1α,25-Dihydroxyvitamin D_3 Stimulates Activator Protein 1 DNA-Binding Activity by a Phosphatidylinositol 3-Kinase/Ras/MEK/Extracellular Signal Regulated Kinase 1/2 and c-Jun N-Terminal Kinase 1-Dependent Increase in c-Fos, Fra1, and c-Jun Expression in Human Keratinocytes

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1α,25-Dihydroxyvitamin D_3 added to human keratinocytes increases differentiation through an activation of the transcription factor activator protein 1. We have previously reported that the 1α,25-dihydroxyvitamin D_3-induced increase of activator protein 1 DNA binding activity is mediated by a protein kinase C-independent mechanism. The purpose of this study was to investigate further the mechanisms by which 1α,25-dihydroxyvitamin D_3 modulates activator protein 1 DNA binding activity in cultured normal human keratinocytes. Western blotting experiments revealed that 1α,25-dihydroxyvitamin D_3 caused a rapid and transient activation of the mitogen-activated protein kinases, extracellular signal regulated kinase 1/2 and c-Jun N-terminal kinase 1. 1α,25-Dihydroxyvitamin D_3 also enhanced the expression of the activator protein 1 subunits, c-Fos, Fra1, and c-Jun as determined by northern and western blotting. The 1α,25-dihydroxyvitamin D_3-induced activator protein 1 DNA binding activity was completely blocked by the MEK inhibitor PD 98059 indicating that the MEK/extracellular signal regulated kinase pathway is involved in the activation of activator protein 1. Transfection experiments showed that 1α,25-dihydroxyvitamin D_3 also increased the activator protein 1-dependent transactivation, which was completely blocked by expression of a dominant negative Ras, suggesting that the 1α,25-dihydroxyvitamin D_3-induced activator protein 1 activity involves Ras-dependent signaling. Furthermore, preincubation of the keratinocytes with the specific phosphatidylinositol 3-kinase inhibitors, Wortmannin and LY294002, demonstrated that the 1α,25-dihydroxyvitamin D_3-induced activation of extracellular signal regulated kinase 1/2 and c-Jun N-terminal kinase 1 required phosphatidylinositol 3-kinase activity. Finally, preincubation of keratinocytes with a polyclonal antibody against the membrane receptor annexin II, blocked the 1α,25-dihydroxyvitamin D_3-induced activation of extracellular signal regulated kinase 1/2 and c-Jun N-terminal kinase 1. Taken together, our results indicate that 1α,25-dihydroxyvitamin D_3, via binding to the membrane receptor annexin II, induces activation of the phosphatidylinositol 3-kinase/Ras/MEK/extracellular signal regulated kinase 1/2 and c-Jun N-terminal kinase 1 signal transduction pathway resulting in increased expression of c-Fos, Fra1, and c-Jun, and subsequently increased activator protein 1 DNA binding activity and gene transcription. ***Key words: activator protein 1/annexin II/1α,25-dihydroxyvitamin D_3/keratinocytes/mitogen-activated protein kinase. J Invest Dermatol 120:561–570, 2003***

The stratified epidermis is characterized by the migration of keratinocytes from the proliferative basal compartment to the upper, terminally differentiated cornified layers of the skin. As the cells migrate from the basal layer, the keratinocytes undergo a distinct pattern of differentiation that is essential for the function of the skin. 1α,25-Dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$), the hormonally active form of vitamin D, decreases proliferation and increases terminal differentiation of preconfluent cultured human keratinocytes (Hosomi *et al*, 1983; Smith *et al*, 1986). The cellular actions of 1α,25$(OH)_2D_3$ have primarily been attributed to its intracellular receptor, the vitamin D receptor (VDR), which belongs to the superfamily of nuclear steroid/thyroid receptors (Haussler, 1986). Like several of these receptors, VDR is known to bind as a heterodimer with the retinoid X receptor (RXR) to DNA sequences in the promoter region of vitamin D responsive genes (Demay *et al*, 1990; Noda *et al*, 1990; Zierold *et al*, 1994). Recent studies have indicated that 1α,25$(OH)_2D_3$ also rapidly instigates several, apparently VDR independent processes by a number of signal transduction pathways, including altered

Manuscript received May 1, 2002; revised September 30, 2002; accepted for publication December 3, 2002

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Abbreviations: 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; AP-1, activator protein 1; ERK, extracellular signal regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; VDR, vitamin D receptor; PI3, phosphatidylinositol 3.

0022-202X/03/$15.00 ·

phosphoinositide metabolism (Sorensen and Baran, 1995), increased concentration of intracellular calcium (Jones and Sharpe, 1994), altered calcium transport (Nemere *et al*, 1984), protein kinase C (PKC) activation (Hanafin *et al*, 1995; Slater *et al*, 1995; Chen *et al*, 1999), and activation of mitogen-activated protein kinase (MAPK) (Marcinkowska *et al*, 1997; Chen *et al*, 1999). How $1\alpha,25(OH)_2D_3$ mediates these rapid actions is still not fully elucidated. Recent results have shown that $1\alpha,25(OH)_2D_3$ can bind to the annexin II membrane receptor (Baran *et al*, 2000a) and it has been speculated that annexin II is the surface membrane receptor mediating the rapid actions of $1\alpha,25(OH)_2D_3$ (Baran *et al*, 2000b).

The MAPK cascade is a signaling pathway intimately involved in cellular proliferation, differentiation, and apoptosis (Xia *et al*, 1995; Whitmarsh and Davis, 1996; Chang and Karin, 2001). It consists of three distinct layers of protein kinases, including a MEK kinase (MEKK), which activates a MAPK kinase (MEK), which in turn activates a MAPK (Robinson and Cobb, 1997). Three distinct MAPK signal transduction pathways have been identified in mammalian cells, leading to activation of the MAPK the extracellular signal regulated kinases (ERK), the c-Jun N-terminal kinases (JNK, also known as stress-activated protein kinases; SAPK), and p38 (Davis, 1993, 1994; Waskiewicz and Cooper, 1995). Once activated the MAPK may translocate to the nucleus where they phosphorylate and activate a number of target proteins (Cano and Mahadevan, 1995; Hill and Treisman, 1995; Karin and Hunter, 1995; Robinson and Cobb, 1997).

Activator protein 1 (AP-1) is a nuclear transcription factor, known to play an important part in keratinocyte differentiation (DiSepio *et al*, 1995; Rutberg *et al*, 1996; Sark *et al*, 1998; Johansen *et al*, 2000). AP-1 is formed as homodimers between Jun (c-Jun, JunB, JunD) proteins or heterodimers between Fos (c-Fos, FosB, Fra1, Fra2) and Jun proteins (Angel and Karin, 1991). Once activated, the AP-1 complex binds to the 12-*O*-tetradecanoylphorbol-13-acetate response element, which is found in the promoter region of several genes, including genes involved in keratinocyte differentiation, such as keratin 1 (Lu *et al*, 1994), keratin 5 (Casatorres *et al*, 1994), transglutaminase 1 (Yamada *et al*, 1994), and involucrin (Welter *et al*, 1995).

We have previously shown that $1\alpha,25(OH)_2D_3$ increases the AP-1 DNA binding activity in cultured human keratinocytes (Johansen *et al*, 2000). In contrast to calcium, this induction was PKC independent (Johansen *et al*, 2000). The exact mechanism by which $1\alpha,25(OH)_2D_3$ increases the AP-1 DNA binding activity in human keratinocytes, however, was not elucidated. Recent results have shown that $1\alpha,25(OH)_2D_3$-induced AP-1 DNA binding activity in a human colonic carcinoma cell line is mediated by the MAP kinases, ERK and JNK (Chen *et al*, 1999). Furthermore, it has been demonstrated that the calcium-induced differentiation of keratinocytes is mediated by a Ras-independent activation of the Raf/MEK/ERK1 pathway (Schmidt *et al*, 2000). It is therefore possible that activation of the MEK/ERK or JNK pathway plays an important part in keratinocyte differentiation.

