1,25-Dihydroxyvitamin D₃ Stimulates Activator Protein-1-dependent Caco-2 Cell Differentiation

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1,25-Dihydroxyvitamin D₃ (1,25(OH)₂D₃) is a potential chemopreventive agent for human colon cancer. We have reported that 1,25(OH)₂D₃ specifically activated protein kinase C-α (PKC-α) and also caused a reduction in proliferation while increasing apoptosis and differentiation in CaCo-2 cells, a cell line derived from a human colon cancer. The mechanisms by which this secosteroid influences these important cellular processes, however, remain unclear. The transcription factor, activator protein-1 (AP-1), regulates many genes involved in these processes. Therefore, we asked whether 1,25(OH)₂D₃ activated AP-1 in CaCo-2 cells and, if so, by what mechanisms? 1,25(OH)₂D₃ caused a time-dependent increase in AP-1 DNA binding activity and significantly enhanced the protein and mRNA abundance of c-Jun, a component of AP-1. 1,25(OH)₂D₃ also induced a rapid and transient activation of ERK2 (where ERK is extracellular signal-regulated kinase) and a more persistent activation of JNK (where JNK Jun N-terminal kinase). Transfection experiments revealed that 1,25(OH)₂D₃ also increased AP-1 gene-transactivating activity. This AP-1 activation was completely blocked by PD 098059, a specific mitogen-activated protein kinase/ERK kinase inhibitor, as well as by a dominant negative JNK or a dominant negative Jun, indicating that the AP-1 activation induced by this secosteroid. Inhibition of JNK activation or c-Jun protein expression significantly reduced 1,25(OH)₂D₃-induced alkaline phosphatase activity, a marker of CaCo-2 cell differentiation, in secosteroid-treated cells. Taken together, the present study demonstrated that 1,25(OH)₂D₃ stimulated AP-1 activation in CaCo-2 cells by a PKC-α and JNK-dependent mechanism leading to increases in cellular differentiation.

1,25-Dihydroxyvitamin D₃ (1,25(OH)₂D₃),¹ the major active biological metabolite of vitamin D₃, has been suggested to be a potential chemopreventive agent of human colon cancer (reviewed in Ref. 1). The cellular actions of 1,25(OH)₂D₃, and other active metabolites of vitamin D₃, are thought to be transduced by the vitamin D receptor (VDR) upon binding to these secosteroids. The VDR-secosteroid complex heterodimerizes with the retinoid X receptor to bind to unique promoter sequences within the genome, i.e. vitamin D response elements, which have been demonstrated to alter the expression of genes involved in the regulation of cell growth, differentiation, and apoptosis (2–4). Recent studies have also indicated that 1,25(OH)₂D₃ induced several rapid, apparently non-genomic biological effects via a number of signal transduction pathways, including ceramide/phosphoinositide signaling, increases in the concentration of intracellular calcium, as well as by activation of protein kinase C (PKC) (5–8). The PKC family of closely related serine/threonine protein kinases includes at least 11 isoforms. These isoforms can be divided into three group as follows: Ca²⁺-dependent (α, β₁, and γ), Ca²⁺-independent (δ, η, and θ), and atypical PKCs (ζ, η, and ξ) (7). Although these isoforms of PKC share highly conserved domains, they differ in substrate specificity, tissue expression, and cellular distribution, indicating that they likely play different roles in the regulation of important cellular processes (7). We have previously shown that PKC-α was specifically activated by 1,25(OH)₂D₃ in CaCo-2 cells, a human colon adenocarcinoma-derived cell line (9). We have also shown that 1,25(OH)₂D₃ caused a dose-dependent inhibition of proliferation and an enhancement of differentiation (10), as well as induced the apoptosis of CaCo-2 cells (11). The mechanisms by which this secosteroid caused these important cellular processes are currently unknown.

