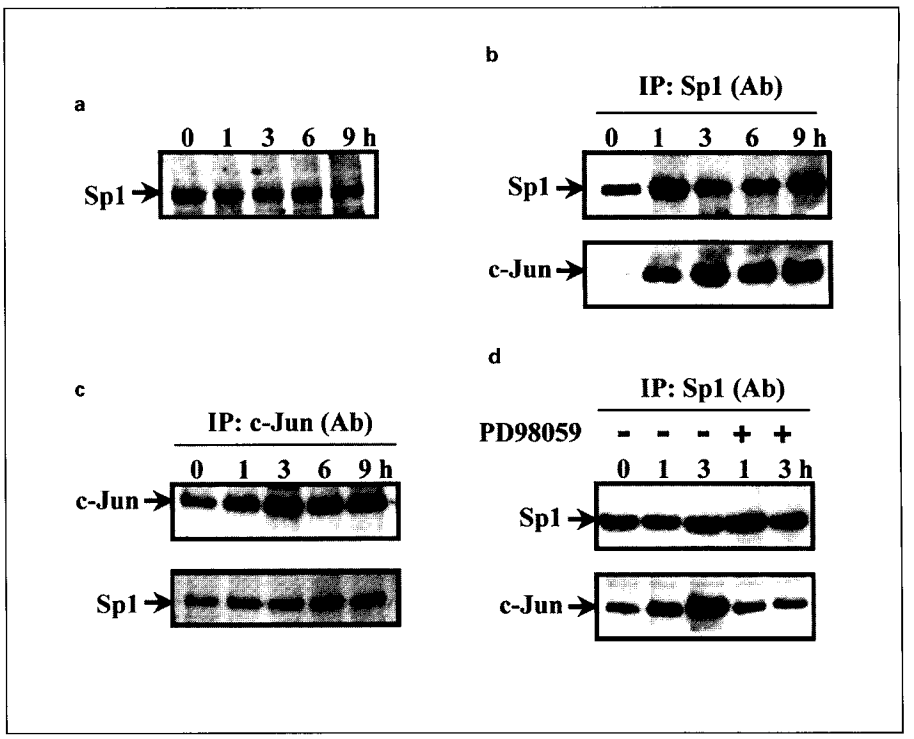

Fig. 5. Time-dependent effects of PMA on the interaction between Sp1 and c-Jun in cells. Confluent cells were starved for 24 h in serum-free culture medium before PMA treatment and then treated with PMA in culture medium without serum for different time periods as indicated. **a** Nuclear extracts were prepared and subjected to Western blotting using antibodies specific for Sp1. **b** Nuclear extracts from PMA-treated cells were immunoprecipitated (IP) with agarose-bearing antibodies (Ab) against Sp1. Immunoprecipitates were subjected to 10% SDS-PAGE followed by Western blotting with antibodies against Sp1 and c-Jun. **c** Immunoprecipitates (IP) were obtained using agarose-bearing antibodies (Ab) against c-Jun, and the amount of c-Jun and Sp1 in immunoprecipitates was analyzed by Western blotting. **d** Cells were pretreated with 30 μM PD98059 for 30 min before PMA treatment, and the interaction between Sp1 and c-Jun of cell nuclear extracts in the immunoprecipitated (IP) pellet obtained using agarose-bearing antibodies (Ab) against Sp1 was analyzed.

in cells, which then persisted for up to 9 h. When agarose-bearing c-Jun antibodies were used, both c-Jun and Sp1 in the immunoprecipitated complex increased in a time-dependent manner upon PMA treatment (fig. 5c). These results support the notion that treatment of cells with PMA induced expression of c-Jun, followed by the enhancement of the interaction between c-Jun and Sp1. Since the treatment of cells with PD98059 inhibited the PMA-induced expression of c-Jun (fig. 4c), inhibition of the formation of Sp1 and c-Jun complex by PD98059 treatment in PMA-treated cells further confirmed the observation that the treatment of cells with PD98059 reduced the amount of c-Jun interacting with Sp1 (fig. 5d).