Phosphatidylinositol 3 kinase (PI3 kinase) is a lipid kinase composed of an 85 kDa (p85) regulatory subunit and a 110 kDa (p110) catalytic subunit. The PI3 kinase is known to play a part in a wide variety of cellular processes, including organization of the cytoskeleton, cell growth, transformation, and prevention of apoptosis (Fry, 1994; Toker and Cantley, 1997). Also it has been suggested that the PI3 kinase may play a part in the $1\alpha,25(OH)_2D_3$-induced monocyte differentiation (Hmama *et al*, 1999), and that PI3 kinase activity is essential for all-*trans*-retinoic acid-induced granulocytic differentiation of HL-60 cells (Bertagnolo *et al*, 1999). The PI3 kinase is also required for an activation of the Ras/c-Rafl/MEK/ERK signaling cascade induced by vanadyl sulfate in Chinese hamster ovary cells (Pandey *et al*, 1999).

In this study we addressed the question whether the $1\alpha,25(OH)_2D_3$-induced AP-1 DNA binding activity seen in cultured human keratinocytes after 36 h of incubation was due to a PI3 kinase dependent activation of the MEK/ERK cascade. We found that addition of $1\alpha,25(OH)_2D_3$ to cultured normal human keratinocytes resulted in a rapid but transient phosphorylation of ERK1, ERK2, and JNK1, which was accompanied by increased expression of both c-Fos, Fra1, and c-Jun as determined at the mRNA and protein levels. In addition, $1\alpha,25(OH)_2D_3$ increased the AP-1 transcriptional activities in a Ras-dependent manner. The $1\alpha,25(OH)_2D_3$-induced activation of ERK1/2 and JNK1 was downregulated by inhibition of both the MEK and the PI3 kinase, demonstrating that the PI3 kinase/Ras/MEK/ERK1/2 and JNK1 pathway is the principal pathway in the $1\alpha,25(OH)_2D_3$-induced activation of AP-1 and in the resulting differentiation. Finally, we present evidence suggesting that $1\alpha,25(OH)_2D_3$ activates the MAPK signal transduction pathway through the surface membrane receptor, annexin II.

MATERIALS AND METHODS

Materials Keratinocyte serum-free medium, keratinocyte basal medium, and dominant negative Ras (Ras-N17) cloned into pcDNA3 and pcDNA3-Ras-N17 were obtained from Invitrogen (Carlsbad, CA). $1\alpha,25(OH)_2D_3$ dissolved in 2-propanol was kindly provided by Leo Pharmaceuticals Products (Ballerup, Denmark). Wortmannin, LY294002 and PD 98059 were purchased form Calbiochem (San Diego, CA). AP-1 Consensus Oligo (5′-CGCTTGATGAGTCAGCCGGAA-3′), Gel Shift Binding $5\times$ Buffer, T4 Polynucleotide Kinase and T4 Polynucleotide Kinase $10\times$ Buffer were obtained from Promega (Madison, WI). [γ-^{32}P]adenosine triphosphate, Enhanced Chemiluminescence Detection System (ECL), microspin G-25 columns and Nick Spin Columns Sephadex G-50 fine DNA-grade were provided by Amersham/Pharmacia Biotech (Uppsala, Sweden). Complete (a protease inhibitor cocktail) was purchased from Boehringer Mannheim (Mannheim, Germany). Trypsin, ethylenediamine tetraacetic acid (EDTA), Tris base, Tris–HCl, dithiothreitol (DTT), and boric acid were obtained from Sigma (St Louis, MO). Antibodies against ERK, p38α (no cross-reactivity with p38β, whereas cross-reactivity with p38γ and p38δ has not been ruled out), phospo-c-Jun, JNK, and protein kinase B were purchased from Cell Signaling Technology (Beverly, MA) and antibodies against TFIIB, c-Fos, c-Jun, JunB, JunD, Fra1, Fra2, FosB, and annexin II were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Fractionated rabbit immunoglobulins were from DAKO (Glostrup, Denmark). DNA probes recognizing c-*Fos*, *Fra1*, c-*Jun*, and *GAPDH* were from Geneka Biotechnology Inc. (Montreal, Canada). The reporter construct p(AP-1)$_3$-luc$^+$ containing three tandem copies of the AP-1 site was from Stratagene (La Jolla, CA), and the CMV-β-galactosidase expression vector used for normalization was from Clontech (Palo Alto, CA). SeaKem Gold agarose gel were obtained from BioWhittaker Molecular Applications (Rockland, ME).

Cell cultures Normal adult human keratinocytes were obtained by trypsinization of skin samples from patients undergoing plastic surgery as previously described (Kragballe *et al*, 1985). First-passage keratinocytes were grown in keratinocyte serum-free medium. Twenty-four hours before stimulation the medium was changed to keratinocyte basal medium (without growth factors) in which the cells were stimulated. Cells were grown at 37 C and 5% CO_2 in an incubator. At 50–60% confluency, as judged by light microscopy, $1\alpha,25(OH)_2D_3$ 10^{-7} M final concentration was added. The $1\alpha,25(OH)_2D_3$ stock solution was diluted in 2-propanol and the final 2-propanol concentration in medium was 0.025‰. In separate experiments specific inhibitors were added 30 min before stimulation.

Transient transfection assays Normal human keratinocytes were transiently transfected in 12 well plates using Effectene (Qiagen, Valencia, CA). Cells were maintained in keratinocyte basal medium for 24 h prior to transfection. Transfections were performed with 75 ng p(AP-1)$_3$-luc$^+$ (Stratagene) reporter construct together with 0, 0.008, 0.04, 0.2, or 1 ng pcDNA3-Ras-N17. Two nanograms of CMV-β-galactosidase were used for normalization and empty expression vector was added to ensure equal promoter load. Six hours following transfection fresh medium was added and cells were subsequently incubated for 36 h, 48 h, or 60 h in keratinocyte basal medium containing either vehicle (2-propanol) or $1\alpha,25(OH)_2D_3$ 10^{-7} M. Luciferase and β-galactosidase activities in cell lysates were determined by standard techniques. Luciferase activities were normalized to β-galactosidase activities. All transfections were made in triplicate and repeated three times.

Isolation of the nuclear fraction Nuclear extracts were prepared as previously described (Rosette and Karin, 1995; Johansen *et al*, 2000).

Briefly, cells were released from the culture flask by a rubber policeman followed by dilution in a hypoton buffer (10 mM HEPES, 10 mM KCl, 0.2 mM EDTA, 0.1 mM ethyleneglycol-bis-(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid, 1 mM DTT, pH 7.9). Then the suspension was passed through a 27 gauge needle six times and centrifuged at 10,000 × *g* for 1.5 min. Pellet was resuspended in a hyperton buffer (20 mM HEPES, 0.4 mM NaCl, 1 mM EDTA, and 1 mM DTT, pH 7.9) and rotated at 4°C for 30 min before being centrifuged at 10,000 × *g* for 5 min. The supernatant was considered the nuclear extract. Protein concentration was determined as previously described by Bradford (1976) with bovine serum albumin as standard.

Labeling of consensus oligonucleotides for AP-1 Four microliters of AP-1 consensus oligonucleotide (1.75 pmol per μl), 2 μl of T4 polynucleotide kinase 10 × Buffer (700 mM Tris–HCl, 100 mM $MgCl_2$, and 50 mM DTT), 4 μl of [γ-^{32}P]adenosine triphosphate (3000 Ci per mmol at 10 mCi per ml), 8 μl nuclease-free water, and 2 μl T4 polynucleotide kinase were added to an Eppendorf tube and incubated for 10 min at 37°C before the reaction was stopped by the addition of 1 μl 0.5 M EDTA. Finally, 200 μl of TE-Buffer (10 mM Tris–HCl, pH 8 and 1 mM EDTA) was added.

Labeled and unlabeled oligonucleotides were separated on a Nick spin column at 300 × *g* for 5 min.