Nuclear receptors, such as the VDR and retinoid X receptor, also interact with the transcription factor activator protein-1 (AP-1) in a complex manner (12–14). AP-1 has been described as a major modulator of cell growth, differentiation, and apoptosis (15–17). AP-1, a homo- or heterodimeric complex, is composed of Jun/Jun, Jun/Fos, or Jun/activating transcription factor-2. The AP-1 complex binds to the palindromic 12-O-tetradecanoylphorbol-13-acetate response element (TRE) with the nucleotide sequence TGA(C/G)TCA, which is found in the promoter region of many genes, including the c-jun gene, and regulates their expression. The Jun family includes c-Jun, JunB, and JunD, and the Fos family includes c-Fos, FosB, Fra1, Fra2 (18, 19). The N terminus of c-Jun includes regulatory phosphorylation sites, which are required for AP-1-mediated gene transcription (19, 20). The C terminus of c-Fos contains negative Jun; CAT, chloramphenicol acetyltransferase; AP-1, activator protein-1; PKC, protein kinase C; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; VDR, vitamin D receptor; bp, base pair; TRE, 12-O-tetradecanoylphorbol-13-acetate response element; MAP, mitogen-activated protein.

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1,25-Dihydroxyvitamin D₃ (1,25(OH)₂D₃),¹ the major active

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The abbreviations used are: 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; RPA, RNase protection assay; EMSA, electrophoretic mobility shift assay; DMEM, Dulbecco’s modified Eagle’s medium; ERK, extracellular-signal regulated kinase; JNK, Jun N-terminal kinase; dn-Jun, dominant negative Jun; CAT, chloramphenicol acetyltransferase; AP-1, activator protein-1; PKC, protein kinase C; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; VDR, vitamin D receptor; bp, base pair; TRE, 12-O-tetradecanoylphorbol-13-acetate response element; MAP, mitogen-activated protein.
tains autonomous activation domains and phosphorylation of its C terminus influences its transactivating activity (21). Members of the Fos and Jun gene families are often classified as “immediate early response genes” since they are rapidly activated by a number of growth factors (18, 19). AP-1 DNA binding and transcriptional activities generally correlate with an increase in the abundance of the AP-1 complex, as well as with changes in the phosphorylation of the regulatory sites of its subunits (15, 22).

Based on these observations, we therefore asked whether AP-1 and its upstream kinases were activated by 1,25(OH)2D3 in CaCo-2 cells and, if so, by what mechanisms? The present studies demonstrated that 1,25(OH)2D3 rapidly increased c-jun gene expression at both transcriptional and translational levels and induced rapid PKC-dependent activation of ERK2 and JNK1. In addition, 1,25(OH)2D3 increased AP-1 transcriptional activities in an ERK- and JNK-dependent manner. AP-1 activation by this secosteroid was also PKC-α-dependent. Furthermore, inhibition of JNK activation or suppression of c-Jun expression demonstrated that AP-1 activation by 1,25(OH)2D3 played an important role in stimulating cell differentiation.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents—**1,25(OH)2D3 was purchased from Steroids LTD Laboratory (Chicago). PD 098059, a specific inhibitor of MEKs, was purchased from Biomol Research Laboratories, Inc. (Plymouth, PA). The broad spectrum PKC inhibitor, GF109203x, was obtained from LC Services (Woburn, MA). The specific Ca2⁺-dependent PKC isofrom inhibitor, G66796, was purchased from Calbiochem. The synthetic vitamin D₃ analog, 1,25-dihydroxy-16-ene-23-yn6-26,27-hexafluorocholecalciferol (F₂₆-D₃), was kindly provided by Dr. M. R. Uskokovic (Hoffmann-La Roche). Curcumin and other chemicals were of the highest purity available and purchased from Sigma, unless otherwise indicated. Anti-c-Jun and anti-sense c-jun oligonucleotides were synthesized and purchased from Life Technology Inc.

