Phosphorylation of activation transcription factor-2 (ATF-2) at serine 121 by protein kinase C controls c-Jun-mediated activation of transcription

(Running title: Phosphorylation of ATF-2 at Ser 121)

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Activation transcription factor-2 (ATF-2) is phosphorylated by various protein kinases, such as JNK/p38/ERK, calmodulin kinase IV, protein kinase A and protein kinase C (PKC), in response to a variety of stimuli. However, the role of the phosphorylation of ATF-2 by PKC in vivo in the transcriptional control of genes that include the activation protein-1 (AP-1)/cyclic AMP-response element (CRE) remains to be defined. Using antibodies against the phosphorylated serine residue (p-Ser) at position 121 of ATF-2, we have demonstrated that PKC phosphorylates ATF-2 at serine residue 121 (Ser121) and that phosphorylation of Ser121 (to yield ATF-2pS121) becomes detectable at the late stage of the response of HeLa cells to 12-O-tetradecanoylphorbol-13-acetate (TPA) and is maintained for more than 2 h. By contrast, phosphorylation of ATF-2 at threonine residues 69 and 71 (Thr69/71; to yield ATF-2pT69/71) and at Ser340 and Ser367 (to yield ATF-2pS340 and ATF-2pS367) is detectable as an immediate early response. Unlike levels of ATF-2pT69/71 and ATF-2pS340, the level of ATF-2pS121 increases in the nuclei of HeLa cells in response to TPA. A serine-to-alanine mutation at position 121 of ATF-2 represses the c-Jun-dependent transcription of AP-1/CRE reporter genes and, also, the p300-mediated activation of a Gal4-reporter gene in response to TPA. Our results suggest that the phosphorylation of ATF-2 at Ser121 plays a key role in the c-Jun-mediated activation of transcription that occurs in response to TPA.

Abbreviations used are: AP-1, activation protein-1; ATF-2, activation transcription factor-2; CRE, cAMP-response element; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; GPDH, glyceraldehyde-3-phosphate dehydrogenase; MEF, mouse embryonic fibroblasts; TPA, 12-O-tetradecanoylphorbol-β-acetate.

INTRODUCTION

The phosphorylation of transcription factors is critical for the expression of genes that are involved in the proliferation, differentiation, and survival of cells, and such phosphorylation can be induced by many types of stimulus, such as growth factors, stressors and viral infection (1, 2). Moreover, phosphorylated transcription factors are not only involved in a variety of biological reactions in molecular association with other nuclear factors and cofactors, they are also involved in the degradation and oligomerization of proteins for transmission of signals to the nucleus (1, 2).

Activating transcription factor-2 (ATF-2) is a member of the family of ATF/cAMP-response element-binding (CREB) proteins and is characterized by a basic-zipper (b-ZIP) domain that consists of basic amino acids and a leucine zipper region that acts as a DNA-binding region. Those members of the ATF-2 family have been identified to date, namely, ATF-2, ATF-7 (ATFa) and CREBPa (3). ATF-2 regulates gene expression via the binding of ATF-2, as a homodimer, to the recognition sequences of the cAMP-response element (CRE) or via formation of a heterodimer with activation protein-1 (AP-1) that binds to CRE sequences. Thus, ATF-2 can
interact with other members of the ATF family and with members of the AP-1 family (4). The heterodimerization of ATF-2 with c-Jun is critical for the nuclear localization of c-Jun-ATF-2 heterodimers and the transcriptional activity of this complex (5). Studies of ATF-2-null mice have shown that ATF-2 is essential to animal development (6, 7), and recent reports have shown that the gene for ATF-2 is a tumor-susceptibility gene associated with mammary tumors (8, 9) and also plays a role in suppression of skin cancer (10).

Stress-activated protein kinases (SAPKs), such as Jun NH2-terminal protein kinases 1 and 2 (JNK1 and JNK2) and p38, phosphorylate ATF-2 at Thr69 and Thr71 (to yield ATF-2pT69/71), which are close to its amino-terminal transcriptional activation domain and, in this way, they enhance its transactivation capacity (11-13) in response to stresses such as UV light, osmotic stress, hypoxia and inflammatory cytokines (14). It has been also proposed that activation of ATF-2 by p38/JNK might play a role in apoptosis (15). A recent study of “knock-in” mice showed that phosphorylation of both Thr69 and Thr71 of ATF-2 by p38 kinase is required for the initiation of anti-apoptotic activity in embryonic liver and heart in conjunction with ATF-7 (16).

Both insulin and serum can activate ATF-2 via a two-step mechanism that involves two distinct Ras effector pathways: the phosphorylation (p-) of Thr71 via the Ras-Raf-MEK-ERK pathway; and the subsequent phosphorylation of Thr69 via the Ras-RalGDS-Ral-Src-p38 pathway (17). Phosphorylation of Thr69 and Thr71 stabilizes ATF-2 and modulates AP-1/CRE-dependent transcription (18, 19). Thus, ATF-2 is responsive to a variety of external and internal stimuli, which range from mitogenic signals that are mediated via ERK to stress signals that are mediated via p38 (11-14).

It was reported several years ago that induction of transcription of the c-jun gene in response to 12-O-tetradecanoylphorbol-13-acetate (TPA) is mediated by the upstream AP-1-like site (jun2 site) in the promoter of the c-jun gene (20-22). Moreover, a TPA-induced protein kinase, which is distinct from SAPKs, phosphorylates c-Jun but not ATF-2 in HeLa cells (12, 23). However, phosphorylated amino acid residues in ATF-2 and the protein kinase responsible for the TPA-mediated phosphorylation of ATF-2 remain to be identified.

We reported previously that ATF-2 associates with p300 to regulate the differentiation-responsive element-dependent transcription of the c-jun gene during the retinoic acid-induced (RA-induced) differentiation of F9 cells (24, 25) [the differentiation-response element (DRE) resembles an AP-1-like sequence, a CRE-like sequence and a TPA-responsive element (TRE)]. However, the molecular mechanisms underlying the kinase-mediated activation of transcription of the c-jun gene and the molecular signaling associated with activation of RA signals via PKC remain to be clarified.

We report here that the phosphorylation of the serine residue at position 121 of ATF-2, catalyzed by, at least, PKCα, is one aspect of the response to TPA and that this phosphorylation is involved in the regulated transcription of genes that include an AP-1-like or CRE-like sequence.

**EXPERIMENTAL PROCEDURES**
Plasmids and Antibodies—A series of human ATF-2-expression vectors derived from pcDNA3-Flag (Invitrogen Co., Carlsbad, CA, USA), pET15b (Novagen, Darmstadt, Germany), pGEX-4T-1 (GE Healthcare, Uppsala, Sweden) and pFR-Luc (Stratagene, La Jolla, CA, USA) were obtained from the RIKEN DNA Bank (Tsukuba, Ibaraki, Japan). Plasmids that encoded Flag-ATF-2WT (wild type) and Gal4-ATF-2WT were used to generate the corresponding alanine-substitution mutants, pcDNA-Flag-ATF-2T69/71A, pcDNA-Flag-ATF-2S121A, pcDNA-Flag-ATF-2S340A, pcDNA-Flag-ATF-2S367A, pGal4-ATF-2S121A, pGal4-ATF-2S121A, pGal4-ATF-2S340A, pGal4-ATF-2S367A and pGal4-ATF-2T69/71A by site-directed mutagenesis (24, 25). To generate His-tagged proteins and glutathione S-transferase (GST) fusion proteins, cDNAs that encoded ATF-2WT and ATF-2S121A, and cDNAs that encoded ATF-2WT, ATF-2(1-125), ATF-2S121A, ATF-2(1-125)T69/71A, ATF-2(1-125)T69/71A, S121A and ATF-2(1-125)S121A were generated by ligation of the full-length coding sequence and PCR-amplified DNA that corresponded to a mutated site(s) into the respective vectors, in-frame, and proteins fused to GST were purified as described previously (24, 25). Amino acid substitutions were verified by nucleotide sequencing. The CRE-luc construct was generated by amplification of CRE from CRE-tk-CAT (provided by Dr. M. R. Montiminy, Salk Institute, San Diego, CA, USA, and ligation to a Renilla reporter vector (Promega, Madison, WI, USA). DRE-luc, mDRE-luc and mCRE-luc were prepared as described elsewhere (25). pGal4-p300N, pGal4-p300M, pGal4-p300C, jun2-luc (encoding firefly luciferase), pGal4 tk-luc, pGal4-ATF-2WT, pCMV-Flag-c-junWT, pCMV-Flag-c-jun(S63A/S73A), pCMV-Flag-PKCα and pCMV-Flag-PKCαKD (“kinase dead”) were kindly donated by Dr. Y. Shi (Harvard Medical School, Boston, MA, USA), Dr. H. van Dam (University of Leiden, Leiden, The Netherlands), Dr. S. Ohno (Yokohama City University, Yokohama, Japan) and Dr. K. Iwai (Osaka University, Osaka, Japan). The PKC inhibitor Gö6976, the p38 inhibitor SB202190 and the control compound SB202474 were obtained from Calbiochem (Darmstadt, Germany). Rabbit polyclonal antibodies specific for human ATF-2 (N-96), for phosphorylated human ATF-2 (p-Thr69/71), for human PKCα, PKCβI and PKCβIII, for human β-actin, and HRP-IgG-F(ab)_2 (HRP, horseradish peroxidase) were obtained from Santa Cruz Biotech. Inc. (Santa Cruz, CA, USA), and mouse monoclonal antibody against phosphorylated human ATF-2 (p-Thr71) was purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against ATF-2-pS490/498 and FITC-IgG-F(ab')_2 (FITC, fluorescein isothiocyanate) were obtained from Colorado Bioscience (Aurora, CO, USA) and BioSource International Inc. (Camarillo, CA, USA), respectively. TRITC-IgG (TRITC, tetramethyl rhodamine isothiocyanate) and FITC-IgG second antibodies and antibodies against Flag (M2) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of Antibodies Against Phosphorylated Serine Residues at Positions 121, 340 and 367 of ATF-2—Antibodies against phosphorylated peptides that included,
respectively, a phosphorylated (p-) amino acid residue at position 121, 340 and 367 of ATF-2 were raised in rabbits, which were immunized with keyhole-limpet hemocyanin-conjugated phosphopeptides [NH₂-TPIIRpSKIEC-COOH (TB0608; 116-125), NH₂-QTQSTpSGRRRC-COOH (TB0609; 335-344) and NH₂-NRAAApSRC-COOH (TB0610; 362-368)]. The antibodies were affinity-purified on columns prepared with the respective immobilized phosphopeptides according to the protocol from the manufacturer (MBL, Inc., Ina, Nagano, Japan).