Electrophoretic mobility shift assay Binding reactions consisted of 3–5 μg of nuclear protein, preincubated for 10 min at room temperature in the incubation buffer [5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris–HCl, pH 7.5, 20% glycerol, 0.25 mg per ml poly(dI-dC)]. Then, 1 μl of ^{32}P-labeled AP-1 probe was added and incubations were carried out for additional 20 min. The binding complexes were resolved on a 4% polyacrylamide gel in 0.5 × TBE buffer (22.5 mM Tris base, 4 mM boric acid, and 1 mM EDTA). Supershifts were performed by the addition of 2 μg of the corresponding commercially available antibodies specific for Fra1 and c-Jun to the binding reactions, 15 min after the addition of the ^{32}P-labeled probe. Incubation was then allowed to proceed for an additional 30 min.

In control experiments a specific competitor (unlabeled AP-1 oligo) or a nonspecific competitor (unlabeled SP-1 oligo), were added 10 min before addition of labeled AP-1 oligo.

Western blotting Keratinocytes were washed twice with ice-cold phosphate-buffered saline, lyzed with cold lysis buffer [50 mM Tris–HCl, pH 6.8, 10 mM DTT, 10 mM β-glycerophosphate, 10 mM NaF, 0.1 mM Na orthovanadate, 10% glycerol, 2.5% sodium dodecyl sulfate (SDS), 25 mM phenylmethylsulfonyl fluoride and 50 × complete (Boehringer Mannheim)], scraped from the plate and boiled for 3 min. Then the lysate was centrifuged at 13,000 × *g* for 3 min and the supernatant was collected and assayed for protein concentration as described by Bradford (1976). Equal protein amounts were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and blotted on to nitrocellulose membranes. Membranes were incubated with the appropriate primary antibody and detected with either anti-rabbit IgG-horseradish peroxidase (Santa Cruz) or anti-rabbit IgG-horseradish peroxidase (DAKO) in a standard ECL reaction (Amersham) according to the manufacturers' instructions.

RNA purification Cultured human keratinocytes were washed twice with ice cold phosphate-buffered saline, before 1 ml of TRIZOL reagent was added for the isolation of total RNA as recommended by the manufacturer (Gibco BRL/Life Technologies).

Northern blotting Total RNA (10 μg) was separated on a 1.25% SeaKem Gold agarose gel and transferred to Hybond N membranes by capillary blotting. The integrity of RNA was assessed by methylene blue staining of the membrane. [γ-^{32}P]adenosine triphosphate-labeled probes were generated using human c-Fos, Fra1, c-Jun, and GAPDH fragments according to the manufacturer's protocol (Geneka Biotechnology Inc.). The probes were purified using microspin G-25 columns. Hybridization with the ^{32}P-labeled probes was carried out as recommended by the manufacturer.

Statistics Results were expressed as mean ± SD. For statistical analysis a one-way ANOVA was done followed by a Student–Newman–Keuls test. To test for normal distribution, a probability test was made. A probability of $p < 0.05$ was regarded as statistically significant.

RESULTS

1α25(OH)2D3 induces a rapid and transient activation of the MAPK ERK1, ERK2, and JNK1 in cultured human keratinocytes

We have previously shown that 1α,25(OH)$_2$D$_3$ increases the AP-1 DNA binding activity in human keratinocytes in a concentration-dependent manner (Johansen *et al*, 2000). Furthermore, MAPK signaling pathways, including both ERK and JNK, have been known to influence AP-1 transcriptional activity by increasing both the expression of AP-1 proteins and altering their phosphorylation (Whitmarsh and Davis, 1996). Therefore, studies were performed to assess the effect of 1α,25(OH)$_2$D$_3$ on the MAPK. After incubation of cultured human keratinocytes with 1α,25(OH)$_2$D$_3$ 10^{-7} M or vehicle (2-propanol) for the indicated time periods (**Fig 1**), activated ERK, JNK, and p38 were determined in whole cell lysates by western blotting using specific antibodies recognizing specifically the dually phosphorylated active forms of ERK1/2, JNK1/2, and p38. Within 2 min 1α,25(OH)$_2$D$_3$ induced a rapid activation of both ERK1 and ERK2, which returned to the basal level within 1 h (**Fig 1*a***). Equal protein loading, was determined by assessing the total amount of ERK1 and ERK2. Only the phosphorylated forms of ERK1 and ERK2 were increased after incubation with 1α,25(OH)$_2$D$_3$ (**Fig 1*a***). JNK1, but not JNK2, was also rapidly activated by 1α,25(OH)$_2$D$_3$. As seen in **Fig 1(*b*)** the phosphorylated form of JNK1 was detectable 2 and 5 min after the addition of 1α,25(OH)$_2$D$_3$, whereas the phosphorylated form of JNK2 was undetectable at any time points. Total JNK1 and JNK2 expression were similar at all time points investigated (**Fig 1*b***). In control experiments keratinocytes were exposed to ultraviolet (UV)B irradiation,

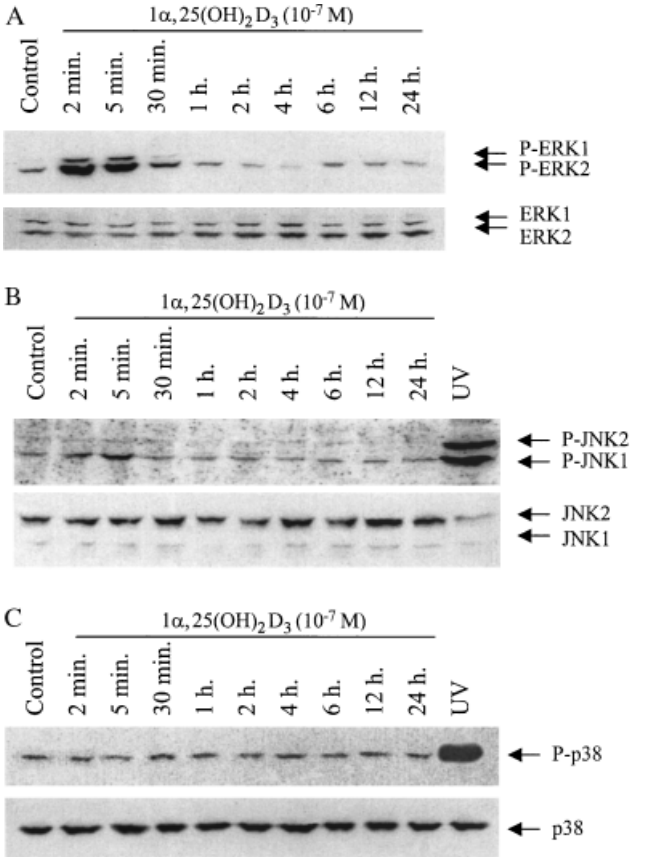

Figure 1. Time course study of the effects of 1α,25(OH)$_2$D$_3$ on the expression and activation of ERK1/2, JNK1/2, and p38 in cultured human keratinocytes. Keratinocytes were treated with 1α,25(OH)$_2$D$_3$ 10^{-7} M or vehicle for the indicated time periods. Whole cell extracts (50 μg of protein) were separated by SDS–PAGE on a 8–16% gradient gel and blotted on to a nitrocellulose membrane. After blotting, specific antibodies were used to detect both phosphorylated and total ERK1/2 (*a*), JNK1/2 (*b*), and p38 (*c*). Whole cell extracts from human keratinocytes irradiated with UVB for 1 min served as a positive control for phosphorylated JNK1/2 and p38. Representative results of at least three separate experiments are shown.

which potently increased the levels of the phosphorylated form of both JNK1 and JNK2 (**Fig 1*b***). No significant changes of the phosphorylated form of p38, as determined by densitometry, was seen after incubation with $1\alpha,25(OH)_2D_3$ (**Fig 1*c***). Again UVB irradiation was used as a positive control to induce phosphorylation of p38.