**Cell Culture, Transfection, and CAT Assay—**CaCo-2 cells, derived from a human colonic carcinoma cell line, were cultured at 37 °C in 5% CO₂ in Dulbecco’s modified Eagle medium (DMEM), as described previously (8, 9, 23). CaCo-2 cells, with stably transduced human PKC-α cDNA, in sense or antisense orientation, have previously been described in detail (24). Cells were treated with 1,25(OH)2D3 or vehicle (EtOH) for indicated times and protected from fluorescent light. Sixty to eighty percent confluent cells in 6-well cell culture plates were transfected with LipofectAMINE™ following the protocol provided by the manufacturer (Life Technologies, Inc.). Each transfection was performed in triplicate and repeated 3–4 times. The β-galactosidase expression plasmid βSV-β-gal (Promega, Madison, WI) was included to normalize for transfection efficiency. CAT assays were performed as described previously (23). The CAT activity of each transfection was expressed as relative units after normalization for transfection efficiency by β-galactosidase activity.

**Plasmid Constructions—**The plasmid, pBAC-J-C, was used for the c-Jun RNA probe in the RNAse protection assay (RPA), and the dominant negative Jun expression plasmid, dn-Jun, were kindly provided by Dr. John Kokokides (University of Chicago). The dominant negative JNK expression plasmid (dn-JNK) was a gift from Dr. R. J. Davis (University of Massachusetts, Worcester). The AP-1 reporter plasmid 3xTRE-CAT contains three AP-1-binding sites upstream of a CAT reporter gene. The empty control vector, pBL-CAT, has no AP-1-binding sites. Both plasmids were kindly provided by Dr. E. Fuchs (University of Chicago) (26).

**RNA Isolation and RNAse Protection Assay (RPA)—**Total RNA was isolated by the TRI-Reagent, following the protocol recommended by the manufacturer (Sigma). The 216-bp antisense c-jun RNA probe and the 115-bp antisense 28S rRNA probe (Ambion, Austin, TX) were synthesized and 32P-labeled by MAXIscript™ (Ambion). RPA was carried out with RPA II™ kits (Ambion) following the protocol provided by the manufacturer. The radioactivity in each band was measured by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA), as described previously (25).

**Electrophoretic Mobility Shift Assay (EMSA)—**Nuclear proteins were prepared and stored at −70 °C until used as described previously (25).

**RESULTS**

**1,25(OH)₂D₃ Stimulates AP-1 DNA Binding Activity in CaCo-2 Cells—**To determine whether the AP-1 DNA binding activity was induced in CaCo-2 cells by 1,25(OH)₂D₃, electrophoretic mobility shift assays (EMSA) were performed using 32P-labeled oligonucleotides containing a consensus sequence for the AP-1-binding site. As shown in Fig. 1, 1,25(OH)₂D₃ caused a detectable increase in AP-1 binding as early as 15 min. This activation was maximal by 3 h and persisted for at least 24 h (Fig. 1, lanes 1–8). Unlabeled oligonucleotides inhibited this binding in competition assays (Fig. 1, lanes 9 and 10). Anti-c-Jun antibody (α-c-Jun), but not normal rabbit serum, induced a significant shift in the EMSA (supershift) (Fig. 1, lanes 11 and 12). A consensus c-Myc binding sequence was used in a c-Myc EMSA to demonstrate specificity of the increases in DNA binding by AP-1, but not c-Myc, induced by 1,25(OH)₂D₃ (data not shown). Taken together, these observations demonstrated that 1,25(OH)₂D₃ increased AP-1 DNA binding activity.

**1,25(OH)₂D₃ Increases the Abundance of Both the c-Jun Protein and the c-jun mRNA—**Western blots were used to assess alterations in the major components of AP-1 in response to 1,25(OH)₂D₃ treated CoCa-2 cells. The protein abundance of Jun rapidly increased within 15–30 min after exposure of CaCo-2 cells to 1,25(OH)₂D₃ (Fig. 2, A and B). In contrast, 1,25(OH)₂D₃ did not change the protein abundance of JunB, JunD, or c-Fos (Fig. 2A). The RPA demonstrated that the steady state level of c-jun mRNA transcript also rapidly increased within 15 min (Fig. 3) and was maximal at approximately 1 h after exposure of CaCo-2 cells to 1,25(OH)₂D₃ (Fig. 3B).
1,25(OH)₂D₃ Induces AP-1 Transactivating Ability in CaCo-2 Cells—Previous studies have demonstrated that changes in AP-1 DNA binding activity do not necessarily mirror the transcriptional activity of this complex (18). To assess the potential ability of 1,25(OH)₂D₃ to induce AP-1-mediated gene transcription, pre-confluent CaCo-2 cells were transfected with an empty vector plasmid, pBL-CAT, or an AP-1 reporter plasmid, 3x-TRE-CAT, that contains three TRE sites for AP-1 binding upstream of a CAT reporter gene (25, 26). Since we have previously shown that 1,25(OH)₂D₃ had no detectable effect on the CAT activity in cells transfected with c-Myc-CAT, but not vehicle (EtOH), significantly increased CAT activity was observed in cells transfected with 3x-TRE-CAT (p < 0.05), compared with cells transfected with the empty vector, pBL-CAT, and compared with 3x-TRE-CAT-transfected cells treated with EtOH (Fig. 4). In contrast, 1,25(OH)₂D₃ had no effect on the CAT activity in cells transfected with c-Myc-CAT, indicating the specificity of the AP-1 activation induced by 1,25(OH)₂D₃.