Cell Culture—Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Gibco-Invitrogen, Grand Island, NY, USA), 100 U of penicillin plus streptomycin and glutamine (Gibco-Invitrogen) at 100 μg per ml each (25). Cells were serum-starved in DMEM plus 0.5% FBS for 12-16 h at 37 °C in an atmosphere of 5% CO₂ in air before stimulation. Then cells were treated with TPA (Sigma-Aldrich) at 60-200 nM for 6 h at 37 °C. Induction of the differentiation of F9 cells by retinoic acid (RA) was performed as described elsewhere (25).

Primary mouse embryonal fibroblasts (MEF) derived from mice with null alleles for ATF-2 and ATF-7 or for ATF-2 only were generated from E11.5 and E12.5 embryos that were derived from crosses of heterozygous animals as follows: ATF-2⁻/⁺/ATF-7⁻/⁺x ATF-2⁻/⁺/ATF-7⁻/⁺ (16).

Western Blotting—HeLa cells or HeLa-S cells were serum-starved, incubated with TPA (60-200 nM) for 6 h and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 25 mM NaF, 25 mM β-glycerophosphate, and 0.1 mM Na₃VO₄) that contained a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). After incubation for 30 min on ice and centrifugation at 15,000 rpm for 15 min, supernatants were saved and analyzed by SDS-PAGE (4-12% polyacrylamide) and Western blotting with the appropriate phosphorylation-specific antibodies (see above). In some experiments, cells were washed three times for 3 min each at 4 °C with CSK (cytoskeleton) buffer, which contained 10 mM piperazine-N,N’-bis(2-ethanesulfonic acid) (PIPES), pH 6.8, 100 mM NaCl, 300 mM sucrose, 7 mM MgCl₂, 1 mM EDTA, 0.5% Triton X-100, 1 mM Na₃VO₄, 50 mM NaF and 20 mM β-glycerophosphate, and then lysed in RIPA buffer as described above. For peptide blocking, antibodies against ATF-2pS121 (1:100) were preincubated with or without 200 μg/ml p-Ser121 phosphorylated peptide (TB0608-1; MBL) or 200 μg/ml Ser121 non-phosphorylated peptide (TB0608-2; MBL). The antibodies against ATF-2pS340 (1:100) were incubated with or without 200 μg/ml p-Ser340 phosphorylated peptide (TB0609-1; MBL) or 200 μg/ml Ser340 non-phosphorylated peptide (TB0609-2; MBL). The antibodies against ATF-2pS367 (1:100) were incubated with or without 200 μg/ml p-Ser367 phosphorylated peptide (TB0610-1; MBL) or 200 μg/ml Ser367 non-phosphorylated peptide (TB0610-2; MBL), for 60 min at room temperature. Phosphatase inhibitors (1 mM Na₃VO₄, 50 mM NaF and 20 mM β-glycerophosphate) were included during blotting procedures.

Transfection and Assays of Luciferase Activity—From 2 to 5 x 10⁴ HeLa cells, F9 cells...
and MEF, respectively, were subcultured in 500 μl of DMEM plus 10% FBS in individual wells of 24-well plates (Greiner-Japan, Tokyo, Japan) and in collagen-coated or gelatin-coated wells of 24-well plates (IWAKI, Tokyo, Japan). The indicated amount of pcDNA-Flag-ATF2WT or one of its mutated derivatives and 0.4 μg of AP-1-luc or 0.2 μg of CRE-luc and pBSK-II(-) (to give a total final amount of DNA of 1 μg) were used for transfections with Lipofectamine™ 2000 (Invitrogen BV, Groningen, The Netherlands) according to the manufacturer’s protocol. Six hours after transfection, fresh DMEM plus 0.5% FBS was added and incubation was continued for another 16 h. Cells were then exposed to 60 nM TPA for 6 h and assayed for luciferase activity with a Renilla (for CRE-luc) or a firefly (for AP-1-luc) luciferase reporter assay kit (Promega). Inhibitors Gö6976 (10 μM), SB202190 (20 μM) and SB202474 (20 μM), as indicated, were added to the culture medium 5 h before the treatment with RA or TPA. In mammalian two-hybrid assays, the luciferase reporter (pFR-Luc; 400 ng) was introduced into F9 cells, HeLa cells or MEF with the pGal4-ATF-2 expression vector (100 or 200 ng) and the internal control pRL-CMV (100 ng), as well as pBSK-II(-) (to give a total final amount of DNA of 1 μg) by the Lipofectamine™ method (see above). Luminescence was measured immediately with a luminometer (AROVO™ Light, model 1420; Perkin Elmer, Turku, Finland). Luciferase activity was normalized by reference to the protein concentration of the sample, as determined with a protein assay system from Bio-Rad (Hercules, CA, USA) with BSA (bovine serum albumin) as the standard. At least three cell-culture wells were subjected to each treatment. All experiments were performed as least three times.

**Immunofluorescence**—HeLa cells were cultured in Iscove’s modified DME (GIBCO-Invitrogen) plus 5% FBS. For stimulation of phosphorylation at Ser121 of ATF-2, cells were treated with 200 nM TPA for 30 min or 60 min. PKC-mediated phosphorylation of serine was blocked by treatment with 10 μM Gö6976 (PKC inhibitor; Calbiochem) for 30 min prior to the treatment with TPA. Confocal and Nomarski differential-interference-contrast (DIC) images were obtained with a confocal laser scanning microscope with a 40x 1.00 NA oil immersion objective (Fluoview FV500; Olympus, Tokyo; ref. 26). Cells were treated in *situ* with 0.5% Triton X-100 at 4 °C for 3 min prior to fixation in 4% paraformaldehyde for 20 min (27), and they were permeabilized in phosphate-buffered saline (PBS) plus 0.1% saponin and 3% BSA at room temperature (28). Cells were stained with primary antibody for 1 h, washed with PBS plus 0.1% saponin, and then stained with FITC- or TRITC-conjugated second antibody for 1 h. For DNA staining, cells were treated with 200 μg/ml RNase A for 1 h and 20 μg/ml propidium iodide (PI) for 30 min. Stained cells were mounted with ProLong™ antifade reagent (Molecular Probes, Eugene, OR, USA). For peptide blocking, antibodies against ATF-2pS121 (1:100) were preincubated with or without 200 μg/ml p-Ser121 phosphorylated peptide (TB0608-1; MBL) or 200 μg/ml Ser121 non-phosphorylated peptide (TB0608-2; MBL). The antibodies against ATF-2pS340 (1:100) were preincubated with or without 200 μg/ml p-Ser340 phosphorylated peptide (TB0609-1; MBL), 200
μg/ml Ser340 non-phosphorylated peptide (TB0609-2; MBL) or 200 μg/ml p-Ser121 phosphorylated peptide (TB0608-1; MBL) for 30 min at room temperature. Phosphatase inhibitors (1 mM Na3VO4, 50 mM NaF and 20 mM β-glycerophosphate) were included during in situ extraction and peptide blocking. One planar (xy) section slice (thickness, 1.0 μm) was examined in each replicate of each experiment. To ensure that there was no bleed-through from the fluorescein signal into the red channel, fluorescein and/or PI and rhodamine were excited independently at 488 nm and 543 nm, respectively (29). The mean fluorescence intensity of phosphorylated ATF-2 in nuclei was measured with Fluoview version 4.3 software (Olympus).

Statistical Analysis—Statistical significance was determined by Student’s t-test for experiments with two groups and by an analysis of variance followed by Fisher’s protected least-significant-difference (PLSD) test for experiments with more than two groups. A value of p<0.05 was considered to represent statistical significance.