$1\alpha,25(OH)_2D_3$ induced phosphorylation of c-Jun To determine whether the $1\alpha,25(OH)_2D_3$-induced phosphorylation of the ERK and JNK1 led to phosphorylation of the c-Jun protein, western blotting was performed using a specific antibody recognizing only the phosphorylated form of c-Jun. When cultured human keratinocytes were incubated with $1\alpha,25(OH)_2D_3$ 10^{-7} M, phosphorylation of c-Jun was seen as early as 2 min after the incubation was started (**Fig 2**). The phosphorylation of c-Jun was transient and lasted for 20 min (**Fig 2**). Only the level of the phosphorylated form of c-Jun was increased, whereas there was no regulation of total c-Jun at this early time point (**Fig 2**).

$1\alpha,25(OH)_2D_3$ increases the expression of c-Fos, Fra1, and c-Jun in human keratinocytes We then determined whether the incubation with $1\alpha,25(OH)_2D_3$ resulted in any changes in the expression of the various Jun and Fos proteins. Whole cell extracts were prepared from cultured human keratinocytes incubated with $1\alpha,25(OH)_2D_3$ or vehicle (2-propanol). c-Fos (62 kDa) expression was significantly increased by $1\alpha,25(OH)_2D_3$. This increase was seen after 6 h and it reached a maximum after 24 h (2.6-fold, $p<0.01$) (**Fig 3*a***). Also, the 43 kDa Fra1 protein was detected in the keratinocytes. The amount of Fra1 increased significantly after 12 h (1.8-fold, $p<0.05$) of incubation with $1\alpha,25(OH)_2D_3$, and reached a maximum after 36 h (3.2-fold, $p<0.01$) (**Fig 3*a***). After 72 h the amount of Fra1, in both vehicle and $1\alpha,25(OH)_2D_3$ stimulated cells, was barely detectable. Fra2 (38 kDa) was also detected in human keratinocytes, but the expression was not changed by $1\alpha,25(OH)_2D_3$ (**Fig 3*a***). FosB was not detected in whole cell extracts from keratinocytes incubated with or without $1\alpha,25(OH)_2D_3$ (data not shown).

The expression of the 39 kDa c-Jun protein was also increased by $1\alpha,25(OH)_2D_3$ (**Fig 3*b***). The level of c-Jun reached a maximum after 24 h (2.4-fold, $p<0.01$) and had almost returned to the control level after 72 h (**Fig 3*b***). In contrast no significant changes in the expression of JunB (43 kDa) and JunD (39 kDa) were seen (**Fig 3*b***). This conclusion was corroborated by densitometric analysis (numerical data not shown).

To determine whether the $1\alpha,25(OH)_2D_3$-induced expression of c-Fos, Fra1, and c-Jun was MEK dependent, the keratinocytes were preincubated for 30 min with a specific MEK inhibitor, PD 98059 (50 μM) (Alessi *et al*, 1995) before the addition of $1\alpha,25(OH)_2D_3$ for 24–48 h. Western blotting demonstrated that PD 98059 completely blocked the $1\alpha,25(OH)_2D_3$-induced increase in both c-Fos, Fra1, and c-Jun expression (**Fig 3*c***).

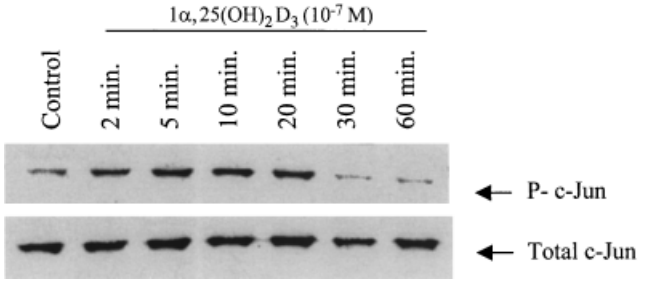

Figure 2. Time course study of the $1\alpha,25(OH)_2D_3$-induced phosphorylation of c-Jun in human keratinocytes. Whole cell extracts (50 μg of protein) from human keratinocytes treated with $1\alpha,25(OH)_2D_3$ 10^{-7} M or vehicle were isolated and separated by SDS–PAGE on a 8–16% gradient gel. After blotting the separated proteins were probed with a specific antibody recognizing only c-Jun phosphorylated on serine 63. Equal loading was confirmed by incubation with an antibody recognizing total c-Jun protein. Representative results of four separate experiments are shown.

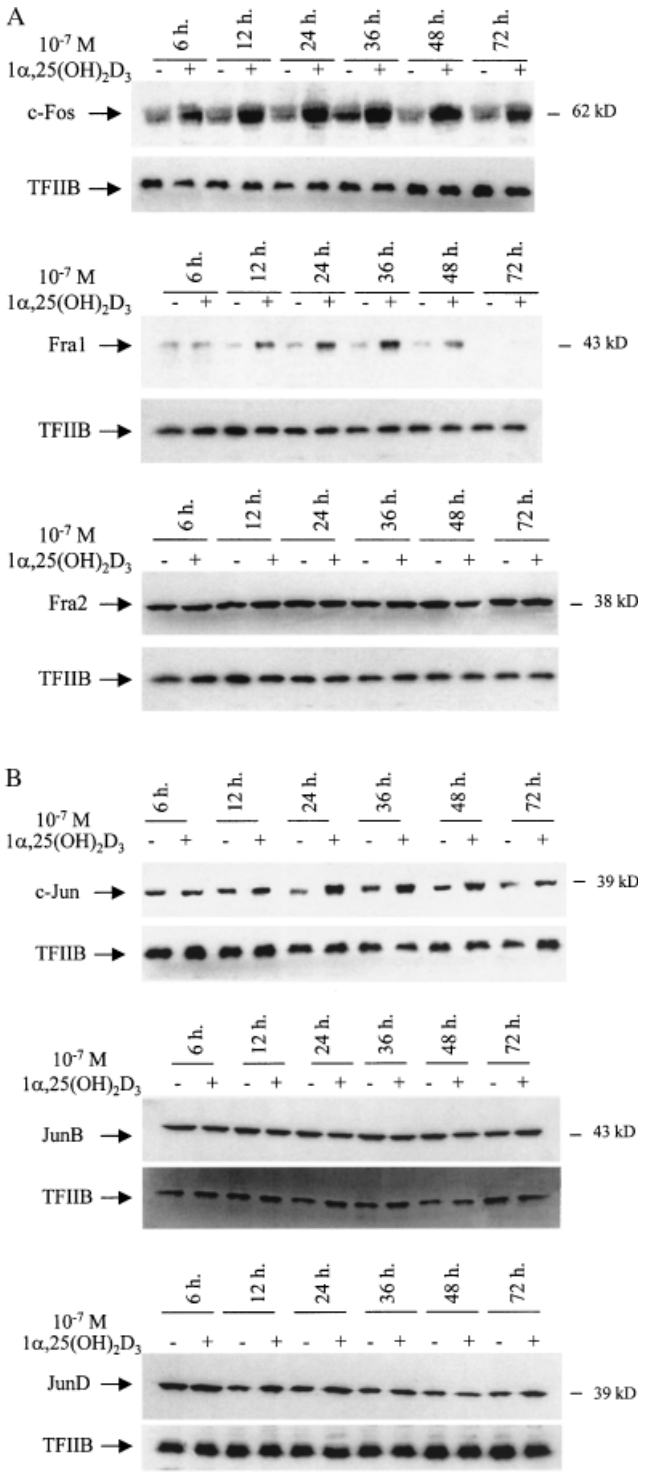

Figure 3. Western blot analysis of the various AP-1 proteins in human keratinocytes. Whole cell extracts (50 μg of protein) from human keratinocytes treated with $1\alpha,25(OH)_2D_3$ 10^{-7} M for the indicated time periods were isolated and separated by SDS–PAGE on a 8–16% gradient gel. After electroblotting, the separated proteins were probed with anti-c-Fos, anti-Fra1, or anti-Fra2 (*a*) or anti-c-Jun, anti-JunD, or anti-JunB antibodies (*b*). (*c*) Human keratinocytes were pretreated with or without PD 98059 before stimulation with $1\alpha,25(OH)_2D_3$ 10^{-7} M for 24–48 h. Equal loading was confirmed by incubation with an anti-TFIIB antibody. Representative results of four separate experiments are shown.

