Extracellular Signal-regulated Kinase 1/2-mediated Phosphorylation of JunD and FosB Is Required for Okadaic Acid-induced Activator Protein 1 Activation

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Previously, we reported that in papilloma-producing 308 mouse keratinocytes, the tumor promoter okadaic acid, a serine-threonine phosphatase inhibitor, increased binding of activator protein 1 (AP-1) to a consensus 12-O-tetradecanoylphorbol-13-acetate-responsive element (Rosenberger, S. F., and Bowden, G. T. (1996) Oncogene 12, 2301–2308). In this study, we investigated the correlation between AP-1 DNA binding and transactivation and examined molecular mechanisms involved in this process. Using a luciferase reporter driven by region −74 to +63 of the human collagenase gene, we demonstrated induction of AP-1-mediated transcription following okadaic acid treatment. By performing in vitro kinase assays, we found elevated activities of extracellular signal-regulated kinase (ERK) 1/2, c-Jun N-terminal kinase, and p38 mitogen-activated protein kinase. The ERK-1/2-specific inhibitor PD 98059 completely abrogated okadaic acid-induced AP-1 transactivation without altering AP-1 expression, DNA binding, or complex composition. Phosphorylation analyses indicated that inhibition of ERK-1/2 decreased okadaic acid-elevated phosphorylation of JunD and FosB. To further examine the role of JunD and FosB in okadaic acid-induced AP-1 transactivation, we generated fusion proteins of the DNA-binding domain of the yeast transcription factor Gal4 and the transactivation domain of either JunD or FosB. Cotransfection experiments of these constructs with a Gal4-luciferase reporter demonstrated that both JunD and FosB are required for okadaic acid-induced AP-1 transactivation. Treatment with PD 98059 reduced JunD/FosB-dependent transactivation, suggesting that ERK-1/2-mediated phosphorylation is a critical component in this process.

Okadaic acid is a polyether compound of a C30 fatty acid and is produced by certain dinoflagellates that concentrate in marine sponges and shellfish. It is the major toxic component responsible for diarrhetic shellfish poisoning (1, 2). Okadaic acid inhibits serine-threonine phosphatases 1, 2A (3), and 3 (4) by binding to their catalytic subunit and leads to the accumulation of hyperphosphorylated proteins (5, 6). Okadaic acid is also a potent tumor promoter in mouse skin initiated with 7,12-dimethylbenz[a]anthracene (7).

Tumor promoters lead to alterations in gene expression modulating biological processes, such as proliferation, differentiation, and cell death, which are involved in expansion of “initiated” cells and tumor development. Gene expression is regulated by transcription factors that bind to specific cis-elements in the promoter region of genes, and these factors either induce or repress their transcription.

Several lines of evidence indicate the transcription factor complex AP-1 as a critical component in epidermal tumor development in mouse skin. In this system, elevated AP-1 activity has been correlated with increased neoplastic transformation. In mouse epidermal JB6 cells, AP-1 activity increases with progression from a tumor promotion-resistant to a tumor promotion-sensitive phenotype (8). Tumor promoters stimulate AP-1 expression and modulate AP-1-regulated gene expression in cultured mouse keratinocytes and in mouse skin (9). More direct evidence for a causative role of AP-1 activation in tumor promotion comes from the finding that acquisition of a tumor promotion-resistant phenotype is consistent with a loss of responsiveness to tumor promoter-induced AP-1 activation (10). Furthermore, down-regulation of AP-1 by “anti-tumor promoters” such as aspirin and aspirin-like salicylates (11) or retinoic acid (12) correlates with inhibition of tumor promoter-induced transformation and tumor development. The same effects are observed when AP-1 activity is blocked by the dominant-negative c-Jun mutant TAM67 (13), whereas overexpression of c-Jun leads to increased neoplastic transformation (14).