1,25(OH)₂D₃ Induces a Rapid but Transient Activation of ERK2 and a More Persistent Activation of JNK1 by a PKC-dependent Mechanism—MAP kinase signaling pathways, including both ERKs and JNKs, influence AP-1 transactivation activity by increasing both the abundance of AP-1 components and altering the phosphorylation of their subunits (34). Further studies were, therefore, performed to assess the effect of 1,25(OH)₂D₃ on the ERK and JNK pathways. After treatment with 1,25(OH)₂D₃ (100 nM) or vehicle (EtOH) for the indicated times, whole cell lysates were analyzed by Western blots for activated ERK1,2 and JNK1,2, respectively. These antibodies recognize only the dual phosphorylated active forms of ERK1,2 and JNK1,2, respectively. 1,25(OH)₂D₃ induced a rapid activation of ERK2 within 3 min, which returned to the control level by 3 h (Fig. 5A). In contrast, 1,25(OH)₂D₃ did not stimulate ERK1. JNK1, but not JNK2, was also rapidly (3 min) and more persistently (24 h) activated by 1,25(OH)₂D₃ (Fig. 5A). To determine whether the activation of either ERK2 and/or JNK1 was PKC-dependent, pre-confluent CaCo-2 cells were pretreated for 3 h with Gö6976, a specific inhibitor of Ca²⁺-dependent PKC isoforms, and then exposed to 1,25(OH)₂D₃ (100 nM) for the indicated times. As shown in Fig. 5B, pretreatment with Gö6976 completely blocked the activation of ERK2 and JNK1 by 1,25(OH)₂D₃, indicating that stimulation of ERK2 and JNK1 by this secosteroid is mediated by one or more Ca²⁺-dependent PKC isoforms.

Both ERK and JNK Cascades Are Required for AP-1 Activation Induced by 1,25(OH)₂D₃.—To elucidate the effects of ERK activation by 1,25(OH)₂D₃ on AP-1 activation, CaCo-2 cells, transfected with 3x-TRE-CAT, were pretreated with different concentrations of PD 098059, a specific inhibitor of MEKs, before exposure of these cells to 1,25(OH)₂D₃. Inhibition of
shown that 1,25(OH)₂D₃ specifically activated PKC-α. As noted earlier, previous studies from our laboratory have induced by 1,25(OH)₂D₃ was PKC-dependent, additional studies were conducted. After transfection with 3x-TRE-CAT, cells were pretreated with GF109203x, a broad spectrum inhibitor of PKC isoforms, before exposure to 1,25(OH)₂D₃ or ethanol for 36 h. Pretreatment of CaCo-2 cells with either of these PKC inhibitors significantly reduced the 1,25(OH)₂D₃-induced AP-1 activation in these PKC-α-overexpressing cells, this increase did not reach statistical significance (p = 0.1). AP-1 activation in these cells may already be nearly maximally driven by increases in basal PKC-α expression. As shown in Fig. 5C, 1,25(OH)₂D₃ was, however, unable to activate AP-1 in cells stably transfected with antisense PKC-α cDNA, indicating that the AP-1 activation induced by this secosteroid in CaCo-2 cells is PKC-α-dependent.