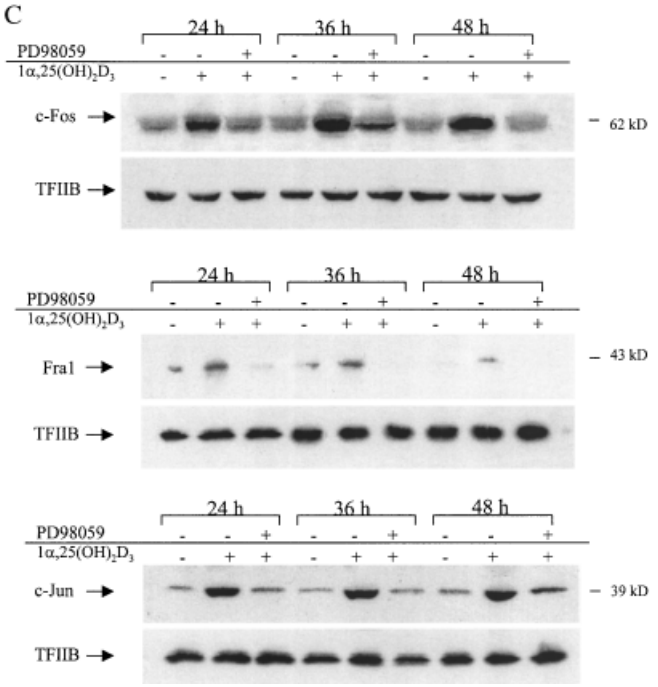

Figure 3. *Continued*

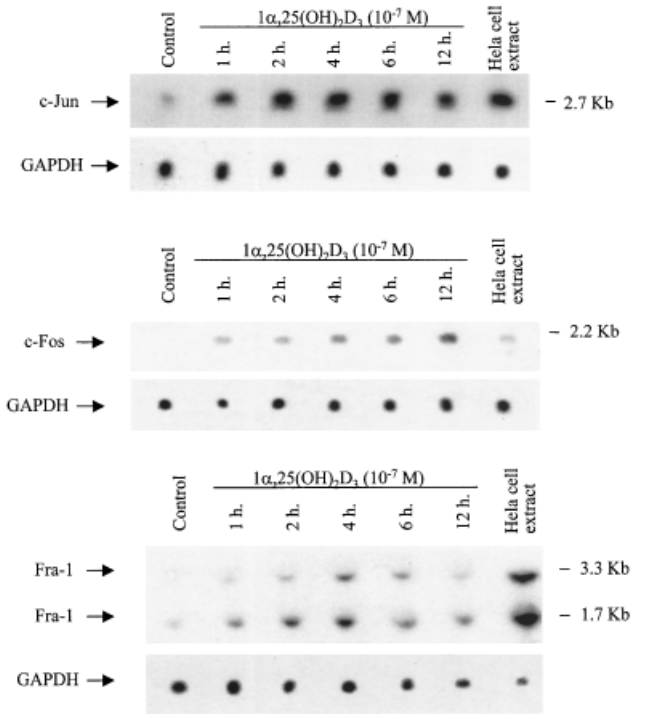

Figure 4. c-Fos, Fra1, and c-Jun mRNA expression in human keratinocytes after incubation with 1α,25(OH)$_2$D$_3$. Total RNA 10 μg from human keratinocytes incubated with 1α,25(OH)$_2$D$_3$ 10^{-7} M for the indicated time periods, were isolated and northern blotting was carried out. Total RNA was hybridized with c-Fos, Fra1, and c-Jun specific probes. Equal loading of the RNA was verified by GAPDH. The gels shown are one representative of four separate experiments.

Incubation with PD 98059 (50 μM) alone did not cause any regulation in the expression of the Jun and Fos proteins (data not shown).

In all experiments the membranes were stripped by boiling for 10 min, before incubation with a specific antibody recognizing TFIIB. This was used as a control for equal protein load.

c-Fos, Fra1, and c-Jun mRNA levels Northern blotting was performed to investigate whether the increased expression of the AP-1 subunits c-Fos, Fra1, and c-Jun was paralleled by an increased accumulation of the corresponding mRNA. Total RNA was isolated from human keratinocytes incubated with 1α,25(OH)$_2$D$_3$ 10^{-7} M or vehicle (2-propanol) for the indicated time periods (**Fig 4**). A significant increase in the expression of c-Fos (1.8-fold, $p < 0.01$), 3.3 kb Fra1 (3.8-fold, $p < 0.01$), 1.7 kb Fra1 (5.2-fold, $p < 0.01$), and c-Jun (3-fold, $p < 0.01$) mRNA were seen after the addition of 1α,25(OH)$_2$D$_3$. The levels of these mRNA were increased after 1 h and continued to increase during the following 2–12 h (**Fig 4**). In all experiments the membranes were stripped by boiling for 10 min, before hybridization with a GAPDH specific probe to demonstrate equal RNA load.

MEK-dependent ERK1/2 and JNK1 activation Western blotting analysis showed that 1α,25(OH)$_2$D$_3$-induced phosphorylation of ERK1 and ERK2 was completely blocked when keratinocytes were preincubated with PD 98059 (50 μM) for 30 min before stimulation with 1α,25(OH)$_2$D$_3$ 10^{-7} M (**Fig 5*a***). No regulation on total ERK1 and 2 expressions was seen. Also the 1α,25(OH)$_2$D$_3$-induced JNK1 activation was blocked by MEK inhibition, whereas total JNK1 and 2 expression was unchanged (**Fig 5*b***). UVB irradiation was included as a positive control. Incubation with PD 98059 (50 μM) alone did not result in any phosphorylation of ERK and JNK (data not shown). Taken together, these results indicate that 1α,25(OH)$_2$D$_3$-induced activation of ERK1/2 and JNK1 requires MEK activation.

MEK-dependent activation of AP-1 We have previously shown that the 1α,25(OH)$_2$D$_3$-induced AP-1 DNA binding activity was PKC independent (Johansen *et al*, 2000). In order to determine whether this activation was MEK dependent, AP-1 DNA binding activity was determined by electrophoretic mobility shift assays of nuclear extract from keratinocytes pretreated with 50 μM of PD 98059 for 30 min, before stimulation with 1α,25(OH)$_2$D$_3$ 10^{-7} M. As shown in **Fig 6(*a*)** and 1α,25(OH)2D3 significantly increased the AP-1 DNA binding activity after 48 h, with a maximal increase of approximately 130% compared with vehicle-treated cells. Interestingly, when keratinocytes were preincubated with PD 98059 the 1α,25(OH)$_2$D$_3$-induced increase in AP-1 DNA binding activity was completely blocked (**Fig 6*a***), demonstrating that MEK activity is an essential step in the 1α,25(OH)$_2$D$_3$-induced signal transduction pathway resulting in increased AP-1 DNA binding activity in cultured human keratinocytes. Incubating the keratinocytes with PD 98059 alone did not affect the AP-1 DNA binding activity (data not shown).

In control experiments, preincubation with an unlabeled AP-1 oligo completely abolished binding of the ^{32}P-labeled AP-1 oligo, whereas addition of a control oligo, SP-1, did not affect the binding of the ^{32}P-labeled AP-1 oligo, indicating that the AP-1 DNA binding activity was specific for the AP-1 target sequence (**Fig 6*b***). Furthermore, the addition of c-Jun or Fra1 antibodies resulted in a supershifted band (**Fig 6*b***) demonstrating the presence of these two proteins in the detected AP-1 DNA binding complex.