Effect of PMA on the Interaction between c-Jun-Sp1 Complex and Sp1 Oligonucleotide

In order to study the binding of nuclear c-Jun-Sp1 prepared from PMA-treated cells with Sp1 binding sequence DNA, an in vitro assay using the immunoprecipitation method was used. An Sp1 oligonucleotide radiolabeled with ^{32}P-ATP was incubated with cell nuclear extracts prepared from control and PMA-treated cells, respectively. A complex of the Sp1 oligonucleotide and c-Jun-Sp1 was immunoprecipitated with agarose-bearing c-Jun antibodies and the immunoprecipitated radiolabeled Sp1 oligonucleotide was measured. As shown in figure 6, treat-

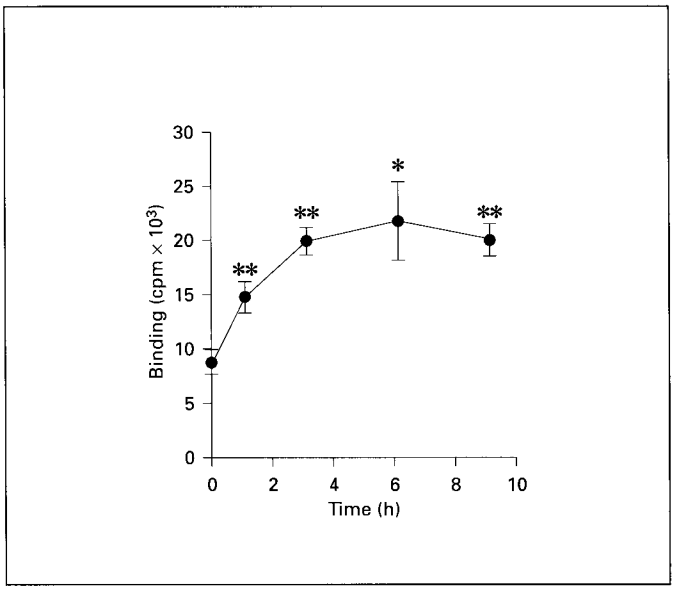

Fig. 6. Effects of PMA on the interaction between Sp1 oligonucleotides and c-Jun-Sp1. Nuclear extracts from PMA-treated cells were prepared and subjected to the assay for binding of the c-Jun-Sp1 complex to Sp1 consensus sites. ^{32}P-Radiolabeled Sp1 oligonucleotide was used as a probe for binding. Values are means ± SEM of three determinations. Statistical significance between control and treatment groups was analyzed by Student's t test (* $p < 0.05$; ** $p < 0.01$).