Recent studies have examined the ability of synthetic ana-
logs of vitamin D₃ to inhibit colonic tumorigenesis (1, 37). Our laboratory has reported, in fact, that a synthetic fluorinated vitamin D₃ analog, F₆-D₃, significantly reduced the tumor incidence in the azoxymethane model of rat colonic tumorigenesis (37). It was, therefore, of interest to determine whether this analog of vitamin D₃ also stimulated AP-1 activity. As shown in Fig. 8, F₆-D₃ (100 nM) caused similar changes in AP-1 activation as those induced by 1,25(OH)₂D₃ in CaCo-2 cells with stably transfected PKC-α cDNA in sense or antisense orientation or in those transfected with an empty vector.

Inhibition of JNK Activation Reduces Alkaline Phosphatase Activity in CaCo-2 Cell—To evaluate the role of JNK activation by 1,25(OH)₂D₃ in stimulating cell differentiation, curcumin, a specific inhibitor of JNK (29–32), was employed. Previous studies indicated that the JNK pathway was more sensitive than the ERK pathway to this agent (29–32). We also observed that curcumin at 15 μM inhibited most of JNK activity without detectable effect on ERK activity stimulated by 1,25(OH)₂D₃ in CaCo-2 cells (data not shown). Our previous study demonstrated that 1,25(OH)₂D₃ significantly reduced CaCo-2 cell growth and enhanced alkaline phosphatase activity, one of the recognized differentiation markers of CaCo-2 cells (10). In the present study curcumin significantly reduced (~76%) alkaline phosphatase activity induced

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**Fig. 5. 1,25(OH)₂D₃ activates ERK2 and JNK1 in CaCo-2 cells in a PKC-dependent manner.** Preconfluent CaCo-2 cells were treated with 1,25(OH)₂D₃ for the indicated times or pretreated for 3 h with Go6976 (2 μM), a specific inhibitor of Ca²⁺-dependent PKC isoforms. Whole cell extracts (20 μg of protein) were separated by SDS-PAGE on a 10% resolving gel. After electrophoresis, active JNK1,2 and ERK1,2 were detected by anti-active JNK and anti-active ERK antibodies (1:5000 final dilution), respectively (A indicates activated). Whole cell extracts from CaCo-2 cells, treated with UV irradiation for 2 min (U.V.), served as positive controls for active-JNK1,2 and ERK1,2. A, 1,25(OH)₂D₃-treated; B, Go6976 pretreated followed by 1,25(OH)₂D₃ treatment.

**Fig. 6. JNK and ERK are required for 1,25(OH)₂D₃-induced AP-1 transcriptional activity.** CaCo-2 cells were transfected with the AP-1 reporter plasmid, 3×TRE-CAT, or co-transfected with 3×TRE-CAT, or the empty vector, pBL-CAT, and the indicated dominant negative plasmid (dn-JNK, or dn-Jun), or a control vector (pMNC), using LipofectAMINE™ as described under “Experimental Procedures.” The CAT activities were measured and normalized to β-galactosidase activity. Values were expressed as means ± S.D. (n = 6). A, cells transfected with 3×TRE-CAT and pretreated with the indicated concentrations of PD 098059, a specific inhibitor of MEK, for 1 h before exposure to 1,25(OH)₂D₃ (100 nM) for an additional 36 h. *p < 0.05, compared with cells transfected with 3×TRE-CAT without pretreatment with PD 098059. B, cells co-transfected with 3×TRE-CAT, or pBL-CAT and a dominant negative Jun (dn-Jun) or a dominant negative JNK (dn-JNK), or the control pMNC, and treated with 1,25(OH)₂D₃. **p < 0.05, compared with cells co-transfected with dn-Jun, or dn-JNK, or the empty vector pMNC.
by 1,25(OH)2D3 in CaCo-2 cells 14 days postplating (Fig. 9). This finding suggests that activation of JNK contributes to cell differentiation induced by this secosteroid.