For Phosphorylation of (His)6-ATF-2WT, (His)6-ATF-2S121A and Synthetic Peptides by in vitro PKC, Gene Silencing with siRNA and Real Time RT-PCR, and Assays of Kinase Activity in vitro, Immunokinase Assays and Phosphatase Treatment, see Supplemental Data.

RESULTS

Protein Kinase C (PKC) Is Involved in AP-1/CRE-Dependent Transcription during the Retinoic Acid-Induced Differentiation of F9 Cells—We reported previously that phosphorylation of activation transcription factor 2 (ATF-2) by protein kinase C (PKC) is critical for DRE-dependent transcription during the differentiation of F9 cells in response to retinoic acid (RA) or adenovirus E1A (24). Talmage’s group showed that PKCα and PKCβ exhibit different patterns of induced expression and that each plays a distinct role during such RA-induced differentiation (30, 31). Moreover, RA triggers the transition of expression from PKCβ to PKCα in differentiated F9 cells. However, protein kinase Cα is not sufficient for induction of the full differentiation of F9 cells but is involved in pathways that lead to the expression of differentiation-associated genes (32). To investigate the roles of PKC in AP-1/CRE-dependent transcription during the response to RA, we used siRNAs to suppress the expression of genes for PKCα, PKCβI and PKCβII. As shown in Fig. 1A, the expression of a CRE reporter gene was decreased six-fold and five-fold by siRNA specific for PKCα and by siRNA specific for PKCβI, respectively, during the response of F9 cells to RA. However, expression of the reporter gene was unaffected by siRNA directed against PKCβII and by the negative control siRNA (N.C.; random siRNA). We observed a similar response by the RA-inducible AP-1 reporter gene with siRNAs against PKCα and against PKCβI but not against PKCβII (Fig. 1B). Each siRNA reduced the expression of the respective endogenous PKC but the control siRNA did not (Fig. 1C). Thus, at least, the signal cascade of PKCα and PKCβI appeared to be involved in the AP-1/CRE-related response of F9 cells that are exposed to RA. To investigate, in further detail, the effect of each
siRNA on the RA-induced differentiation of F9 cells, as described above, we treated cells with 1 μM RA to induce differentiation and then monitored morphological changes. Compared with morphological changes in cells that had been pretreated with control siRNA (N.C.), morphological changes in cells that had been pretreated with siRNAs against PKC\(\alpha\) and against PKC\(\beta/II\) were significantly delayed or absent. By contrast, changes in cells pretreated with siRNA against PKC\(\beta/II\) were unaffected (Fig. 1D).

We next compared, by real time RT-PCR, the levels of expression of RA-inducible genes and of genes whose expression is a marker of differentiation and non-differentiation after the exposure of cells to 1 μM RA. The levels of the induced expression of genes for laminin B1 and collagen type 4\(\alpha/1\), two markers of endodermal differentiation, were 85-90% lower in cells that had been pretreated with siRNAs against PKC\(\alpha\) and against PKC\(\beta/II\) than in control cells 72 h after treatment with RA (Fig. 1E). These results indicated that the “knock-down” of PKC\(\alpha\) and PKC\(\beta/II\) inhibited the RA-induced differentiation of F9 cells.

We also studied the effects of the PKC inhibitor Gö6976 and the p38 inhibitor SB202190 on the transcription of AP-1/CRE reporter genes in F9 cells. Figures 1F shows that pretreatment with an inhibitor for 2 h prior to the exposure of cells to RA resulted in 50-70% lower transcription of the respective reporter genes, as compared to transcription in the presence of the control reagent (SB202474). These results indicated that pathways involving PKC and p38 might both be involved in the transcription of AP-1/CRE reporter genes during the differentiation of F9 cells in response to RA.

**Phosphorylation of ATF-2 at Amino Acid Residue 121**—To examine the phosphorylation of ATF-2 by PKC in vitro, we used recombinant ATF-2 and corresponding peptides that included the possible sites of phosphorylation in ATF-2. Sakurai et al. reported that serine residues at positions 340 and 367 are critical for PKC\(\beta\)-induced phosphorylation in vitro (33). By contrast, we showed previously that the serine residue at position 121 is crucial for PKC\(\alpha\)-induced phosphorylation and p300-dependent transcription (24). However, the exact roles of these residues in ATF-2 remain unknown. We examined, first, the relative levels of phosphorylation by PKC of the recombinant wild-type His-ATF-2 protein (His\(_{56}\)-ATF-2WT) and a serine-to-alanine mutant at residue 121 (His\(_{56}\)-ATF-2S121A). As shown in Figures 2A and 2B, the extent of phosphorylation by rat PKC of His\(_{56}\)-ATF-2S121A was 30 to 50% lower than that of His\(_{56}\)-ATF-2WT. We also observed a similar relative reduction in phosphorylation of His\(_{56}\)-ATF-2S121A by human PKC\(\alpha\) and rabbit PKC\(\alpha\) (data not shown). Our results indicated that the serine residue of position 121 (Ser121) of ATF-2 is a possible site of phosphorylation by PKC. We next examined the phosphorylation of peptides that corresponded to positions 121, 340 and 367 of ATF-2, in vitro, at pH 7.0 and at pH 4.5 (Fig. 2, C-L). In the analysis of the phosphorylation of Ser121 of ATF-2 by PKC, we included peptide P4, which did not include the two glutamic acid residues normally found at positions 124 and 125, adjacent to Ser121, because these glutamic acid residues are negatively charged at neutral pH and such charged glutamic acid residues might prevent
access by PKC (34). We examined peptides P1 and P4 as substrate peptides at both pH 7.0 and pH 4.5. Conventional PKCs, such as α, βI, and βII, and PKCs from human and rat were all able to phosphorylate Ser121 of human ATF-2 to some extent. The extent of phosphorylation of peptides that included Ser121 was 5- to 10-fold lower than that of peptides that included Ser340 or Ser367. At acidic pH (pH 4.5), the phosphorylation of Ser121 was significantly enhanced in some cases in the presence of the glutamic acid residues at positions 124 and 125, adjacent to Ser121. We found that Ser121 was clearly phosphorylated in vitro by all tested PKCs with the exception of PKCy.

**Antibodies Directed Against Phosphorylated Ser121, Ser340 and Ser367**—We generated antibodies that specifically recognized ATF-2 that had been phosphorylated at positions 121, 340 and 367, respectively (see refs. 24 and 33 and, also, Fig. 3A). Bands of proteins of approximately 68-70 kDa were detected after SDS-PAGE of extracts of HeLa cells with antibodies against ATF-2 (N-96; Fig. 3B). Similar mobilities of phosphorylated ATF-2 in HeLa cells were revealed by antibodies specific for p-Ser121, p-Ser340 and p-Ser367 (Fig. 3B). As shown in Figure 3C, recognition of phosphorylated ATF-2 by antibodies against p-Ser121 was blocked by the addition of 200 μg/ml p-Ser121 peptide (TB0608-1) but not by addition of the nonphosphorylated peptide (TB0608-2). Moreover, the signals due to antibodies against p-Ser340 and p-Ser367 were blocked by peptides that corresponded to the respective phosphorylated peptides (TB0609-1 and TB0610-1) but not by those that corresponded to the non-phosphorylated peptides (TB0609-2 and TB0610-2). These results indicated that each preparation of antibodies was specific for the phosphorylation of the respective serine residue (p-Ser121, p-Ser340 and p-Ser367). In some cases, two bands corresponding to p-Ser121, p-Ser340 and p-Ser367, respectively, were evident and the intensity of each band seemed to depend on the extent of phosphorylation and on cell type. We obtained similar results after Western blotting of lysates from cell lines derived from rabbit cornea and kidney, and mouse teratocarcinoma (supplemental Fig. S1).

The recognition, in ELISAs, of phosphorylated peptides of ATF-2 by antibodies against p-Ser121, p-Ser340 and p-Ser367, respectively, was inhibited by the phosphorylated peptides, but not by the nonphosphorylated peptides, that included the respective sites of phosphorylation in ATF-2 (supplemental Fig. S2, A, B and C), confirming the specificity of each antibody. Each major band of protein of 68-70 kDa disappeared when the relevant phosphopeptide was included as a competitor. However, the bands of the lower-molecular-mass protein visualized with p-Ser367-specific antibodies did not disappear, suggesting that these bands might represent unrelated cross-reacting proteins. We treated nuclear extracts with protein phosphatase-1A (PP1A) and examined its effects by Western blotting. As shown in Figure 3D, the bands of phospho-ATF-2 disappeared after such treatment. Thus, the respective bands were specific for phosphorylated ATF-2 and each individual residue of ATF-2 was indeed a site of phosphorylation in vivo. To confirm this conclusion, we performed immunoprecipitation and Western blotting to demonstrate the
cross-reactivity of antibodies specific for ATF-2 in vivo (supplemental Fig. S2D). First, we used antibodies specific for each phospho-ATF-2 to immunoprecipitate the protein from lysates of HeLa cells and then we performed immunoblotting with N-96 antibodies that are specific for general preparations of ATF-2. Each preparation of phospho-ATF-2-specific antibodies cross-reacted with ATF-2 in the reaction with N-96 antibodies in extracts of HeLa cells.