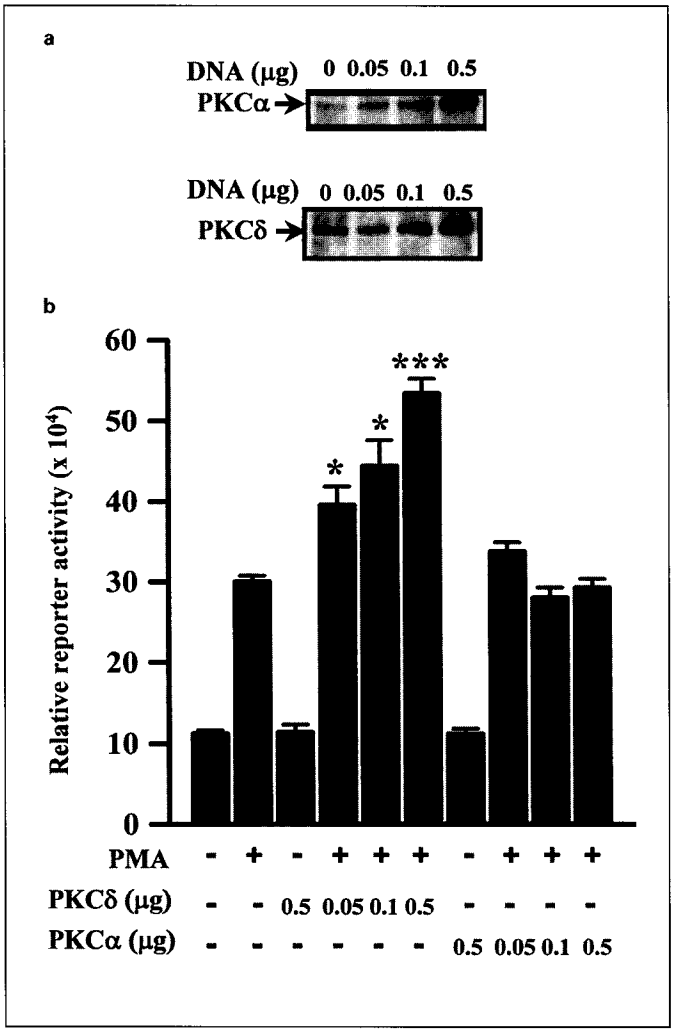

Fig. 7. Effects of PKC overexpression on the promoter activity of 12(*S*)-lipoxygenase. Cells were cotransfected with pXLO-7-1 (–224 bp) luciferase plasmid (0.5 μg), β-galactosidase plasmid (0.2 μg) and PKCα or PKCδ plasmids by the lipofection method. After replacing the Opti-MEM medium with 2 ml of fresh culture medium, cells were incubated for an additional 24 h and then treated with 5 n*M* PMA. After 30 min of PMA treatment, the medium was removed and then the cells were further cultured in fresh medium for up to 18 h. Medium containing 10% fetal bovine serum was used throughout the experiment. **a** Expression of PKCα and PKCδ proteins was analyzed by Western blotting with anti-PKCα and anti-PKCδ antibodies. **b** The expression of luciferase activity and β-galactosidase activity was also determined. Values shown in **b** are means ± SEM of three determinations. Statistical significance between PKC overexpression and control cells upon PMA treatment was analyzed by Student's t test (* $p < 0.05$; *** $p < 0.005$).

ment of the cells with PMA increased the interaction between Sp1 oligonucleotide and nuclear c-Jun-Sp1 in a time-dependent manner. A significant effect was observed in cells treated with PMA for 1 h, and a maximum increase was found in cells treated with PMA for 3 h, which persisted for up to 9 h after PMA treatment.

Functional Role of PKC Subspecies in PMA-Induced Activation of 12(S)-Lipoxygenase Gene Promoter

We previously reported that PKCα and PKCδ were two PKC subspecies among the classic and novel PKCs which are expressed in A431 cells using a Northern blot analysis [28]. In order to study the functional role of PKCα and PKCδ in PMA-induced activation of 12(*S*)-lipoxygenase gene promoter, cells were transiently cotransfected with luciferase reporter pXLO-7-1 and PKC expression vector. Effects of PMA treatment on the activation of 12(*S*)-lipoxygenase gene reporter activity in cells were then studied. Overexpression of PKCα and PKCδ in cells was confirmed using Western blot analysis as shown in figure 7a. Control cells and those overexpressing PKC were then stimulated with PMA. A stimulatory effect on the activation of the 12(*S*)-lipoxygenase gene promoter was only observed in cells that overexpressed PKCδ but not in cells that overexpressed PKCα (fig. 7b). The treatment of cells with PD98059 inhibited the promoter activation in cells that overexpressed PKCδ upon PMA treatment (fig. 8). These results suggested that PKCδ was involved in the mediation of the gene activation of PMA-induced 12(*S*)-lipoxygenase.

Discussion

In this study, activation of MAPK signal pathways followed by the induction of c-Jun in PMA-induced activation of human 12(*S*)-lipoxygenase gene transcription were analyzed. Previously, we reported that the promoter activation of the 12(*S*)-lipoxygenase gene by PMA treatment was similar to that induced by EGF and Ha-ras overexpression [26]. The two Sp1 binding sequences residing at –158 to –150 bp and –123 to –114 bp in the 12(*S*)-lipoxygenase gene promoter were requisite for all PMA, EGF and Ras activation responses [8, 26, 27]. We recently found that the activation of Ras-Raf-MEK-ERK and Ras-Rac-JNK signal pathways was directly involved in EGF-induced expression of 12(*S*)-lipoxygenase [7]. In this report, we provided new evidence suggesting that the induction of 12(*S*)-lipoxygenase expression by PMA was mediated mainly through the activation of Raf followed