PI3 kinase-dependent activation of ERK1/2 and JNK1 To determine whether the PI3 kinase plays a part in 1α,25(OH)$_2$D$_3$-induced phosphorylation of ERK1/2 and JNK1, keratinocytes were preincubated with the PI3 kinase inhibitor Wortmannin (Powis *et al*, 1994) for 30 min before the addition of 1α,25(OH)$_2$D$_3$. Wortmannin 100 nM completely abolished the 1α,25(OH)$_2$D$_3$-induced phosphorylation of ERK1/2 (**Fig 7*a***) and JNK1 (**Fig 7*b***). No regulation on total ERK1/2 and JNK1/2

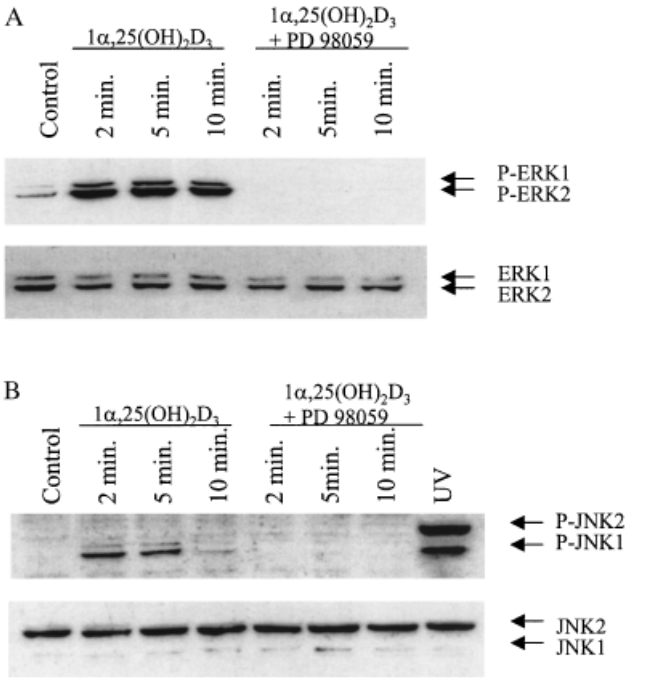

Figure 5. MEK-dependent activation of ERK1/2 and JNK1 in human keratinocytes. Keratinocytes were preincubated with or without PD 98059 for 30 min before incubated with $1\alpha,25(OH)_2D_3$ 10^{-7} M for the indicated time periods. Whole cell extracts (50 μg of protein) were separated on a 8–16% gradient gel before being electroblotted on to a nitrocellulose membrane. Active ERK1/2 and JNK1/2 were detected by anti-phospho-ERK1/2 antibodies (*a*) and anti-phospho-JNK1/2 antibodies (*b*), respectively. Whole cell extracts from human keratinocytes irradiated with UVB for 1 min served as a positive control for phosphorylated JNK1/2. Representative results of four separate experiments are shown.

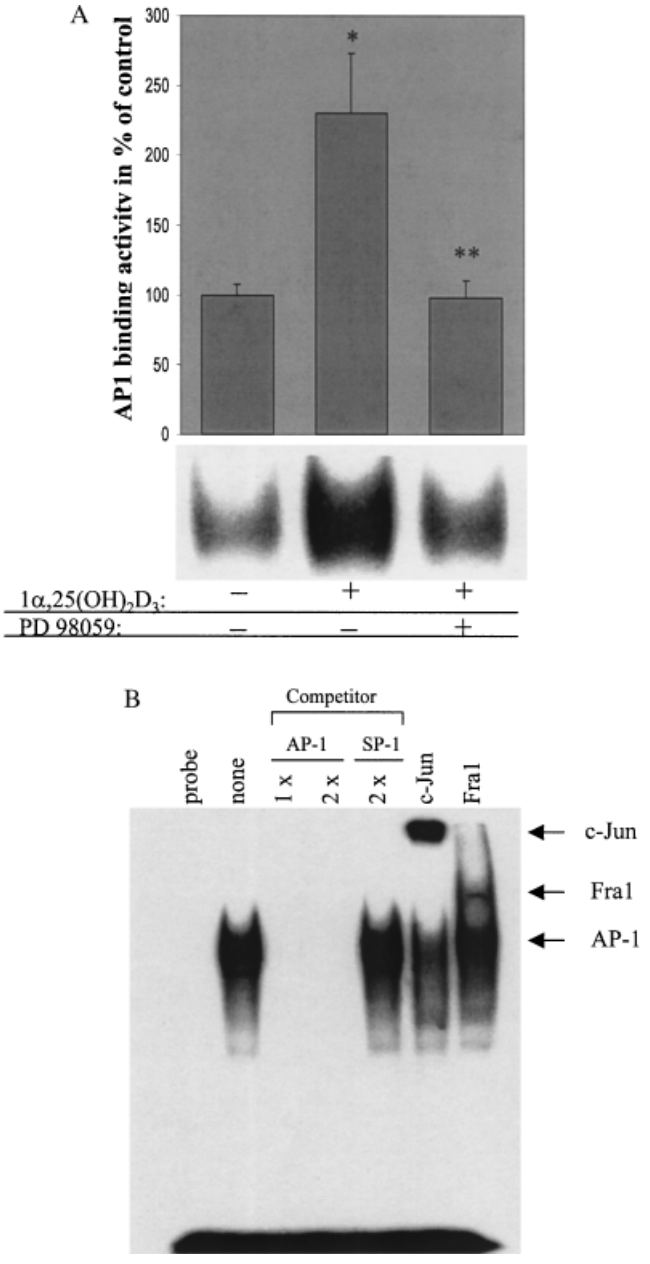

Figure 6. $1\alpha,25(OH)_2D_3$ induced AP-1 DNA binding activity is sensitive to MEK inhibition. Human keratinocytes were incubated with $1\alpha,25(OH)_2D_3$ 10^{-7} M for 48 h before AP-1 DNA binding activity was assayed by electrophoretic mobility shift assay. In separate experiments keratinocytes were pretreated with PD 98059 (50 μM) for 30 min and then incubated with $1\alpha,25(OH)_2D_3$ for 48 h before AP-1 DNA binding activity was determined. $^{*}p < 0.05$ compared with keratinocytes cultured without $1\alpha,25(OH)_2D_3$. $^{**}p < 0.05$ compared with keratinocytes cultured with $1\alpha,25(OH)_2D_3$ and without PD 98059. Each bar represents the mean ± SD from five separate experiments (*a*). Keratinocyte nuclear extracts were incubated with the indicated amounts of the indicated nonlabeled competitor oligonucleotides or with anti-c-Jun or anti-Fra1 antibodies for 10 min prior to the addition of a ^{32}P-labeled AP-1 binding sequence (*b*).

expression was seen. Similar results were obtained with another PI3 kinase inhibitor, LY294002 (10 μM) (Vlahos *et al*, 1994) (data not shown). Wortmannin (100 nM) or LY294002 (10 μM) added to the keratinocytes alone did not result in any phosphorylation of ERK and JNK (data not shown).

$1\alpha,25(OH)_2D_3$ increases AP-1-mediated transactivation in human keratinocytes by a Ras-dependent mechanism To assess the potential ability of $1\alpha,25(OH)_2D_3$ to induce AP-1-mediated gene transcription, human keratinocytes were transfected with an AP-1 responsive reporter construct, containing three AP-1 binding sites upstream of a luciferase reporter gene, p(AP-1)$_3$-luc$^+$. After transfection the keratinocytes were incubated with $1\alpha,25(OH)_2D_3$ or vehicle (2-propanol). $1\alpha,25(OH)_2D_3$, increased the luciferase activity significantly ($p < 0.05$) at 36 h, 48 h, and 60 h, where the $1\alpha,25(OH)_2D_3$-treated cells exhibited a 65% increase in the normalized luciferase activity compared with cells receiving vehicle (**Fig 8**). To evaluate the role of Ras in AP-1 activation by $1\alpha,25(OH)_2D_3$, keratinocytes were cotransfected with p(AP-1)$_3$-luc$^+$ and a dominant negative Ras expression plasmid (Ras-N17). First the dose–response of transfected Ras-N17 was established and used to define a window in which no significant effects on expression of the normalizing vector or cell morphology were observed. Surprisingly, we found that the amount of cotransfected Ras-N17 should be kept below 1 ng per well of a 12-well plate. Using this window we next showed that cotransfected Ras-N17 inhibited AP-1-mediated transactivation in a concentration-dependent manner (**Fig 8**). This indicates that the $1\alpha,25(OH)_2D_3$-induced AP-1 transactivating activity is dependent on Ras activation.