The transcription factor AP-1 (15) is a dimeric complex formed by Jun or Jun and Fos proteins and binds to a promoter element that is referred to as the 12-O-tetradecanoylphorbol-13-acetate-responsive element (TRE). There are three different Jun proteins, c-Jun, JunB, and JunD, and four different Fos proteins, c-Fos, FosB, Fra-1, and Fra-2. AP-1 activity is regulated at various levels, including transcriptional and post-transcriptional mechanisms leading to increased AP-1 expression and post-translational modifications, such as phosphorylation and oxidation/reduction, altering DNA binding affinity and transactivation potential. Since DNA binding affinity and transactivation potential are different for the various AP-1 proteins, AP-1 activity is also determined by its composition. AP-1 composition, on the other hand, is dependent not only on the sequence of the actual TRE, but also on the sequence of adjacent bases (16).

Previously, we presented experimental evidence that in the papilloma-producing mouse keratinocyte cell line 308, the principal mechanism of okadaic acid-stimulated AP-1 DNA binding

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† The abbreviations used are: AP-1, activator protein 1; TRE, 12-O-tetradecanoylphorbol-13-acetate-responsive element; MAPK, mitogen-activated protein kinase; ATF-2, activating transcription factor 2; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MEK, MAPK/ERK kinase; MEM, minimal essential medium; PCR, polymerase chain reaction; DTT, dithiothreitol.
activity is increased AP-1 expression (17). Furthermore, we were able to show that this increased AP-1 expression is at least partly due to increased AP-1 transcription. This increased AP-1 transcription could be the result of post-translational modifications of transcription factors regulating AP-1 gene expression. Mitogen-activated protein kinases (MAPKs) have been discussed as important mediators of signal transduction regulating gene expression, and activation of these enzymes has been implicated in altered phosphorylation of several transcription factors regulating AP-1 gene expression. We generated the fusion protein constructs pBindJunD and pBindFosB.

**EXPERIMENTAL PROCEDURES**

Cell Culture and Treatment—The papilloma-producing 308 mouse keratinocytes were kindly provided by Dr. Stuart H. Yuspa (23). The cells were maintained in minimal essential medium (MEM) supplemented with 5% fetal calf serum, 2.5% calf serum, and 100 units/ml penicillin/streptomycin (all purchased from Life Technologies, Inc.) at 37 °C in a humidified atmosphere containing 7.5% CO2. For experiments, cells were grown to 75–90% confluency. Before treatment, cells were washed once with serum-free MEM. Cells were treated with okadaic acid (sodium salt, LC Laboratories) dissolved in dimethyl sulfoxide added to serum-free MEM to a final concentration of 100 ng/ml; controls were treated with equivalent amounts of MeSO (0.1%, v/v). In the indicated experiments, prior to the addition of okadaic acid/MeSO, cells were pretreated for 1 h with 50 μM PD 98059 (2'-amino-3'-methoxyflavone, Alexis Corp.), a specific inhibitor of the ERK-1/2 activators MK-2-1, dissolved as 50 μM stock in MeSO; controls were treated with MeSO (0.1%, v/v).

**Plasmids**—To study TRE-dependent transactivation, a luciferase reporter construct driven by TRE region -74 to +63 of the human collagenase gene containing a TRE was used. This construct was kindly provided by P. Brown and L. M. Yang. To determine the role of JunD and FosB in the involvement of ERK-1/2-mediated phosphorylation in this process, we generated the fusion protein constructs pBindJunD and pBindFosB in okadaic acid-mediated TRE-dependent transactivation and to analyze the involvement of ERK-1/2-mediated phosphorylation in this process, we generated the fusion protein constructs pBindJunD and pBindFosB. pBindJunD was generated by PCR using 30 ng of upstream sense primer 5'-CGAAGCGCTCCGGGGAGGTGGGATGAAAC-3' (mismatches shown in boldface; base pairs 9-39; GenBank TM X15358), creating a MluI site, and 150 ng of downstream antisense primer 5'-GACGCTTACAGGGCCGAGCCGCAGGG-3' (base pairs 633-662), creating an MluI site, and 150 ng of downstream antisense primer 5'-CCCTGCGCGCCGCA-TCTCCTCCTCC-3' (base pairs 2260-2285), creating a NotI site. The PCR product was carried out in 50 μl of UTTma buffer with 50 μM dNTPs, 2 μM Mg2+, 15 ng of JunD-KSBluescript (XHJ-12.4, Promega), and 6 units of UTTma polymerase (Perkin-Elmer) (35 cycles for 1 min at 98 °C and 2 min at 73 °C). The PCR product was digested with MluI and Acc65I, gel-purified, and ligated into the MluIAcc65I-linearized pBind plasmid (Promega), creating a fusion protein of the Gal4-binding domain and the JunD transcription activation domain. pBindFosB was generated by PCR using 30 ng of upstream sense primer 5'-GGCTCGAGAAGGCGCAGAGG-3' (base pairs 1862–1888; GenBank TM X41897), creating an MluI site, and 30 ng of downstream antisense primer 5'-CCCTGCGCGCCGCA-TCTCCTCCTCC-3' (base pairs 2260–2285), creating a NotI site. The PCR product was carried out in 60 μl of UTTma buffer with 50 μM dNTPs, 1.6 μM Mg2+, 15 ng of fosB-pGem1 (kindly provided by R. Bravo), and 6 units of UTTma polymerase (35 cycles for 1 min at 97 °C, 1 min at 65 °C, and 1 min at 72 °C). The PCR product was digested with MluI and NotI, gel-purified, and ligated into the MluINotI-linearized pBind plasmid, creating a fusion protein of the Gal4-binding domain and the FosB transactivation domain.