Sequential immunoprecipitations revealed that the intensity of the slowly migrating band of phosphorylated ATF-2, as visualized with antibodies against p-Ser121, fell by 40-50% when extracts had been treated with ATF-2pT69/71-specific antibodies (supplemental Fig. S2E). These results indicated that specificity, in terms of the present assays, for phosphorylated ATF-2pS121 overlapped by 40-50% with that for ATF-2pT69/71.

**Phosphorylation Assays**—To confirm the results of phosphorylation of Ser121 of ATF-2, we performed a kinase assay in vitro with GST-fused ATF-2(1-125), namely, the amino-terminal-half protein, and its serine-to-alanine variant at position 121 as substrates. Each substrate was mixed with recombinant human PKC and then incubated with [$\gamma^{32}$P]-ATP. As shown in Figure 4A, GST-ATF-2(1-125) was phosphorylated by PKCα but the GST-ATF-2(1-125)S121A mutant of this fragment of ATF-2 was phosphorylated only 40-60% as efficiently as wild-type GST-ATF-2(1-125).

We also performed an immuno-kinase reaction using immunoprecipitates obtained with PKCα-specific antibodies and GST-ATF-2(1-125)WT or GST-ATF-2(1-125)S121A as substrate for the kinase reaction. HeLa cells were transfected with pCMV-Flag-PKCα and the plasmid that encoded the “kinase-dead” form, namely, pCMV-Flag-PKCα KD and then, after or without treatment with TPA, cell extracts were immunoprecipitated with Flag-specific antibodies. Immunoprecipitates were incubated with GST-ATF-2(1-125), as substrate, and [$\gamma^{32}$P]ATP and then each reaction mixture was subjected to SDS-PAGE (Fig. 4B). As we had anticipated, GST-ATF-2(1-125)WT was phosphorylated by PKCα but not by PKCαKD (lanes 6-10). The serine-to-alanine mutant GST-ATF-2(1-125)S121A was not phosphorylated to a significant extent, as compared with the wild type, in response to TPA (lanes 3, 4, 8 and 9). Thus, we were able to conclude definitively that PKCα phosphorylates ATF-2 at Ser121.

**Induction of Phosphorylation of Serine 121 in ATF-2 in Response to TPA**—As shown in Figure 1, PKC plays a critical role in RA signaling during the differentiation of F9 cells and the phosphorylation of Ser121 of ATF-2, as well as in the transcription of AP-1/CRE reporter genes. We next examine the molecular mechanism of TPA-induced phosphorylation of Ser121 of ATF-2 by PKC and the effect of such phosphorylation on the transcription of AP-1/CRE reporter genes in HeLa cells, the so-called “TPA-response.” To investigate the role of each site of phosphorylation in ATF-2, we stimulated HeLa cells with TPA and examined levels of phosphorylation of ATF-2, after induction, by Western blotting with antibodies specific for the various sites of phosphorylation of ATF-2. Figure 4C shows that TPA induced the
time-dependent phosphorylation of the threonine residues at positions 69 and 71 of ATF-2 (to yield ATF-2pT69/71). Such phosphorylation was detectable within 15 min, peaked at 30 min, then declined at 60 min and returned to the basal level. A similar pattern was detected in the case of phosphorylation of Ser340 and Ser367 of ATF-2. By contrast, the phosphorylation of Ser121 began 30 min after the start of exposure to TPA, increased gradually, and remained strong for more than 2 h.

In F9 cells, the phosphorylation of the threonine residues at positions 69 and 71 (Thr69/71) was enhanced 24 h after the start of incubation with RA and then the level of phosphorylated ATF-2 decreased (supplemental Fig. S1). Such temporary induction was detected similarly in the case of phosphorylation of Ser340. The phosphorylation of Ser367 also continued into the late phase of the induction of differentiation by RA. However, the phosphorylation of Ser121 of ATF-2 was initially induced at 30 h and then increased gradually until 117 h. These results indicated clearly that, in both HeLa cells and F9 cells, induction of the phosphorylation of Ser121 in ATF-2 is distinct and delayed as compared to the phosphorylation of other sites in ATF-2 in response to TPA and to RA.

The temporary induction of phosphorylation of Thr69/71 might be due to the induction of JNK and p38, which respond to stress signals, or of ERK, which responds to mitogenic signals. PKC is also involved in phosphorylation during the RA-induced activation of AP-1/CRE genes in F9 cells (24, 30-32). To clarify the distinct roles of JNK/p38/ERK and PKC, we used selective inhibitors to identify the major upstream kinases that activate ATF-2. Pretreatment of HeLa cells with SB202190 caused an average of decline of approximately 40% in TPA-induced CRE-mediated transcription, as compared with negative-control pretreatments (supplemental Fig. S3). Gö6976, an inhibitor of PKC, depressed TPA-induced CRE-mediated transcription by more than 85%. In addition, the negative-control compound SB202474 did not significantly alter the extent of TPA-induced transcription. Thus, it is likely that the PKC and JNK/p38/ERK pathways are both involved in TPA-induced transcription.

Nuclear Localization of Ser121-Phosphorylated ATF-2 and Ser340-Phosphorylated ATF-2—To examine the distribution of endogenous Ser121-phosphorylated and Ser340-phosphorylated ATF-2 in HeLa cells, we used the antibodies specific for the respective phosphorylated peptides. As shown in Figure 5A, immunostaining with antibodies against p-Ser121 was blocked by the addition of 200 μg/ml p-Ser121 peptide (TB0608-1) but not by addition of the nonphosphorylated peptide (TB0608-2) or of the phosphorylated Ser340 (p-Ser340) peptide (TB0609-1). Similarly, signals due to antibodies against ATF-2pS340 were blocked by peptides that corresponded to p-Ser340 (TB0609-1) but not by those that corresponded to nonphosphorylated Ser340 (TB0609-2) and p-Ser121 peptides (TB0608-1) (Fig. 5B). Both forms of phosphorylated ATF-2 protein were detected throughout nuclei but strong signals were observed only in regions of decondensed chromatin. Antibodies against p-Ser121 peptide yielded strong signals both inside and outside nuclei. The signals that we
obtained outside nuclei seemed to be due to nonspecific cross-reacting proteins since these signals did not disappear after treatment of cells with the PKC inhibitor Gö6976, while, by contrast, the signals within nuclei disappeared completely after such treatment (see Figs. 5 and 6).

Co-Localization of ATF-2pS121 and ATF-2pT69/71 in the Nuclei of HeLa Cells—Signals due to ATF-2pT69/71 were localized in regions of decondensed chromatin and mainly as nuclear foci (Fig. 6A). Signals due to ATF-2pT69/71 were detected in all regions of nuclei with the exception of the nucleoli. The entire nuclear region was immunostained with antibodies against ATF-2 (N-96), as was the cytoplasm (5). Treatment with TPA did not significantly alter the localization of signals due to ATF-2pT69/71 and N-96-immunostained ATF-2. Moreover, in most cases, the signals due to ATF-2pS121 partially overlapped the signals due to ATF-2pT69/71. Incubation with TPA resulted in increasingly intense signals due to ATF-2pS121, leading to even more conspicuous co-localization of ATF-2pS121 with ATF-2pT69/71. Signals due to ATF-2pS340 were co-localized to some extent with those due to ATF-2pT69/71; however treatment with TPA did not alter the localization of the respective signals in nuclei. Phosphorylation of ATF-2 at positions 490 and 498 is critical for the stress response to DNA damage that involves ATM kinase (35). Signals due to ATF-2pS490/498 were evident throughout nuclei but were not co-localized with those due to ATF-2pT69/71. Treatment with TPA did not alter the localization of the respective signals in nuclei.

We also examined the specificity of signals due to p-Ser121, p-Ser340 and p-Thr69/71 by pre-incubating cells with the PKC inhibitor Gö6976 for 30 min. The intensity of signals increased significantly upon incubation with TPA but signals were abolished by the pre-treatment with Gö6976 and remained at basal levels (Fig. 6B). By contrast, signals due to ATF-2pS340 were unchanged by TPA but pre-treatment with Gö6976 resulted in signals below basal levels. Moreover, treatment with TPA slightly decreased the intensity of signals due to ATF-2pT69/71, and Gö6976 reduced these signals to basal levels. These data indicate that TPA affects the phosphorylation of ATF-2 at Ser121 in the nucleus specifically, but it does not similarly affect ATF-2pS340 and ATF-2pT69/71. TPA increases the nuclear accumulation of ATF-2pS121 and decreases the level of ATF-2pT69/71. The dissimilar profiles of phosphorylation of ATF-2pS121 and ATF-2pT69/71 in response to TPA might be due to different respective actions of PKC.