$1\alpha,25(OH)_2D_3$-induced ERK1/2 and JNK1 activation is inhibited by preincubation with an antibody against annexin II Annexin II has been suggested to act as a surface membrane receptor for $1\alpha,25(OH)_2D_3$ (Baran *et al*, 2000b). To determine whether the $1\alpha,25(OH)_2D_3$-induced ERK and JNK activation is mediated through annexin II, human keratinocytes were pretreated with or without a rabbit polyclonal antibody to

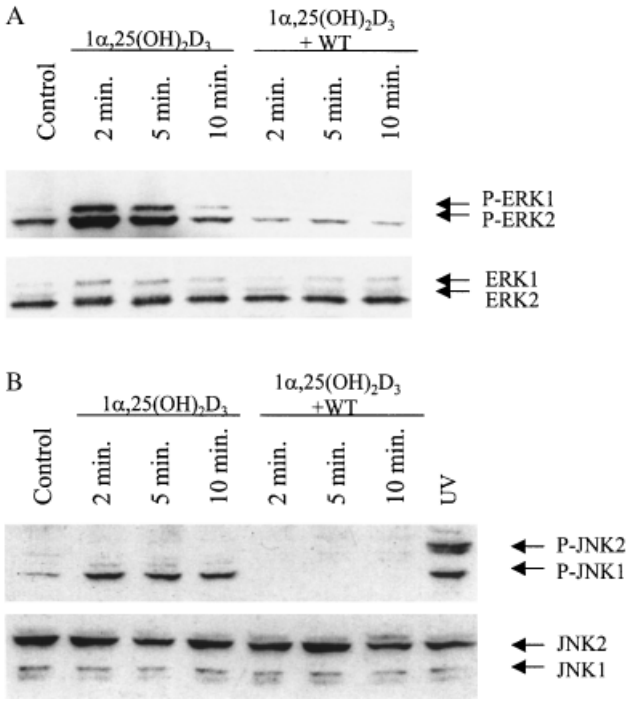

Figure 7. PI3 kinase dependent activation of ERK1, ERK2, and JNK1. Human keratinocytes were preincubated with 10 nM Wortmannin (WT) for 30 min before incubation with 1α,25(OH)$_2$D$_3$ 10^{-7} M for the indicated time periods. Whole cell extracts (50 μg of protein) were isolated and separated by SDS–PAGE on a 8–16% gradient gel. Activated ERK1/2 and JNK1/2 were detected by anti-phospho-ERK1/2 antibodies (*a*) and anti-phospho-JNK1/2 antibodies (*b*), respectively. Whole cell extracts from keratinocytes treated with UVB irradiation served as a positive control for phosphorylated JNK1/2. Data shown are one representative of four separate experiments.

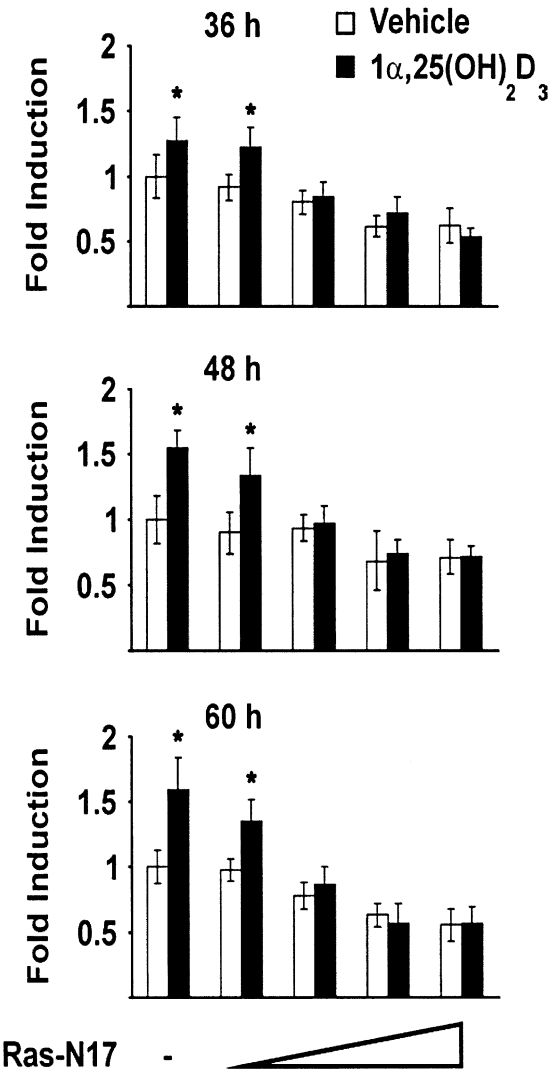

Figure 8. Dominant negative Ras represses 1α,25(OH)$_2$D$_3$-induced AP-1 transactivating activity in normal human keratinocytes. Normal human keratinocytes were transiently transfected with 75 ng p(AP-1)$_3$-luc$^+$ reporter construct and increasing amounts of the pcDNA3-Ras-N17 encoding the dominant negative Ras-N17 mutant protein (0.008 ng, 0.04 ng, 0.2 ng, or 1 ng). Cells were subsequently treated with medium containing either vehicle (2-propanol) or 10^{-7} M 1α,25(OH)$_2$D$_3$ for 36 h, 48 h, or 60 h prior to harvest. In all transfections empty expression vector was added to ensure equal promoter load, and CMV-β-galactosidase was used for normalization. Reporter activity was normalized to β-galactosidase values, and fold induction is presented as the mean ± SD. *$p < 0.05$ compared with p(AP-1)$_3$-luc$^+$-transfected cells treated with vehicle.

annexin II for 30 min before incubation with 1α,25(OH)$_2$D$_3$ 10^{-7} M for 2–10 min. In control experiments, human keratinocytes were pretreated with fractionated rabbit immunoglobulins, prepared from sera of nonimmunized rabbits, for 30 min before incubation with 1α,25(OH)$_2$D$_3$ 10^{-7} M for 2–10 min. Whole cell extracts (50 μg of protein) were isolated and western blotting was performed. Preincubation with an antibody against annexin II completely abolished the 1α,25(OH)$_2$D$_3$-induced phosphorylation of ERK1/2 and JNK1 (**Fig 9**), whereas no changes were seen in control experiments with rabbit immunoglobulins from nonimmunized rabbits. Furthermore, treatment of the cells with the annexin II antibody alone did not affect the phosphorylation of ERK and JNK (data not shown). These results suggest that annexin II may serve as a surface membrane receptor for 1α,25(OH)$_2$D$_3$ causing activation of the signal transduction pathway leading to phosphorylation of ERK1/2 and JNK1.

DISCUSSION

In this study we have shown that 1α,25(OH)$_2$D$_3$ induces activation of the PI3 kinase/Ras/MEK/MAPK (ERK1/2 and JNK1) signaling pathway in cultured human keratinocytes resulting in increased expression of c-Fos, Fra1, and c-Jun and subsequently increased AP-1 DNA binding and AP-1-mediated transactivation. We furthermore suggest that this signal transduction cascade is activated by 1α,25(OH)$_2$D$_3$ through binding to the membrane receptor annexin II.