Transactivation Analysis—308 cells were transiently transfected using N1-1(2,3-dioleoyl-1-phosphotidyl-N,N,N-trimethylammonium methyl 1-chloroacetate) liposomal transfection reagent (Boehringer Mannheim). Cells (1–1.5 × 10^6) were seeded in a six-well plate and transfected after 24 h under serum-free conditions with 5 μg of the human collagenase TRE-luciferase construct or 5 μg of the various pBind constructs together with 5 μg of the pG5Luc luciferase reporter containing five Gal4-binding sites upstream of a minimal TATA box fused to the luciferase gene (pG5L). Cells were transfected to serum-containing MEM. After another 12 h, the cells were treated. At various times, cells were lysed in 1% Triton X-100, 25 mM glycyglycine, 15 mM MgSO4, 4 mM EGTA, and 1 mM DTT, and protein concentration was determined with Bio-Rad Dye Reagent. 180 μl of assay buffer (25 mM glycyglycine, 15 mM K2PO4, 15 mM MgSO4, 4 mM EGTA, 1 mM DTT, and 2 mM ATP) were added to 40 μg of protein, and the luminescence reaction was initiated with a Monolight 2010 luminescence fluorimeter with the injection of 100 μl of 0.2 mM 3-[1-(2,3-dioleoyloxy)propyl]-N-methylacetamidinium iodide (M Cell) (22). The final concentration of 10 μM was used for all experiments.

**Protein Kinase Assays**—Cells were lysed in 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 25 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium vanadate, 1 mM dl-phenylalanine, 1 mM NaF, 1 μM leupeptin, and 1 μM pepstatin, and 1 mM phenylmethylsulfonyl fluoride. 100 μg of protein were incubated with phospho-specific ERK-1/2 antibody, phospho-specific JNK antibody, or phospho-specific p38 MAPK antibody (New England Biolabs Inc.) with gentle rocking overnight at 4 °C. Protein A-Sepharose beads were added, and the mixture was rotated at 4 °C for 3 h and washed twice in the lysis buffer and twice in kinase buffer (25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM sodium vanadate, and 10 mM MgCl2). Beads were suspended in 50 μl of kinase buffer with 100 μM ATP and 0.5 μg of substrate (ERK-1/2: Elk-1, New England Biolabs Inc.; JNK: c-Jun, Santa Cruz Biotechnology; and p38 MAPK: ATF-2, Santa Cruz Biotechnology) and incubated at 30 °C for 30 min. Samples were boiled in SDS sample buffer (50 mM Tris-HCl (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.001% bromophenol blue) and resolved on 12.5% SDS-polyacrylamide gels. Phosphorylated proteins were analyzed by Western blotting using phospho-specific antibodies for the different substrates (New England Biolabs Inc.).