Reporter Assays with Mouse Embryonal Fibroblasts (MEF) from ATF-2-Null Mice—The DRE locus is necessary and sufficient for the RA-induced expression of the c-jun gene in F9 cells (36, 37) and, in a previous report, we described the transcription of DRE-containing genes that is induced by ATF-2 and p300 in F9 cells (24). In the present study, we focused on the roles of the phosphorylation of individual serine residues at positions 121, 340 and 367 of ATF-2 in the regulated transcription of AP-1/CRE genes. ATF-2 and ATF-7 exhibit considerable sequence homology, in particular, in their amino-terminal activation domains, and their DNA-binding/dimerization domains are almost identical. ATF-7 can form dimers with other
members of the ATF family and with AP-1 proteins and its expression is regulated via INK and p38 (38, 39). ATF-2 and ATF-7 are expressed similarly in various adult and embryonic tissues (Breitwieser and Jones, personal communication). Thus, it is possible that ATF-2 and ATF-7 have overlapping functions in mammalian development. Therefore, in the following experiments, we used mouse embryonic fibroblasts (MEF) from ATF-2-null mice [ATF-2(-/-)] (Fig. 7A) and from ATF-2-null x ATF-7-null mice [ATF-2(-/-)/ATF-7(-/-)] (Fig. 7B) to circumvent any compensatory effects by either partner.

First, to investigate the role of ATF-2pS121, we examined the effects of the transient expression of pCMV-Flag-ATF-2WT and pCMV-Flag-ATF-2S121A on an AP-1 reporter gene in ATF-2(-/-) MEF in response to TPA. As shown in Figure 7A, both ATF-2S121A and ATF-2T69/71A each decreased the TPA-dependent transactivation of pGal4-p300 by 20 to 30%, as compared with the wild-type ATF-2 (p = 0.004 and 0.009, respectively).

The product of the c-jun oncogene forms a complex with ATF-2 (1, 12) and ATF-7 (38, 39), both in vivo and in vitro, to activate the transcription of AP-1 target genes. Efficient activation of transactivational potential in response to TPA has been observed with Gal4-c-Jun chimeric constructs that include the transactivation domain of c-Jun (40, 41). Therefore, we next examined the effect of c-Jun on the activation of an AP-1 gene by ATF-2WT and ATF-2S121A, as well as by other derivatives. As shown in Figure 7B, ATF-2S121A reduced the expression of the AP-1 reporter gene by 30 to 40% (p = 0.003). By contrast, other mutated constructs, namely, ATF-2S340A, ATF-2S367A and ATF-2T69/71A, did not affect the activity of the AP-1 reporter gene. To determine whether our derivatives of ATF-2 could act as transcriptional activators, we fused various forms of ATF-2 to the DNA-binding domain (DBD) of Gal4 and examined their transactivation capacity in HeLa cells (Fig. 7C). In the absence of a c-Jun expression vector, only ATF-2S367A significantly enhanced the Gal4-reporter activity in MEF from ATF-2-null mice [ATF-2(-/-)] and from [ATF-2(-/-)/ATF-7(-/-)] mice (Fig. 7B and C).

We then transfected HeLa cells with the Gal4-site-containing reporter gene together with Gal4-ATF-2 mutants that corresponded to respective sites of phosphorylation and measured the luciferase activity due to Gal4-luciferase in the presence of the pPKCα or the “kinase-dead” pPKCαKD expression vector. Since the amino-terminal half of ATF-2 acts as a transactivation domain and the carboxyl half of ATF-2 has an inhibitory effect on AP-1-dependent transactivation (12, 13, 42), we used a gene that encoded full-length ATF-2 fused to Gal4. We found that the reporter activity of Gal4-luciferase in HeLa cells transfected with ATF-2WT was significantly enhanced in the presence of PKCα. By contrast, the mutant derivative in which Ser121 had been replaced by Ala121 (S121A) repressed expression of the reporter activity (Fig. 7C). Although the transactivational activity with Gal4-ATF-2 was weak, we were able to observe a significant difference in reporter activity between ATF-2WT and ATF-2S121 (p = 0.017). Thus, we can conclude that phosphorylated ATF-2S121 plays a role in the regulation of both TPA- and c-Jun-dependent transcription in HeLa cells.
DISCUSSION

In the present study, we found that the serine residue at position 121 of ATF-2 is phosphorylated by protein kinase C (PKC) and is essential for the nuclear accumulation of ATF-2 in response to TPA, as well as for the transactivation of AP-1 reporter genes. It has been demonstrated that TPA preferentially enhances c-Jun-dependent transcription but does not affect ATF-2-dependent transcription (11-13). Moreover, the TPA-induced protein kinase, which is distinct from SAPKs, phosphorylates c-Jun but not ATF-2 (12). The Raf-MEK-ERK pathway triggers the monophosphorylation of ATF-2T71, providing an explanation for the fact that TPA, a strong activator of the Raf-MEK-ERK pathway, failed to activate an ATF-2-dependent reporter gene (12, 23). Moreover, experiments with kinase inhibitors demonstrated that ATF-2 is phosphorylated at Thr71 by JNK1/2 and by ERK 1/2 (22). In our present study, we used an antibody specific for Ser121 of ATF-2 to clarify the phosphorylation cascade that occurs in the response to TPA and the role of the c-Jun-dependent transactivation of AP-1/CRE reporter genes.

We showed previously that phosphorylation of ATF-2 at Ser121 by PKC is relevant to RA-induced transactivation of AP-1 target genes in co-operation with p300 (24). The predicted sites of phosphorylation of ATF-2 by PKC in vitro were reported to be Ser121 (24), Ser340 and Ser367 (33). We previously performed phosphorylations in vitro with human recombinant ATF-2 as substrate and a mixture of rabbit PKCα, PKCβ and PKCγ, which had been purified by biochemical methods (24). We assumed that phosphorylation of ATF-2 was mediated by PKCα since PKCα is the major kinase in F9 cells during RA-induced differentiation (24, 30-32). By contrast, Sakurai et al. (33) used biochemically purified protein kinase Cβ from rat brain to phosphorylate human recombinant ATF-2 protein and they identified the phosphorylated residues as Ser340 and Ser367. We cannot explain the discrepancy between their report and ours. It is possible that a dipeptide that includes Ser121, generated by digestion with TPCK-trypsin, might have eluted in the void volume from the reversed-phase HPLC column used by Sakurai et al. (33). Alternatively, rat PKCβ might not phosphorylate Ser121 as efficiently as rabbit PKCα, PKCβ and PKCγ. However, we know that this alternative possibility is not relevant because, in our hands, all three sites of phosphorylation of ATF-2 were phosphorylated by PKCα and PKCβ derived from human, rat and rabbit, even though levels of phosphorylation were variable (data not shown). As shown in Figures 2 and 3, the serine residue at position 121 of ATF-2 was clearly phosphorylated by PKCα and PKCβ in vitro. The extent of phosphorylation of the Ser121 peptide by PKC was 5- to 10-fold lower than that of the Ser340 and Ser367 peptides in vitro both at pH 7.0 and at pH 4.5 (Fig. 2). Moreover, the DNA-binding activity of ATF-S121A and its capacity for formation of a heterodimer with c-Jun in vitro were similar to those of ATF-2WT and the T69/71A mutant of ATF-2 (16; data not shown).

To determine whether or not PKCα can phosphorylate Ser121 of ATF-2, we performed in vitro phosphorylation reactions with GST-ATF-2...
and its mutant derivatives as substrates. GST-ATF2(1-125)S121A was phosphorylated approximately three-fold less efficiently by human PKCα than was GST-ATF-2(1-125)WT (Fig. 4A). The weak background signals in the experiments with the GST-ATF-2(1-125) series were due to the non-specific phosphorylation of Ser9 of ATF-2 (data not shown). We also examined immunoprecipitates when TPA-treated cells were used as the source of enzyme. As shown in Figure 4B, at least PKCα, “kinase-dead” PKCα (α), was able to phosphorylate ATF-2 at Ser121. The siRNA “knock-down” experiments also showed that PKCα and PKCβI are involved in the RA-induced transcription of AP-1 genes in F9 cells (Fig. 1). Thus, while PKCα is clearly able to phosphorylate ATF-2, we cannot rule out the possibility that other subspecies of PKC that are sensitive to the PKC inhibitor Gö6976 might be involved in the phosphorylation of ATF-2 in vivo because the nuclear localization of ATF-2 pS121 partially overlapped not only that of PKCα but also that of PKCβI, and these co-localized signals were not significantly enhanced but were maintained upon treatment of cells with TPA (data not shown). We did find evidence for the molecular association of p-Ser121 with PKCα and with PKCβI by immunoprecipitation and Western blotting (IP-Western blots; supplemental Fig. S4). However, we could barely detect the relevant bands when we performed immunoprecipitations with antibodies against p-Ser121 and then blotted with either PKCα or βI-specific antibodies, perhaps because of the low titers of the antibodies against the p-Ser121 peptide or because the sites of association might have hidden the antigenic sites from the antibodies.