by the MEK-ERK signal pathway. First, the PMA-induced enhancement of 12(*S*)-lipoxygenase activity was inhibited by PD98059, an inhibitor of signaling through the ERK cascade (fig. 2), indicating that MEK-ERK activation was essential for this PMA response. Second, PMA treatment activated ERK but not JNK or p38 (fig. 1), indicating that ERK was the only one among the MAPK family activated by PMA treatment. Finally, dose-dependent inhibition of PMA-induced promoter activation of the 12(*S*)-lipoxygenase gene by the overexpression of dominant negative expression vectors of Raf and ERK (fig. 3) pointed to the mediation of the activation of Raf-MEK-ERK signaling in PMA-induced activation of the 12(*S*)-lipoxygenase gene. These results indicate that activation of Raf played a functional role in mediating the PMA-induced promoter activation of the 12(*S*)-lipoxygenase gene. Ueda et al. [35] previously reported that PKC activated the MEK-ERK pathway, which depended on Raf activation. Therefore, activation of Raf by PMA in A431 cells might be partially due to the direct effect of PKC. On the other hand, a partial inhibition of PMA-induced promoter activation of 12(*S*)-lipoxygenase by the overexpression of dominant negative Ras (fig. 3) suggests that PMA may also activate Ras followed by Raf-MEK-ERK signaling in the activation of 12(*S*)-lipoxygenase expression.

While studying the functional role of c-Jun in the gene activation of 12(*S*)-lipoxygenase, we previously found that c-Jun interacting cooperatively with Sp1 stimulated the promoter activation of the 12(*S*)-lipoxygenase gene in cells that overexpressed c-Jun [6]. An interaction between c-Jun and Sp1 was observed in cells after PMA treatment for 1 h, and then persisted for up to 9 h (fig. 5b). This finding supports the notion that PMA-induced gene activation of 12(*S*)-lipoxygenase might be mediated through the cooperative interaction between c-Jun and Sp1. A significant enhancement of binding between the c-Jun-Sp1 complex and Sp1 oligonucleotide, determined using an in vitro assay, was observed in cells treated with PMA for 1 h, and persisted for up to 9 h (fig. 6). This finding suggested that the binding of the c-Jun-Sp1 complex to Sp1-binding sites of promoter may occur in cells upon PMA treatment. To compare the kinetic effects of PMA on the interaction between c-Jun and Sp1 and the activation of 12(*S*)-lipoxygenase gene promoter, we allowed a lag period of 8 h. The amount of c-Jun interacting with Sp1 was significant in cells treated with PMA for 1 h, and then a slight increase for up to 9 h after treatment was observed (fig. 5). However, a significant activation of the gene promoter was observed in cells treated with PMA for 9 h [26].

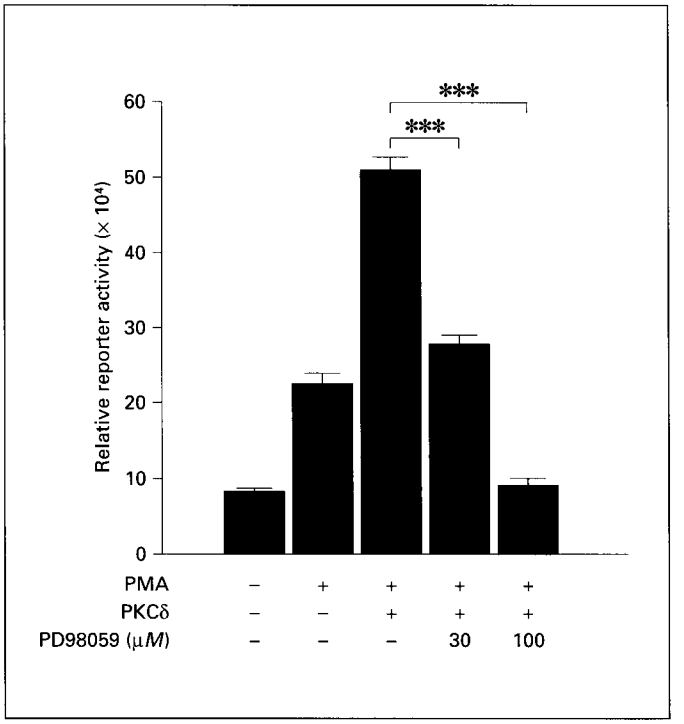

Fig. 8. Effects of PD98059 on the promoter activity of 12(*S*)-lipoxygenase induced by PKCδ overexpression. Cells were cotransfected with pXLO-7-1 (–224 bp) luciferase plasmid (0.5 μg), β-galactosidase plasmid (0.2 μg) and PKCδ plasmid (0.5 μg) by the lipofection method. After replacing the Opti-MEM medium with 2 ml of fresh culture medium, cells were incubated for an additional 24 h and then treated with PD98059 for 30 min followed by 5 n*M* PMA treatment for 30 min. The medium was then switched to PMA-free culture medium containing PD98059 for up to 18 h for the assay of luciferase and β-galactosidase activities. Values are means ± SEM of three determinations. Statistical significance was analyzed by Student's t test (*** $p < 0.005$).