**Isolation of Nuclear Protein**—Cells were rinsed once with phosphate-buffered saline and once with buffer A (10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 50 mM KCl, 0.5 mM DTT). Cells were scraped, pelleted by centrifugation at 1000 rpm for 2 min at 4 °C, and lysed in buffer A containing 0.1% Nonidet P-40 for 30 min at 4 °C. Crude nuclear protein extracts were prepared by the method of Dignam et al. (24). Nuclei were pelleted by centrifugation at 10,000 rpm for 15 min at 4 °C; resuspended in 20 mM HEPES (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 M NaCl at 4 °C. The nuclei were centrifuged at 10,000 rpm for 15 min at 4 °C, and the supernatant was diluted 1:6 with 20 mM HEPES (pH 7.9), 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 M DTT. The protein concentrations of the extracts were determined with Bio-Brad reagent.

**Electrophoretic Mobility Shift Assay**—Oligonucleotide probes were labeled by incorporation of 32P-dCTP (NEL Science Products) into the 5'-overhangs of the annealed human collagenase TRE oligonucleotide 5'-gcctcaTGAGTGACgccac-3' with Klenow DNA polymerase. The oligonucleotide binding assay was performed by mixing 1 × 10^5 probe with 5 μg of nuclear protein extract in the presence of 2 μg of poly(dI-dC)poly(dI-dC) (Amersham Pharmacia Biotech) and gel shift buffer (10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM MgCl2, 0.5 mM DTT, and 4% glycerol) at room temperature for 30 min. In antibody clearing experiments, various amounts of antibodies specific for the various Jun and Fos proteins (Santa Cruz Biotechnology) were preincubated with nuclear extracts at room temperature for 2 h prior to the binding assay. An antibody specific for Nm23-H1 (Santa Cruz Biotechnology) was used as a control; volumes were adjusted with antibody buffer (phosphatebuffered saline, 0.1% sodium azide, and 0.2% gelatin) to deliver 100 μg/ml bovine serum albumin. DNA-binding complexes were resolved by gel electrophoresis on 5% polyacrylamide and 1 × Tris borate electrophoresis buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA) gels. The gels were dried and exposed to Kodak X-AR film at ~80 °C for 2–6 h using an intensifying screen.

**Western Analysis**—20 μg of crude nuclear extract were boiled in SDS...
Okadaic Acid Increases AP-1 Transactivation—Previously, we reported that in papilloma-producing 308 mouse keratinocytes, okadaic acid increases AP-1 binding to a consensus 12-O-tetradecanoylphorbol-13-acetate-responsive element (TRE). To test whether okadaic acid-induced increase in AP-1 activity is mediated by phosphorylation, we transiently transfected 308 cells with a luciferase reporter construct driven by a TRE fragment of the human collagenase gene containing a TRE. Cells were treated with 100 ng/ml okadaic acid (OA) or the solvent dimethyl sulfoxide (DMSO). At the indicated times, cells were lysed, and 40 μg of total protein were analyzed for luciferase activity. The results shown are representative of three independent experiments. RLU, relative light units.

FIG. 1. Okadaic acid increases AP-1 transactivation. 308 cells were transiently transfected with a luciferase reporter construct driven by a fragment of the human collagenase gene containing a TRE. Cells were treated with 100 ng/ml okadaic acid (OA) or the solvent dimethyl sulfoxide (DMSO). The results are shown. 308 cells showed a basal luciferase activity that was significantly increased by okadaic acid. Okadaic acid-mediated stimulation of TRE-luciferase activity was detectable at 4 h and gradually increased further at 6 and 12 h. The level of induction was 1.6 ± 0.3-fold at 4 h, 3.1 ± 1.1-fold at 6 h, and 11.2 ± 3.0-fold at 12 h.