As shown in Figure 3, we prepared antibodies against p-Ser121, p-Ser340 and p-Ser367 (anti-pS121, anti-pS340, anti-pS367) peptides. We detected a major band of protein of 68-70 kDa when we examined the specificity of each of these antibodies (Fig. 3B). Competition experiments with phosphopeptides that corresponded to each respective phosphorylated residue and a phosphatase experiment confirmed the antigenic specificity of each preparation of antibodies (Fig. 3, C and D). The nature of the newly prepared antibodies against the various PKC-phosphorylated sites in ATF-2 was confirmed by cross-reaction with antibodies against phosphorylated ATF-2 (supplemental Fig. S2D). The antigenic specificity of p-Ser121 slightly overlapped that of p-Thr69/71 but not that of p-Ser340 or of p-Ser367, as demonstrated by the results of an absorption experiment (supplemental Fig. S2E).

The specificity of each antibody was also tested in competition experiments that involved immunostaining of cells. As shown in Figure 5, we found that antibodies against p-Ser121 and p-Ser340 could be used successfully for cell-immunostaining experiments. We compared the extent of phosphorylation of Thr71, as well as that of two threonine residues Thr69/71, of ATF-2 with those of Ser121 and Ser340 after stimulation of HeLa cells with TPA (Fig. 4C). In HeLa cells, the phosphorylation of Ser340, Ser367 and Thr69/71 in ATF-2 in response to TPA was transient. The phosphorylation of Thr69/71 appeared at 15 to 30 min and decreased 45 min after the start of treatment with TPA. By contrast, the phosphorylation of Ser121 was induced at 30 min
and was then maintained for another 1.5 h. A similar delay in the phosphorylation of Ser121 was evident during the RA-induced differentiation of F9 cells (Fig. 1). We also detected significant phosphorylation of Ser121, Ser340 and SerS367 in rabbit kidney PK13 cells but only weak phosphorylation of these three sites in rabbit normal cornea RC4 cells. Moreover, exposure of these cells to UV light did not significantly alter the extent of phosphorylation of these residues in ATF-2, suggesting that these residues might not be involved, to any great extent, in the response to UV stress (supplemental Fig. S1A). To confirm a difference between the role of the phosphorylation of Ser121 and that of Ser340, we compared the nuclear localization of the two forms of phospho-ATF-2. As shown in Figure 5, we detected signals due to ATF-2pS121 both inside and outside nuclei. Signals outside nuclei were probably due to noise caused by cross-reacting proteins because nuclear signals due to p-Ser121 were muted in the presence of the p-Ser121 peptide but not in the presence of the unphosphorylated Ser121 peptide or of the p-Ser340 peptide. As shown in Figure 6B, treatment with the PKC inhibitor Gö6976 eliminated only the nuclear signals due to p-Ser121 and did not eliminate cytoplasmic signals. Thus, signals inside nuclei were due to ATF-2pS121. Signals due to p-Ser340 were also specific and muted by the p-Ser340 peptide and not by either the p-Ser121 peptide or the Ser340 peptide (Fig. 6B). It was clear, therefore, that signals due to ATF-2pS121 and ATF-2pS340, respectively, were different and distinct from one another.

The nuclear accumulation of ATF-2, upon stimulation of cells by TPA, was only recognized for ATF-2pS121, and not for ATF-2pS340 and ATF-2pS490/498. ATF-2pS490/498 is a marker of ATM-dependent phosphorylation, while ATF-2pT69/71 is a marker of the JNK/p38/ERK pathway (12, 23, 35). The nuclear accumulation of ATF-2pS121 disappeared in the presence of the PKC inhibitor Gö6976 (Fig. 6B). Some signals due to ATF-2pS121 were found in foci, as were some of those due to ATF-2pT69/71. The total number of foci of ATF-2pS121 was increased by treatment with TPA. These data indicate that ATF-2pS121 is phosphorylated very weakly under steady-state conditions and that treatment with TPA increases the nuclear accumulation of ATF-2pS121. The PKC inhibitor Gö6976 eliminated any enhancement of signals due to ATF-2pS121 above basal levels. By contrast, the extent of phosphorylation of ATF-2S340 was maximal under steady-state conditions, being three times that of ATF-2pS121, but treatment with TPA did not increase the intensity of the former signals (Fig. 6B). In the presence of Gö6976, the signals due to strongly phosphorylated ATF-2pS340 that we detected in steady-state HeLa cells disappeared completely, suggesting that, at steady state, ATF-2pS340 is phosphorylated in HeLa cells and its phosphorylation does not respond to TPA. Thus, the actions of ATF-2pS121 and ATF-2pS340 appear to be separate and different. Signals due to ATF-2pT69/71 were observed mainly as foci in nuclei (Fig. 6, A and B). The intensity of signals due to this form of ATF-2 did not increase significantly upon treatment of cells with TPA, and the inhibitor of PKC also failed to reduce these signals significantly. Thus, we can conclude that, in HeLa cells, PKC only phosphorylates ATF-2 to yield ATF-2pS121 in
response to TPA and then ATF-2pS121 accumulates in the nuclei. Neither ATF-2pS340 nor ATF-2pT69/71 accumulated in the nuclei in response to TPA but the inhibitor Gö6976 abolished the phosphorylation of Ser340 and Thr69/71. As a control we examined the effect of the p38 inhibitor SB202190 on the nuclear accumulation of ATF-2pS121, and we found no significant accumulation of ATF-2pS121 as in the case of ATF-2pT69/71, in nuclei after exposure of cells to SB202190 (supplemental Fig. S5). Liu et al. reported similar observations (5).

The results of Western blotting of whole cell lysates confirmed that levels of ATF-2pS340, ATF-2pS367 and ATF-2pT69/71 were elevated within 15 to 30 min of the start of exposure of cells to TPA, while significant accumulation of ATF-2pS121 was evident only 30 to 120 min after the start of treatment of HeLa cells with TPA (Fig. 4C). Thus, we can conclude that only Ser121 of ATF-2 is responsible for transport of ATF-2 to the nucleus in response to TPA-induced phosphorylation.

TPA does not affect the transactivation potential of ATF-2 to any significant extent in cells that do not express c-Jun, such as F9 cells (11-13). By contrast, in c-Jun-expressing cells, TPA affects the activation of ATF-2 in a c-Jun-dependent manner (5). In such cells, induction is probably mediated by c-Jun/ATF-2 heterodimers, suggesting that the composition of subunits determines the external signals to which the c-Jun promoter responds (5). Thus, the transactivational activity of ATF-2 is dependent on the contribution of c-Jun as a partner, which might reflect the cellular response to TPA.

Disruption of the intramolecular interaction between the bZIP domain and the amino-terminal transcriptional activation domain of ATF-2 is essential for the activation of ATF-2 in the nucleus since exogenously expressed ATF-2 has little transcriptional activation capacity unless it is co-expressed with co-activators (40-42). Hu et al. reported that exogenously expressed ATF-2 accumulates in the cytoplasm and, moreover, that the deletion of 341 amino-terminal residues of ATF-2 enhances the nuclear localization of c-Jun/ATF-2 heterodimers (43). Thus, the 341 amino-terminal residues might influence the structure of ATF-2 to block transport to the nucleus. It seems that ATF-2 has two possible NLS (nuclear localization signal) motifs and that these motifs might be masked by intramolecular interactions that involve the amino terminus and the bZIP domain (41). The c-Jun/ATF-2 dimerization might mask or otherwise inhibit the function of the ATF-2 NESs (nuclear export signals) of ATF-2, so that the complex is retained within the nucleus (5). We followed our demonstration that the phosphorylation of Ser121 is critical for the TPA-induced nuclear accumulation of phosphorylated ATF-2 by examining the effect of a Ser121-to-Ala121 mutation on the transactivation of AP-1/CRE genes.

Our reporter assays with an AP-1 reporter in ATF-2(-/-)/ATF-7(-/-) MEF showed that the ATF-2S121A mutant elicited lower AP-1 reporter activity than did ATF-2WT, ATF-2S340A, ATF-2S367A and ATF-2T69/71A, respectively (Fig. 7B). Moreover, ATF-2S121A failed to transactivate a pG4-p300 reporter gene in response to TPA in ATF-2(-/-) MEF (Fig. 7A). Thus, at least, PKCα affected the transactivation activity of ATF-2, while ATF-2S121A had no such activity (Fig. 7C).
Liu et al. reported that the phosphorylation of c-Jun and of ATF-2 by MAPKs, such as JNK/p38/ERK, is not related to the dimerization and nuclear localization of ATF-2 (5). In fact, we do not yet know the details of the signaling that occurs during the response to TPA and PKC-mediated induction. We also do not know whether dimerization with c-Jun is critical for ATF-2-mediated transactivation, whether activated PKC moves directly to the nucleus to “switch on” AP-1/CRE target genes with the help of c-Jun, and whether PKC activates other kinases, such as Raf-1, to transmit a signal for cross talk with the Raf-MEK-ERK system. It has been proposed that activated PKC in the cytoplasm might be transported directly to the nucleus, where it is involved in the chromatin-mediated regulation of TPA-responsive genes (44). We failed to detect any significant enhancement of the co-localization of ATF-2pS121 and PKC$\gamma$ after treatment of HeLa cells with TPA but we did observe that ATF-2pS121 accumulated in nuclei upon treatment of cells with TPA. Thus, it seems plausible that phosphorylation of ATF-2 by PKC might occur in the cytoplasm and then phosphorylated ATF-2 might be transported to the nucleus with c-Jun.