1,25(OH)2D3-induced AP-1 Activation Plays a Significant Role in Stimulating Cell Differentiation—Prior studies have shown that c-Jun/AP-1 is only one of the downstream substrates of JNK (19, 38). Inhibition of JNK activation could, therefore, not exclude the possibility that other JNK substrates contributed to the reduction of alkaline phosphatase activity in CaCo-2 cells. To study further the role of the secosteroid-induced AP-1 activation in cell differentiation, cells were treated

with 1,25(OH)2D3 plus either antisense or sense c-jun oligonucleotides (Fig. 10). Western blots indicated that at 50 μg/ml c-Jun protein expression was almost completely blocked by antisense c-jun oligonucleotides (Fig. 10A). As expected, the same concentration of sense c-jun oligonucleotides had no effect on c-Jun protein expression (Fig. 10A). Further study in CaCo-2 cells transfected with 3x-TRE-CAT plasmid confirmed that the antisense c-jun oligonucleotides at 50 μg/ml completely inhibited 1,25(OH)2D3-induced AP-1 transacting activity (Fig. 10B). Neither sense nor antisense c-jun oligonucleotides had effects on CAT activity in cells transfected with the control empty vector pBL-CAT (data not shown). The role of AP-1 induced by 1,25(OH)2D3 in cell differentiation was studied in cells 3 days post-plating treated with 50 μg/ml of either antisense or sense c-jun oligonucleotides and 1,25(OH)2D3 (100 nM). The media containing the oligonucleotides and 1,25(OH)2D3 were replaced every other day. Cells were harvested on the indicated days after plating for alkaline phosphatase assay (Fig. 10C). Inhibition of c-Jun/AP-1 by antisense c-jun oligonucleotides significantly reduced alkaline phosphatase activity induced by 1,25(OH)2D3 by ~70% at 14 days postplating, indicating that AP-1 activation plays a significant role in stimulating CaCo-2 cell differentiation by this secosteroid.

DISCUSSION

The present studies have demonstrated that 1,25(OH)2D3 rapidly induced c-jun gene expression at both the transcriptional and the translational levels, as well as stimulated AP-1 transcriptional activity in CaCo-2 cells. The protein abundance of c-Fos, as well as other Jun family members, including JunB and JunD, was not altered by this secosteroid. 1,25(OH)2D3 and other analogs of vitamin D3 have also been found to stimulate differentially Jun and/or Fos family members in other cell types (33, 39–41). Recent studies, moreover, have found that AP-1 transcriptional activity could be regulated by alterations in its subunit composition (19, 33, 41, 42). Different homo- or heterodimeric combinations of AP-1 are likely to have unique functions in regulating cell proliferation, differentiation, and apoptosis (19, 33, 41, 42). In other cells, activation of ERKs has been shown to increase the activity and expression of members of the Jun family.
1,25(OH)2D3 Activates AP-1 in CaCo-2 Cells

FIG. 9. JNK inhibition by curcumin significantly reduces alkaline phosphatase activity in CaCo-2 cells treated with 1,25(OH)2D3. Three days after plating, CaCo-2 cells were incubated in DMEM containing 1,25(OH)2D3 (100 nM) alone or with curcumin (15 μM). Control cells were incubated in DMEM with 0.08% vehicle (EtOH). Alkaline phosphatase assays were performed as described under “Experimental Procedures.” Values were expressed as means ± S.D. (n = 3). At 14 days postplating, compared with CaCo-2 cells treated with 1,25(OH)2D3 alone, curcumin significantly reduced 1,25(OH)2D3-induced alkaline phosphatase activity by ~76% (p < 0.05).