Okadaic Acid Activates ERK-1/2, JNK, and p38 MAPK—Through their ability to phosphorylate transcription factors and modulate their activity, MAPKs have established themselves as key regulators of gene expression. To test whether okadaic acid-induced AP-1-mediated gene expression correlates with increased MAPK activity, we isolated cellular protein from exponentially growing 308 cells that had been treated with 100 ng/ml okadaic acid or with the solvent Me2SO. Using phospho-specific antibodies for the three groups of MAPKs (ERK-1/2, JNK, and p38 MAPK), the activated forms of these kinases were immunoprecipitated. We then performed in vitro kinase assays with recombinant proteins using their major substrates: Elk-1 for ERK-1/2, c-Jun for JNK, and ATF-2 for p38 MAPK. The phosphorylated forms of these substrates were detected by Western blot analysis using phospho-specific antibodies. As shown in Fig. 2, all three MAPKs were efficiently activated by okadaic acid. Okadaic acid stimulated ERK-1/2 activity starting at 1 h and progressively increasing up to 12 h (Fig. 2A). For JNK, a high basal activity was observed. Treatment with okadaic acid led to a further increase in JNK activity. This increase was detectable at 1 h and did not change significantly throughout the time course (Fig. 2B). Okadaic acid-mediated stimulation of p38 MAP kinase was detectable within 2 h, reached a maximum at 4 h, and remained constant up to 12 h (Fig. 2C).

Inhibition of ERK-1/2 Abrogates Okadaic Acid-stimulated Phosphorylation—To further investigate the role of MAPKs in okadaic acid-stimulated AP-1 activation, we tested whether the observed increases in AP-1-dependent transcription are a direct result of MAPK activation. To achieve this goal, we transiently transfected 308 cells with an expression vector for Elk-1, a potent activator of AP-1-dependent transcription. As shown in Fig. 3, Elk-1 expression completely blocked the okadaic acid-mediated induction of AP-1-dependent transcription, indicating that phosphorylation of Elk-1 by ERK-1/2 is a critical step in the regulation of AP-1 activity by okadaic acid.
Figure 3. Inhibition of ERK-1/2 abrogates okadaic acid-stimulated AP-1 transactivation. A, exponentially growing 308 cells were treated with 50 μM MEK-1/2 inhibitor PD 98059 or Me2SO for 1 h. 100 ng/ml okadaic acid (OA) or the solvent dimethyl sulfoxide (DMSO) was added. At the indicated times, cells were lysed, and 100 μg of total protein were immunoprecipitated with a phospho-specific antibody for ERK-1/2. In vitro kinase assays were performed using recombinant Elk-1 as substrate, and phosphorylated Elk-1 was detected by Western blot analysis using a phospho-specific antibody. B, 308 cells were transiently transfected with a luciferase reporter construct driven by a fragment of the human collagenase gene containing a TRE. Prior to the addition of 100 ng/ml okadaic acid or the solvent Me2SO, cells were pretreated with 50 μM MEK-1/2 inhibitor PD 98059 or Me2SO. At the indicated times, cells were lysed, and 40 μg of total protein were analyzed for luciferase activity. Shown is the fold increase in luciferase activity of okadaic acid-treated cells over Me2SO-treated cells. The results are a combination of three independent experiments. The error bars represent 95% confidence.

Inhibition of ERK-1/2 Does Not Alter AP-1 Protein Levels—Inokadaic acid-induced accumulation of AP-1 proteins occurs through an ERK-1/2-independent pathway, we then considered the possibility that ERK-1/2 activity is responsible for post-translational modifications of AP-1 proteins, increasing their DNA binding activity. To test this hypothesis, we performed electrophoretic mobility shift assays of crude nuclear extracts from exponentially growing 308 cells that had been pretreated for 1 h with the MEK-1/2 inhibitor PD 98059 prior to the addition of 100 ng/ml okadaic acid or Me2SO. At 4, 6, and 12 h, TRE binding activity was analyzed using a radiolabeled oligonucleotide of the human collagenase promoter including the TRE. The results are presented in Fig. 5. Basal and okadaic acid-increased TRE DNA binding activities were not compromised by inhibition of ERK-1/2. In contrast, at 4 h, inhibition of these MAPKs induced an increase in DNA binding activity.