This is the first report, to our knowledge, of the unequivocal identification of the residue in ATF-2 that is phosphorylated in HeLa cells in response to TPA. This phosphorylation is mediated a PKC, most likely, PKC$\gamma$. Moreover, the individual replacement by alanine of T69/71, S340 and S367 in ATF-2 did not affect the activation of a Gal4-luciferase reporter by the respective derivatives of pGal4-ATF-2 in the presence of PKCo but the alanine-serine mutation of S121 repressed the expression of the reporter (Fig. 7C). Thus, we can conclude that regulation by TPA of the role of ATF-2 is dependent on the phosphorylation of Ser121 and on the accumulation of ATF-2pS121 in the nucleus, as well as on the formation of heterodimers with partners, such as c-Jun. With respect to the phosphorylation of ATF-2T69/71, we detected the co-localization of ATF-2pT69/71 and ATF-2pS121 in the nucleus. However, TPA did not stimulate the entry of ATF-2pT69/71pS121 into nuclei, but only that of ATF-2pS121 (data not shown). Our results strongly suggest that signaling via p-Ser121 and signaling via p-Thr69/71 are both different and independent.

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FIGURE LEGENDS

FIGURE 1. Effects of siRNA directed against PKC on RA-dependent transcription of an AP-1/CRE-luciferase reporter gene in F9 cells.  

A, CRE reporter assay.  2 x 10⁴ F9 cells were transfected with 20 (odd lanes) and 40 (even lanes) pmole of control siRNA (N.C.), PKCα-specific siRNA, PKCβI-specific siRNA and PKCβII-specific siRNA, respectively, plus 200 ng of CRE-luc, and then they were exposed to RA, as described in the text.  After exposure to RA for 24 h, cells were assayed for luciferase activity.  Values from a representative experiment are given as means ± S.E. (n=3).  

B, AP-1 reporter assay.  2 x 10⁴ F9 cells were transfected similarly with 20 (odd lanes) and 40 (even lanes) pmoles of siRNA, as described above, plus 200 ng of AP-1-luc.  After exposure to RA for 24 h, cells were harvested for assays of luciferase activity.  Values from a representative experiment are given as means ± S.E. (n=3).  

C, Inhibition of the expression of PKCs by the respective siRNAs.  2 x 10⁴ F9 cells were transfected with 40 pmole of an active siRNA or the control siRNA together with reporter plasmids.  After treatment of cells for 24 h with RA, cells extracts were analyzed by SDS-PAGE (4-12% acrylamide) and Western blotting (WB) with respective antibodies (Anti-), as indicated on the right and described in the text.  

Lane 1, without siRNA; lane 2, with siRNA; lane 3, with control N.C.; and lane 4, RA-treated F9 cells.  

D, “Knock-down” of PKC suppresses RA-mediated morphological changes in F9 cells that are associated with differentiation.  F9 cells that had been treated with indicated siRNAs directed against expression of PKCα, PKCβI and PKCβII, as well as with the negative control (N.C.), were incubated with 1 μM RA.  The cells were photographed 72 h after the start of exposure to RA under a phase-contrast microscope (original magnification, x125).  

E, Quantitation of levels of transcripts of RA-inducible genes and of genes that are markers of cell differentiation.  The relative levels of expression of genes for laminin B1 and collagen 4α1 in F9 cells that had been treated with indicated siRNAs, with or without treatment with RA (1 μM), were obtained by determining Ct values from the standard curve.  All experiments were performed in triplicate (results are means and standard deviations), and negative controls without template RNA were always included in each experiment.  All results were normalized by reference to the values for glyceraldehyde-3-phosphate dehydrogenase mRNA.  

F, Effects of inhibitors of PKC and p38 on expression of AP-1/CRE reporter genes.  We examined the effects of Gö6976 and SB202190 on the TPA-induced transcription of AP-1/CRE-reporter genes.  CRE-luc, or AP-1-luc, or the mutant derivative (mCRE-luc and mAP-1-luc) reporters (0.2 μg)
and pBSK II(-) plasmid, included to give a total amount of DNA equal to 1 μg, were used for transfection of HeLa cells as indicated in the text. Starved cells were pre-treated with Gö6976 (10 μM), SB202190 (20 μM) or the control compound SB202474 (20 μM) for 2 h prior to treatment with 150 nM TPA or DMSO (vehicle), as a control, for 6 h. Luciferase activities were measured. All experiments were performed three times and average results with standard deviations are shown.

FIGURE 2. PKC phosphorylates recombinant ATF-2 at Ser121. A and B, His-ATF-2 and its mutant derivative (S121A; 1.0 and 0.5 μg) were incubated with 15 ng of rat protein kinase C (PKC) for 10 min at 30 °C. The phosphorylation reaction was performed as described in the text. A, Proteins were then fractionated by SDS-PAGE (10% acrylamide). ATF-2 was visualized by staining with Coomassie brilliant blue. Lanes 1 and 2, 0.5 μg and 1.0 μg of wild-type His-ATF-2 protein; lanes 3 and 4, 0.5 μg and 1.0 μg of mutated His-ATF-2 (S121A). B, Phosphorylation of His-ATF-2 and its mutant derivative (S121A) by PKC. Lanes 1 and 2, 0.5 μg and 1.0 μg of wild-type His-ATF-2; lanes 3 and 4, 0.5 μg and 1.0 μg of His-ATF-2 (S121A); lanes 5 and 6, no added protein substrate. (C-L), Phosphorylation of synthetic peptides by PKC. Individual peptides (1.25 pmole), encompassing the serine residues at positions 121 (Ser121), 340 (Ser340) and 367 (Ser367), were incubated with 15 ng of rat protein kinase C (C and D), and with recombinant human protein kinase Co. (E and F), βI (G and H), βII (I and J) and γ (K and L), in the respective and appropriate PKC buffers [50 mM Tris-HCl buffer adjusted to pH 7.0 (panels C, E, G, I and K) and 50 mM sodium citrate buffer adjusted to pH 4.5 (panels D, F, H, J and L)] for 10 min at 30 °C. The PKC-catalyzed phosphorylation reactions were performed according to the instructions from the manufacturer of the enzymes (Upstate Biotechnology Inc.). The resultant phosphorylated peptides were fractionated by SDS-PAGE (18% polyacrylamide). In all panels, lane 1, Ser121 peptide (amino acid residues 111 to 130; P1); lane 2, mutant derivative of Ser121 peptide (S121A; P1m); lane 3, Ser340 peptide (amino acid residues 332 to 351; P2); lane 4, mutant derivative of Ser340 peptide (S340A; P2m); lane 5, Ser367 peptide (amino acid residues 357 to 376; P3); lane 6, mutant derivative of Ser367 peptide (S367A; P3m); lane 7, Ser121 peptide (amino acid residues 111 to 123; P4); lane 8, mutant derivative of Ser121 peptide (S121A; P4m); and lane 9, no peptide added as substrate.

FIGURE 3. Characterization of antibodies specific for phosphorylated ATF-2. A, Schematic representation of ATF-2 and its domains, with sites of phosphorylation (as indicated) by JNK/p38/ERK, ATM and PKC. N, Amino terminus; C, carboxyl terminus; ZNF, zinc finger motif; AD, activation domain; NLS, nuclear localization signal; NES, nuclear exclusion signal; S/TQ, serine-, threonine-, and glutamine-rich region; DBD, DNA-binding domain. B and C, Western blotting of phosphorylated ATF-2 with antibodies against phosphopeptides that included Ser121, Ser340, Ser367 and ATF-2 (N-96). B, Lysates were prepared from HeLa-S cells as described in the text. The lysates were incubated with antibodies [anti-p-Ser121, anti-p-Ser340, anti-p-Ser367, anti-ATF-2 (N-96)] in the presence of goat IgG (to reduce nonspecific binding) and Western blotting (WB) was performed as described in the text. β-actin was included as a control. Each sample was analyzed in duplicate (odd lanes; 20 μg, even lanes;
10 μg. C, In competition experiments, lysates were incubated with 200 μg of an antigenic phosphorylated or non-phosphorylated peptide, which corresponded to p-Ser121 (TB0608-1 and TB0608-2), p-Ser340 (TB0609-1 and TB0609-2) and p-Ser367 (TB0610-1 and TB0610-2), respectively, with respective primary antibodies against phosphorylated ATF-2. Other procedures were the same as described in (B). Each sample was analyzed in duplicate (odd lanes; 10 μg, even lanes; 20 μg). β-actin was included as a control. D, Treatment with protein phosphatase-1A (PP1A) of phosphorylated ATF-2. HeLa cells, transfected with pcDNA-Flag-ATF-2, were treated with TPA for 6 h and then cell lysates, containing phosphorylated ATF-2, were prepared and immunoprecipitated with Flag-specific antibodies. Immunoprecipitates were treated with protein phosphatase-1A (PP1A; lane 2) or control buffer in the absence (lanes 1 and 2) or presence (lane 3) of phosphatase inhibitor, as described in the text. Western blotting with indicated antibodies was performed as described in the text.