The transcription apparatus required the assembly of a very large multiprotein complex composed of RNA polymerase II along with several accessory factors, including some TATA box-binding protein-associated factors. It is still not clear how the c-Jun-Sp1 complex cooperated with the basal transcription factor complex to initiate the gene transcription of 12(*S*)-lipoxygenase during the 8-hour lag period.

The functional interaction between c-Jun and Sp1 was not only observed in the gene activation of 12(*S*)-lipoxygenase induced by PMA and EGF, but also in the transcriptional activation of the human p21^{WAF1} gene, which encodes a cell cycle inhibitor protein. Kardassis et al. [23] recently reported that overexpression of c-Jun transactivated the human p21^{WAF1} gene by acting as a superactiva-

tor of Sp1 in HepG2 cells. Gene expression of $p21^{WAF1}$ is regulated by many inducers under various physiological and pathological conditions. The inducers, such as phorbol esters [1] and TGF-β [12], mediate their effects on $p21^{WAF1}$ gene expression via the proximal region (–210 to +1 bp) of the promoter. The proximal promoter of the $p21^{WAF1}$ gene contains characteristic GC-rich motifs that serve as binding sites for members of the Sp1 family species [12]. Our present findings strongly support the notion that upregulation of $p21^{WAF1}$ gene expression by PMA may also be mediated through the induction of c-Jun followed by the interaction of c-Jun with Sp1.

In addition, we demonstrated the functional role of PKCδ in the mediation of PMA-induced expression of the 12(*S*)-lipoxygenase gene. The presence of PKCα, δ and ζ but not β, γ and ε in A431 cells has been reported [28]. It has been shown that among PKCα, δ and ζ, only PKCα and δ were activated by PMA [30]. In the present study, PMA treatment enhanced the gene activation of 12(*S*)-lipoxygenase in cells overexpressing PKCδ but not PKCα (fig. 7b). While studying the activation of ERK by PMA, overexpression of cells with PKCδ, but not PKCα, activated the Raf-MEK-ERK pathway [35]. Taking these results together, evidence obtained from our study provides further support for the notion that although PKCα can not be ruled out, PKCδ mediated the gene activation of 12(*S*)-lipoxygenase in response to PMA.

In summary, our results indicated that PMA induced gene expression of 12(*S*)-lipoxygenase through the activation of ERK, followed by the induction of c-Jun and enhancement of the interaction between c-Jun and Sp1. Formation of c-Jun-Sp1 complex then mediated the gene activation. Sp1 was initially recognized as a constitutive transcription factor. However, transactivity of Sp1 can be activated by posttranslational modification such as dephosphorylation [25] in the regulation of several genes. These include induction of acetyl-CoA carboxylase by glucose in preadipocytes [11] and endothelial nitric oxide synthase by lysophosphatidic acid in endothelial cells [10]. Dephosphorylation modification of Sp1 increases its binding with Sp1-binding sites of the gene promoter in electrophoretic mobility shift analysis [10, 11]. However, in the gene activation of 12(*S*)-lipoxygenase through the cooperative interaction between c-Jun and Sp1 caused by PMA, no change in binding affinity between Sp1 and Sp1-binding sites was observed [26]. So far, only the induction of 12(*S*)-lipoxygenase and $p21^{WAF1}$ through the mechanism of interaction between c-Jun and Sp1 has been reported. Since a large number of eukaryotic promoters contain proximal Sp1-binding sites, it will be of particular interest to study whether other genes with Sp1-dependent promoters can be transactivated by c-Jun. Gene activation mediated through the interaction between c-Jun and Sp1 may be an important mechanism for transcriptional gene regulation.

Acknowledgements

We are greatly indebted to Dr. Yoshitaka Ono for providing expression plasmids of PKCα and PKCδ. Thanks also go to Drs. Tzeng-Horng Leu, Joseph T. Bruder and Ming-Zong Lai for providing plasmids pMMrasDN, C4B and K52R ERK2, respectively. We would also like to thank Drs. Ushio Kikkawa, Naoaki Saito, Wai-Ming Kan, Patrick Y.-K. Wong and Shen K. Yang for their valuable discussion, and Y.L. Chang for her secretarial assistance. This work was supported in part by grant NSC 88-2314-B-006-001 from the National Science Council of the Republic of China.

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