Inhibition of ERK-1/2 Does Not Alter AP-1 Composition—Since we could not demonstrate a causative relationship between ERK-1/2 activation and increased AP-1 DNA binding, we then hypothesized that ERK-1/2 may increase AP-1-dependent transactivation by modifying interactions between AP-1 proteins and/or between AP-1 proteins and AP-1 inhibitors such as IP-1 (27) and Jif-1 (28). Those altered interactions could then lead to changes in the composition of AP-1 complexes and allow, without altering their DNA binding affinity, for the formation of complexes with higher transactivation potential. To address this idea, we used antibody clearing experiments to analyze the composition of AP-1 complexes bound to the human collagenase TRE oligonucleotide. Nuclear extracts of cells treated for 6 h with okadaic acid with (Fig. 6D) or without (Fig. 6A) prior inhibition of ERK-1/2 by the MEK-1/2 inhibitor PD 98059 were incubated for 2 h with varying amounts of antibodies for the various Jun and Fos proteins and then analyzed in electrophoretic mobility shift assays. An Nm23-H1-specific antibody was used as a control, and protein amounts added to the

Figure 4. Inhibition of ERK-1/2 does not alter AP-1 protein levels. Western analyses were performed with 20 μg of nuclear protein isolated from exponentially growing 308 cells pretreated with 50 μM MEK-1/2 inhibitor PD 98059 or the solvent dimethyl sulfoxide (DMSO) for 1 h and treated with 100 ng/ml okadaic acid (OA) or Me2SO for 6 h. 1:1000–1:3000 dilutions of antibodies specific for the various Jun and Fos proteins were used. Horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2000) was used as secondary antibody. Antigen-antibody complexes were visualized by chemiluminescence. The results shown are representative of three independent experiments.
different samples were adjusted with bovine serum albumin since unspecific inhibition increased with increasing amounts of antibody. The presence of the various AP-1 proteins was evaluated by the ability of the antibodies to form with the AP-1 complexes a more slowly migrating form or a supershift and/or to reduce TRE binding activity. As shown in Fig. 6, JunD and FosB were identified as major components of the AP-1 complexes binding to the human collagenase TRE at 6 h. Inhibition of ERK-1/2 did not alter this composition.

Inhibition of ERK-1/2 Reduces Phosphorylation of JunD and FosB—Since increased AP-1 expression, increased DNA binding, and formation of more potent AP-1 complexes were excluded as mechanisms of ERK-1/2-mediated AP-1 transactivation, we then tested the possibility of ERK-1/2-induced increases in AP-1 transactivation potential through altered phosphorylation of the major components, JunD and FosB. Cells (pretreated with the MEK-1/2 inhibitor PD 98059 and treated with okadaic acid or Me₂SO for 6 h) were labeled with orthophosphate, and phosphorylation of JunD and FosB was examined by immunoprecipitation analyses. Similar results were obtained when equal counts or equal amounts of protein (data not shown) were immunoprecipitated. As shown in Fig. 7, okadaic acid increased phosphorylation of JunD and FosB. Pretreatment with the MEK-1/2 inhibitor significantly reduced okadaic acid-mediated phosphorylation.

Both JunD and FosB Are Required for Okadaic Acid-induced Transcription—To examine the role of JunD and FosB in okadaic acid-induced AP-1 transactivation more closely, we generated fusion proteins of the DNA-binding domain of the yeast transcription factor Gal4 and the transactivation domain of JunD (pBindJunD) or FosB (pBindFosB) and tested directly the ability of okadaic acid to increase the transactivation po-

**FIG. 5.** Inhibition of ERK-1/2 does not reduce AP-1 DNA binding activity. Shown are the results from an electrophoretic mobility shift assay of crude nuclear extracts (5 μg) from exponentially growing 308 cells using a radiolabeled TRE oligonucleotide. Cells were pretreated with 50 μM MEK-1/2 inhibitor PD 98059 (B) or the solvent dimethyl sulfoxide (DMSO) (A) for 1 h and treated with 100 ng/ml okadaic acid (OA) or Me₂SO. The results shown are representative of three independent experiments.