FIGURE 4. Phosphorylation assays with PKC. A, 1.0 μg of GST-ATF-2(1-125)WT, GST-ATF-2(1-125)S121A, GST-ATF-2(1-125)T69/71A, GST-ATF-2(1-125)T69/71AS121A and GST were incubated with 12.5 ng of human PKCα and 5 μCi of [γ-32P]ATP in kinase buffer for 10 min at 30 °C, as described in the text. Loading of equal amounts of GST-ATF-2(1-125) derivatives and GST was confirmed by staining with Coomassie brilliant blue (CBB). B, 1 x 10^6 HeLa cells were transfected with 5 μg of pFlag-PKCαWT or pFlag-PKCαKD plus 3 μg of pRSV-LacZ. After treatment of cells with TPA for 6 h, cell lysates were immunoprecipitated with antibodies against Flag and protein A+G. Immunocomplexes were incubated with 0.5 μg (lanes 6 to 10) or 0.25 μg (lanes 1-5) of GST-ATF-2(1-125)WT or GST-ATF-2(1-125)S121A and 5 μCi of [γ-32P]ATP in kinase buffer for 10 min at 30 °C. The resultant phosphorylated protein complexes were subjected to SDS-PAGE (10% polyacrylamide) and autoradiography. A Western blot with antibodies specific for Flag is shown in the lowest panel. Levels of GST-ATF-2(1-125) and GST were visualized by staining with CBB. C, Kinetics of phosphorylation of ATF-2 in response to TPA in HeLa cells. Analysis of the phosphorylation of ATF-2. HeLa cells were treated with 150 nM TPA for 6 h as described in the text. Cells were harvested and cell lysates were prepared at the indicated time points. Levels of the various forms of phosphorylated ATF-2, ATF-2 (N-96) and β-actin were analyzed by immunoblotting as described in the text.

FIGURE 5. Staining of ATF-2 phosphorylated at Ser121 and at Ser340. A, HeLa cells were double-stained with antibodies specific for ATF-2pS121 and with PI (for DNA). Peptide blocking was performed with the p-Ser121 peptide, the Ser121 (non-phosphorylated) peptide and the p-Ser340 peptide, as described in the text. Asterisks indicate cross-reactive staining in the perinuclear region. B, HeLa cells were double-stained with antibodies specific for ATF-2p340 and PI. Peptide blocking was performed with the p-Ser340 peptide, the Ser-340 (non-phosphorylated) peptide and the p-Ser121 peptide, as described in the text. Scale bars, 10 μm.
A, HeLa cells treated with DMSO (vehicle) alone or with 200 nM TPA for 30 min were double-stained with antibodies against ATF-2 (N-96), ATF-2pS121, ATF-2pS340, ATF-2pS490/498 or ATF-2pT69/71.  Scale bars, 10 μm.  
B, HeLa cells, pretreated with DMSO alone or with 10 μM Gö6976 for 30 min, were incubated with or without 200 nM TPA for 30 min. Cells were stained with antibodies against ATF-2pS121, ATF-2pS340 or ATF-2pT69/71.  Scale bars, 10 μm.  Lower panel, Mean intensities of fluorescence due to various phosphorylated forms of ATF-2 after immunostaining were measured in nuclei; bars indicate means ± S.D. Results shown are from a representative experiment (n = 89 to 134 cells), as indicated; three independent experiments gave similar results.  The dots represent individual values of mean intensity in the nucleus.  Asterisks indicate significant differences (*, p<0.05; **, p<0.01; ***, p<0.001 and N.S., not significant), as determined by Student’s t-test.

FIGURE 7. Mutation of ATF-2 at Ser121 affects the TPA-dependent transactivation of Gal4-p300 and, also, AP-1-mediated transcription in ATF-2(-/-) MEF, ATF-2(-/-)/ATF-7(-/-) MEF and HeLa cells.  
A, Four hundred ng of pGal4-luc reporter plus 400 ng of pG4-p300N and 200 ng of pcDNA-Flag-ATF-2WT, pcDNA-Flag ATF-2S121A, pcDNA-Flag-ATF-2T69/71A, pcDNA-Flag-ATF-2S367A or pcDNA4 empty vector were used for transfection of 5 x 10⁴ ATF-2(-/-) MEF.  Six h after transfection, cells were incubated with DMEM plus 0.5% FBS for another 24 h.  Then TPA (150 nM; even lanes) or DMSO (odd lanes) was added and, after incubation for 6 h, cells were harvested and assayed for luciferase activity.  The average luciferase activities from three experiments are shown with standard deviations (results with asterisks differ significantly from the result for ATF-2WT without TPA; p = 0.004 and 0.009, as indicated).  
B, 5 x 10⁴ MEF from ATF-2(-/-)/ATF-7(-/-) null mice were co-transfected with 400 ng of pG4 reporter construct and pcDNA-Flag-ATF-2WT or a derivative, as indicated, plus 200 ng of pFlag-c-Jun and 300 ng of pRSV-lacZ.  Then, 48 h after transfection, cells were harvested for assays of luciferase activity, as described in (A) (asterisks indicate p = 0.003).  
C, 5 x 10⁴ HeLa cells were transfected with 400 ng of pG4-luciferase, 200 ng of pG4-ATF-2 WT or pG4-ATF-2S121A and 400 ng of pCMV-Flag-PKCα or pCMV-Flag-PKCαKD.  Then, 12 h after transfection, cells were incubated with DMEM for another 12 h and with DMEM plus 0.5% FBS for another 24 h.  Finally, TPA was added for 6 h and cells were harvested for assays of luciferase activity.  The average luciferase activities from three experiments are shown with standard deviations.  (p=0.017 relative to the value for the pG4-ATF-2WT).  pPKCα and pPKCαKD indicate the expression plasmids pCMV-Flag-PKCα and “kinase-dead” pCMV-Flag-PKCαKD (400 ng each).
**Fig. 1**

**A**

F9 CRE-luciferase

RA: + - siPKCα siPKCβI N.C. siPKCβII

1 2 3 4 5 6 7 8 9 10

**B**

F9 AP-1-luciferase

RA: + - siPKCα siPKCβI N.C. siPKCβII

1 2 3 4 5 6 7 8 9 10

**C**

WB: Anti-

PKCα

β-Actin

PKCβI

β-Actin

PKCβII

β-Actin

1 2 3 4

**D**

F9 RA-

F9 RA+

siPKCα

siPKCβI

siPKCβII

N.C.

**E**

Laminin B1 mRNA

Collagen 4α1 mRNA

RA - + siPKCα siPKCβI siPKCβII N.C.

1 2 3 4 5 6

**F**

Relative luciferase activity

RA - + Inhibitor mCRE-luc CRE-luc mAP-1-luc AP-1-luc

Gö6976 SB202190 SB202474

0 2 4 6 8 10 12 14 16
**Fig. 2**

- **A** and **B**: Comparison of WT and S121A proteins under pH 7.0 conditions.
  - Rat PKCa, β, γ (C)
  - Human PKCa (E)
  - Human PKCβI (G)
  - Rat PKCa, β, γ (D)
  - Human PKCα (F)
  - Human PKCβI (H)
  - Human PKCβII (I)
  - Human PKCγ (K)
  - Human PKCβII (J)
  - Human PKCγ (L)

The diagrams illustrate the molecular weight (kDa) of various proteins under different pH conditions (7.0 and 4.5). The pH 4.5 conditions show a shift in the protein bands compared to pH 7.0, indicating changes in protein activity or structure.
Fig. 3
Fig. 4
Fig. 5

A

ATF-2 p-Ser121
Merge

α-ATF-2 p-Ser121
+ no peptide

α-ATF-2 p-Ser121
+ p-Ser121 peptide

α-ATF-2 p-Ser121
+ Ser121 peptide

α-ATF-2 p-Ser121
+ p-Ser340 peptide

B

ATF-2 p-Ser340
Merge

α-ATF-2 p-Ser340
+ no peptide

α-ATF-2 p-Ser340
+ p-Ser340 peptide

α-ATF-2 p-Ser340
+ Ser340 peptide

α-ATF-2 p-Ser340
+ p-Ser121 peptide
Fig. 6A
Fig. 6B

Mean intensity of signal due to phosphorylated ATF-2

- ATF-2 p-Ser121
- ATF-2 p-Ser340
- ATF-2 p-Thr69/71

DMSO  TPA  TPA + GSK976

N.S.

* * * * *

n=119  n=134  n=102

n=107  n=124  n=101  n=114

Fig. 6B
**A**

![Bar graph A](image)

Relative luciferase activity

1. Empty vector
2. ATF-2 wt
3. T69/71A
4. S121A
5. S340A
6. S367A

**B**

![Bar graph B](image)

Relative luciferase activity

1. Empty vector
2. ATF-2 wt
3. T69/71A
4. S121A
5. S340A
6. S367A

**C**

![Bar graph C](image)

Relative luciferase activity

1. pG4dbd
2. ATF-2 WT
3. S121A

**Fig. 7**
Phosphorylation of activation transcription factor-2 (ATF-2) at serine 121 by protein kinase C controls c-Jun-mediated activation of transcription
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