FIG. 10. AP-1 activation by 1,25(OH)2D3 plays a significant role in stimulating cell differentiation. Three days postplating, CaCo-2 cells were incubated in DMEM with 1,25(OH)2D3 alone or containing either antisense or sense c-jun oligonucleotides to suppress c-jun protein expression (see details described under “Experimental Procedures”). All control cells were incubated in DMEM with 0.08% vehicle (EtOH). A, representative Western blots using anti-c-jun in cells treated with 1,25(OH)2D3 alone or plus either antisense or sense c-jun oligonucleotides at the indicated concentrations (μg/ml) for 24 h. B, CAT assay in 3x-TRE-CAT-transfected cells treated with 1,25(OH)2D3 alone or plus either antisense or sense c-jun oligonucleotides at 50 μg/ml for 36 h. The CAT activities were measured and normalized to β-galactosidase activity. Values were expressed as means ± S.D. (n = 6). * p < 0.05, compared with vehicle (EtOH)-treated cells transfected with 3x-TRE-CAT. All the treatments had no effects on CAT activity in cells transfected with the empty control vector pBL-CAT (data not shown); C, alkaline phosphatase assay in cells incubated in DMEM with 1,25(OH)2D3 alone or plus either antisense or sense c-jun oligonucleotides at 50 μg/ml for indicated days postplating. The media containing the oligonucleotides and 1,25(OH)2D3 were replaced every other day. Values were expressed as means ± S.D. (n = 3).

of the Fos family (34). In the present studies, however, 1,25(OH)2D3 failed to increase c-Fos protein abundance. Whether stimulation of one of the MAP kinase members, for example Fos-regulating kinase (38, 43), by 1,25(OH)2D3 may activate c-Fos or other members of the Fos family, via a post-translational change such as phosphorylation, remains to be determined. This will be of interest since the MEK-specific inhibitor, PD 098059, was found to inhibit AP-1 activation by this secosteroid. Activation of JNK1 would be expected to phosphorylate and thereby activate c-Jun. These events would increase the transactivating ability of homo- or heterodimers of Jun on binding to TRE sites in the promoter regions of numerous genes, such as c-jun, thereby enhancing the expression of target genes, including those involved in cell growth, differentiation, and/or apoptosis. Further studies will, therefore, be of interest to determine whether the expression or activity of other members of the AP-1 superfamily, in addition to c-jun, are altered by 1,25(OH)2D3 in CaCo-2 cells.

The transcription of c-jun and the activation of its protein product are regulated, in part, by JNK activation (44). c-Jun can autoregulate its own gene expression by increasing Jun-Jun homodimer binding to the TRE in the promoter region of the c-jun gene (19, 45). MAP kinase signaling pathways, including both ERKs and JNKs, influence AP-1 transcriptional activity by increasing the abundance of AP-1 components and altering the phosphorylation of their subunits (34). 1,25(OH)2D3 caused a rapid but transient activation of ERK2 and a rapid but more persistent activation of JNK1 in CaCo-2 cells. The present studies, utilizing a specific MEK inhibitor or a dominant negative JNK, have demonstrated that inhibition of ERK2 and JNK1 blocked the ability of 1,25(OH)2D3 to activate AP-1. Depending on the cell type and agonist, activation of JNKs and ERKs by a variety of agonists may occur by either PKC-dependent or -independent pathways (46–50). The present experiments using PKC inhibitors have demonstrated that stimulation of both ERK2 and JNK1 by 1,25(OH)2D3 was PKC-dependent in CaCo-2 cells. We have previously demonstrated that 1,25(OH)2D3 specifically activated PKC-α, but not other isoforms of PKC, present in CaCo-2 cells (9). In addition, our laboratory has recently shown that changes in the expression of PKC-α alter the growth and differentiation in CaCo-2 cells, stably transfected with PKC-α cDNA in sense or antisense orientations (24). In the present studies, AP-1 activation induced by 1,25(OH)2D3 in CaCo-2 cells was PKC-α-dependent, as evidenced by experiments utilizing stably transfected Ca-Co-2 cells with inhibited or amplified PKC-α expression. In keeping with our present observations, PKC-mediated AP-1 activation has been reported in other cell lines (7, 50–52). In agreement with our finding, overexpression of PKC-α in rat fibroblast 3Y1 cells resulted in the enhancement of AP-1 transcriptional activity, as well as increased c-jun gene expression (52). Taken together, these results in CaCo-2 cells, as well as those observed in other cell types, demonstrate that activation of AP-1 by 1,25(OH)2D3 is mediated by PKC-α.
**1,25(OH)₂D₃ Activates AP-1 in CaCo-2 Cells**