**FIG. 6.** Inhibition of ERK-1/2 does not alter AP-1 composition. Crude nuclear extracts (5 μg) of exponentially growing 308 cells pretreated with 50 μM MEK-1/2 inhibitor PD 98059 (B) or the solvent Me₂SO (A) for 1 h and treated with 100 ng/ml okadaic acid or Me₂SO for 6 h were preincubated for 2 h at room temperature with varying amounts of antibodies specific for the various Jun and Fos proteins. Controls were preincubated with an antibody specific for Nm23-H1. Electrophoretic mobility shift assays were performed using a radiolabeled TRE oligonucleotide. The results shown are representative of three independent experiments.
tential of these AP-1 proteins. Cells were cotransfected with pBindJunD, pBindFosB, or pBindJunD and pBindFosB and a luciferase reporter construct containing five Gal4-binding sites. As shown in Fig. 8A, okadaic acid affected JunD- or FosB-mediated transcription only slightly, but substantially increased transcription mediated by a combination of JunD and FosB. Treatment with the MEK-1/2 inhibitor PD 98059 had no effect on JunD- or FosB-mediated transcription, but resulted in a statistically significant ($p < 0.05$) reduction of JunD/FosB-dependent transcription (Fig. 8B).

**DISCUSSION**

In this study, we were able to show that in papilloma-producing 308 mouse keratinocytes, the previously reported okadaic acid-increased AP-1 DNA binding to a consensus TRE (17) correlates with increased AP-1 transactivation. Okadaic acid was able to induce transcription of a luciferase reporter construct driven by region −74 to +63 of the human collagenase gene containing a TRE.

This result further supports the idea that increased AP-1 activity plays a critical role in epidermal tumor development in mouse skin. Increased AP-1 activity has been suggested as a prerequisite for tumor promoter sensitivity, and further tumor promoter-induced elevation of AP-1 activity as a requirement for benign tumor development (8). A constitutive increase in AP-1 activity might be necessary for development and maintenance of malignant squamous cell carcinomas (29).

Although we could show that okadaic acid activates all three families of MAPKs (ERK-1/2, JNK, and p38 MAPK), specific inhibition of ERK-1/2 with PD 98059 was sufficient to abrogate okadaic acid-increased AP-1 transactivation. This finding indicates that ERK-1/2 is a critical component in okadaic acid-mediated AP-1 regulation. A similar observation has been made by Frost et al. (30), who demonstrated a requirement for ERK-1/2 in AP-1 activation by Ha-Ras, 12-O-tetradecanoylphorbol-13-acetate, and serum.

ERK-1/2-mediated AP-1 activation appears to occur predominantly at a transcriptional level. c-fos transcription is induced by phosphorylation of Elk-1, which, with the dimeric serum response factor, forms a prebound complex at the serum response element (31, 18). A similar mechanism could be postulated for induction of other AP-1 genes, e.g. junD and fosB, where Ets-binding sites and serum response elements have been identified and implicated in transcriptional regulation (32, 33).

In contrast to these reports, our data suggest that ERK-1/2 acts through a post-translational mechanism, stimulating the transactivation potential of pre-existing AP-1 complexes by alterations in their phosphorylation pattern. We found that inhibition of ERK-1/2 had only slight effects on overall levels of AP-1 proteins. These minor decreases in AP-1 expression were not sufficient to decrease AP-1 DNA binding activity. Using antibody clearing experiments, we demonstrated that the composition of the AP-1 complexes dominating this DNA binding activity was the same in ERK-1/2 inhibitor-treated and untreated cells. Surprisingly and in contrast to our previous study (17), where JunB was identified as major component of the AP-1 complex, JunB did not appear to bind to the human collagenase TRE. A possible explanation for this discrepancy could be the use of a different TRE oligonucleotide. In the present study, we used an oligonucleotide for which the sequence was taken from the human collagenase gene to correspond to the sequence in the luciferase reporter construct used for transactivation studies as opposed to the TRE oligonucleotide described by Angel et al. (34). As reported by Ryseck and Bravo (16), the various AP-1 proteins bind with different affinities to different oligonucleotides that contain identical AP-1-binding sites, implying that adjacent sequences influence the composition of AP-1 complexes.

Our experiments using fusion proteins of the DNA-binding domain of the yeast transcription factor Gal4 and the transactivation domain of either JunD or FosB demonstrated the ability of okadaic acid to increase the transactivation potential of

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**Fig. 7.** Inhibition of ERK-1/2 reduces phosphorylation of JunD and FosB. Exponentially growing 308 cells were pretreated with 50 μM MEK-1/2 inhibitor PD 98059 or the solvent dimethyl sulfoxide (DMSO) for 1 h and treated with 100 ng/ml okadaic acid (OA) or Me2SO. During the last hour of treatment, they were labeled with 100 μCi/ml [32P]orthophosphate. At 6 h, cells were lysed, and equal counts were immunoprecipitated with antibodies specific for JunD or FosB. The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis. The results shown are representative of three independent experiments.

**Fig. 8.** Both JunD and FosB are required for okadaic acid-induced transcription. A, 308 cells were transiently transfected with a construct generating a fusion protein of the Gal4 DNA-binding domain and the transactivation domain of JunD (pBindJunD) or FosB (pBindFosB) and a luciferase reporter construct containing five Gal4-binding sites. Cells were treated with 100 ng/ml okadaic acid (OA) or the solvent dimethyl sulfoxide (DMSO). At 6 h, cells were lysed, and 40 μg of total protein were analyzed for luciferase activity. Shown are relative light units (RLU) of okadaic acid-treated cells versus Me2SO-treated cells. The results are representative of four independent experiments. B, prior to the addition of 100 ng/ml okadaic acid or the solvent Me2SO, cells were pretreated with 50 μM MEK-1/2 inhibitor PD 98059 or Me2SO. Shown is the fold increase in luciferase activity of okadaic acid-treated cells over Me2SO-treated cells. The results are a combination of six experiments. The error bars represent 95% confidence.
these AP-1 proteins and directly implicated them in okadaic acid-mediated TRE-dependent transactivation. Although JunD and FosB each by itself acted as transcriptional activators, only a combination of both was effectively inducible by okadaic acid and mediated okadaic acid effects on transcription. These findings indicate that okadaic acid-induced activation of JunD and FosB may require interactions between their transactivation domains. In the Ga4-luciferase reporter, the five Ga4-binding sites are in close proximity, thus very likely allowing for interactions between monomeric bound Ga4-AP-1 fusion proteins that normally occur through heterodimerization. These interactions may be necessary to introduce activating phosphorylations by recruiting okadaic acid-responsive kinases to their respective phosphorylation sites. A similar observation has been made by Kallunki et al. (35), who reported that JunD becomes a substrate for JNK by heterodimerization with c-Jun or JunB that possesses a JNK-docking site.

Treatment with the MEK-1/2 inhibitor PD 98059 significantly reduced okadaic acid-mediated JunD/FosB-dependent transcription. These data, together with our phosphorylation analyses demonstrating that okadaic acid-increased phosphorylation of JunD and FosB was significantly reduced by inhibition of ERK-1/2, suggest a potential role of increased JunD and FosB phosphorylation in okadaic acid-induced AP-1 transactivation and indicate ERK-1/2 as the responsible kinase. In contrast to c-Jun, whose regulation by phosphorylation is well documented, less is known about phosphorylation changes modulating the activity of JunD and FosB.

Nikolakaki et al. (36) reported that glycogen-synthase kinase 3 phosphorylates JunD in a region proximal to its DNA-binding domain and attenuates its DNA binding capacity. In contrast, the finding that serum-induced phosphorylation of JunD does not affect its ability to bind DNA provides evidence that phosphorylation may also regulate the transactivation potential of this factor (37). Serine 100, homologous to serine 73, one of two serines whose phosphorylation increases the transactivation potential of c-JNKs, has been found to be phosphorylated in response to activation of JNKs. In a similar fashion, phosphorylation of several serine residues in the C-terminal transactivation domain of FosB has been shown to increase its transcriptional activity (38).

In conclusion, these results, together with our previous observations, indicate that in papilloma-producing 308 mouse keratinocytes, okadaic acid stimulates AP-1 activity by two different mechanisms that appear to be mediated by independent signal transduction pathways. Okadaic acid increases TRE DNA binding activity by stimulating expression of AP-1 proteins. This increased TRE DNA binding activity does not necessarily result in increased TRE-dependent transactivation. Further modifications of the pre-existing AP-1 proteins are necessary. In contrast to okadaic acid-increased AP-1 DNA binding activity, which occurs independently of okadaic acid-mediated ERK-1/2 activation, ERK-1/2 activity is required for increased transactivation.

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