**FIG. 11. Schema of AP-1 activation induced by 1,25(OH)₂D₃ in CaCo-2 cells.** Exposure of CaCo-2 cells to 1,25(OH)₂D₃ rapidly activates PKC-α, ERK2, and JNK1. Although it remains unclear whether activated ERK2 alters the transactivating ability of members of the Fos family, activated JNK1 phosphorylates and activates c-Jun. Activated c-Jun may autoregulate its own expression and that of other genes by forming homodimers (Jun-Jun) or heterodimers (Jun-Fos) of AP-1, which bind to TRE sites in their promoter regions. Changes in the expression of these genes will determine the phenotypic characteristics of these cells, including alterations in differentiation, and perhaps in cell growth and apoptosis.

Previous studies have shown that PKC-α can stimulate ERK activity by initially activating Raf-1, a MAP kinase kinase kinase, which, in turn, phosphorylated and activated MEK, a MAP kinase kinase (18, 19). The latter dual functioning kinase then activates the ERKs by phosphorylation of both threonine and tyrosine residues. Once activated, the ERKs translocate to the nucleus of cells, and their phosphorylated substrates include c-Fos, in turn, lead to activation of genes involved in the regulation of cellular proliferation, differentiation, and malignant transformation. It bears emphasizing that activation of the aforementioned cascade may be ras-dependent or -independent (18, 19). Recent studies from our laboratory have shown that 1,25(OH)₂D₃ failed to activate p21<sup>ras</sup> in CaCo-2 cells, indicating that in these cells ERK activation by this secosteroid via PKC-α appears to occur by a ras-independent mechanism (53).

Prior studies in endothelin-stimulated Rat-1 cells demonstrated that activation of PKC inhibited the activity of JNK (54). In contrast to this finding, as noted above, 1,25(OH)₂D₃ activated JNK1 via a PKC-α-dependent mechanism(s). It is unclear at this time how PKC-α activates this kinase in these cells, and future studies will be necessary to address this issue.

We have previously shown that chronic administration of 1,25(OH)₂D₃ was associated with the cessation of logarithmic growth and the onset of differentiation in CaCo-2 cells (10). In the present study utilizing a JNK inhibitor, JNK1 activation induced by 1,25(OH)₂D₃ was shown to play a significant role in enhancing CaCo-2 differentiation, although other, as yet unidentified, pathways may also contribute to these processes. In support of the present findings, other studies have also demonstrated that prolonged JNK activation was associated with cell differentiation (55, 56). Transient activation of ERK2 induced by 1,25(OH)₂D₃ in CaCo-2 cells may play a lesser role in cell differentiation (57). Further study using antisense <i>c-jun</i> oligonucleotides clearly demonstrated the obligate role of AP-1 activation in cell differentiation induced by 1,25(OH)₂D₃. These results were supported by recent studies in CaCo-2 cells that differentiation of these cells was associated with an increase in AP-1 DNA binding activity (58).

Based on our present and prior observations, we have proposed the model depicted in Fig. 11. In this model, treatment of CaCo-2 cells with 1,25(OH)₂D₃ rapidly stimulates PKC-α, which, in turn, activates ERK2 and JNK1, leading to enhanced AP-1 transactivating ability and thus to alterations in genes involved in the control of differentiation and perhaps other important cellular processes regulated by this ubiquitous transcription factor.

In summary, the present study has demonstrated that 1,25(OH)₂D₃ increased the steady state level of the <i>c-jun</i> mRNA transcript and the abundance of the <i>c-jun</i> protein in CaCo-2 cells. This secosteroid also caused a rapid and transient activation of ERK2 and a more persistent activation of JNK1. In addition, 1,25(OH)₂D₃ stimulated both the DNA binding and transcriptional activity of AP-1 via ERK- and JNK-dependent mechanisms. The activation of AP-1 by 1,25(OH)₂D₃ via these kinases was mediated by PKC-α. Finally, the 1,25(OH)₂D₃-induced activation of AP-1, in turn, enhanced the differentiation of CaCo-2 cells. Given the actions of 1,25(OH)₂D₃ to prevent the development of colon cancer, further studies along these lines should be of interest.

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