1α,25(OH)$_2$D$_3$ has previously been shown to induce keratinocyte differentiation (Hosomi *et al*, 1983; Smith *et al*, 1986), and we (Johansen *et al*, 2000) and others (Rutberg *et al*, 1996; Bikle *et al*, 2001) have shown that differentiation involves activation of AP-1. Increased AP-1 DNA binding activity was seen after 24–48 h of incubation with 1α,25(OH)$_2$D$_3$ (Johansen *et al*, 2000), demonstrating that the effect of 1α,25(OH)$_2$D$_3$ was not due to an immediate effect on AP-1. In this study we show that 1α,25(OH)$_2$D$_3$ increases AP-1 DNA binding activity by inducing synthesis of specific Jun and Fos proteins that then form the AP-1 dimers. In accordance with the delayed effect on AP-1 DNA binding activity, increased expression of specific Jun and Fos proteins was seen from 6 to 72 h after 1α,25(OH)$_2$D$_3$ was added.

In accordance with the results obtained in this study ERK1/2 activation has previously been demonstrated in human keratinocytes stimulated with 1α,25(OH)$_2$D$_3$ (Gniadecki, 1996). In other cell types, activation of ERK has been shown to increase the expression of members of the Fos protein family (Whitmarsh and Davis, 1996). In this study we showed that activation of

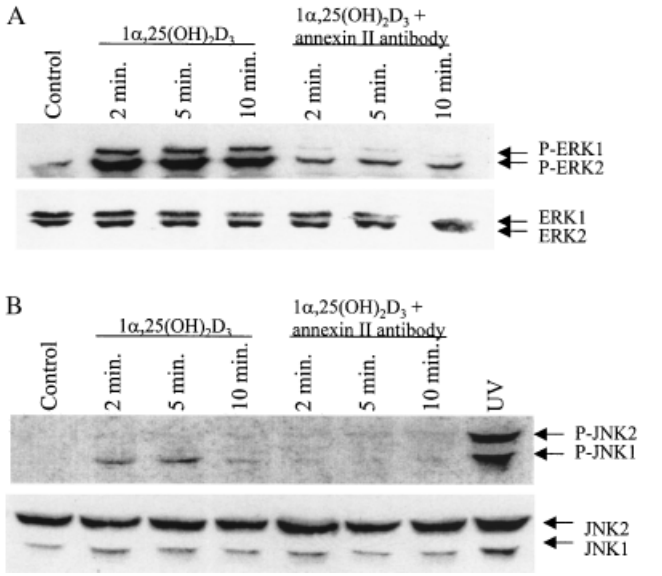

Figure 9. Effect of anti-annexin II antibody on the 1α,25(OH)$_2$D$_3$-induced ERK1/2 and JNK1 phosphorylation. Human keratinocytes were incubated either with 1α,25(OH)$_2$D$_3$ 10^{-7} M or pretreated with an annexin II polyclonal antibody (0.2 μg per ml) for 30 min before incubated with 1α,25(OH)$_2$D$_3$ 10^{-7} M for the indicated time periods. Whole cell extracts (50 μg of protein) were isolated and separated on a 8–16% SDS gel. After blotting specific antibodies were used to detect both phosphorylated and total ERK1/2 (*a*) and JNK1/2 (*b*). Whole cell extracts from keratinocytes treated with UV irradiation served as a positive control for phosphorylated JNK1/2. Results shown are one representative of four separate experiments.

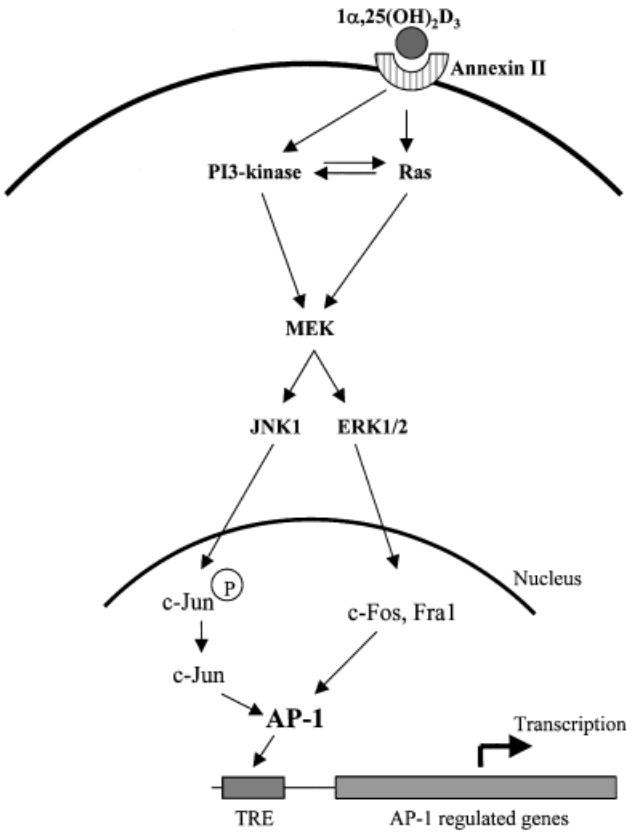

Figure 10. Schematic scenario of AP-1 activation induced by 1α,25(OH)$_2$D$_3$ in human keratinocytes.

ERK1/2 and JNK1 by 1α,25(OH)$_2$D$_3$ led to an increased c-Fos, Fra1, and c-Jun expression at both the mRNA and protein levels. Expression of other Jun and Fos family members was not altered by 1α,25(OH)$_2$D$_3$. The Fos and Jun members that have been shown to be expressed (c-Fos, Fra1, Fra2, c-Jun, JunB, and JunD) all participate in AP-1 formation as previously shown by supershift (Johansen *et al*, 2000). Furthermore, this study showed no changes in the composition of untreated *vs* 1α,25(OH)$_2$D$_3$-treated keratinocytes. Chen *et al* (1999) have shown that 1α,25(OH)$_2$D$_3$ increases the protein level of c-Jun in Caco-2 cells, whereas they did not find any regulation of c-Fos and Fra1 expression. The different cell types investigated may explain these differences.

Recent results have shown that in general c-Jun and JunD act as positive regulators, whereas JunB acts as a suppressor of the expression of epidermal-specific genes (DiSepio *et al*, 1995), indicating that various AP-1 homodimers or heterodimers carry out cell-specific regulation of specific genes. It is therefore noteworthy that incubation of cultured human keratinocytes with 1α,25(OH)$_2$D$_3$ resulted in an increased expression of only c-Jun, c-Fos, and Fra1 demonstrating that 1α,25(OH)$_2$D$_3$ activates expression of specific AP-1 subtypes, which in turn may result in selective activation of AP-1 responsive genes in a manner dependent on the composition of the AP-1 dimers and promoter context.

The MAPK signaling pathways, including both ERK and JNK, influence AP-1 transcriptional activity by increasing specific Jun and Fos proteins and by altering the phosphorylation of AP-1 subunits (Whitmarsh and Davis, 1996). In this study we show that addition of 1α,25(OH)$_2$D$_3$ did not lead to activation of p38 MAPK indicating that p38 MAPK is not involved in 1α,25(OH)$_2$D$_3$-induced keratinocyte differentiation. This is similar to 1α,25(OH)$_2$D$_3$-induced monocytic differentiation of HL-60 cells, which was enhanced by inhibition of the p38 MAPK due to an upregulation of JNK and AP-1 (Wang and Studzinski, 2001).

We have previously demonstrated that 1α,25(OH)$_2$D$_3$-induced differentiation of human keratinocytes is mediated via a PKC-independent upregulation of the AP-1 DNA binding activity, whereas calcium-induced keratinocyte differentiation is PKC dependent (Johansen *et al*, 2000). Moreover, it was recently shown that the calcium-induced differentiation of human keratinocytes is mediated by a Ras-independent activation of the Raf/MEK/ERK signaling pathway (Schmidt *et al*, 2000), whereas this study showed that the 1α,25(OH)$_2$D$_3$-induced increase in AP-1 transactivating activity in human keratinocytes was Ras dependent. These data strongly indicate that the signal transduction pathways leading to AP-1 activation and subsequently differentiation of human keratinocytes is different for calcium and 1α,25(OH)$_2$D$_3$.

PI3 kinase is another kinase that has been shown to play a part in the activation of the MAPK signaling pathway (Baier *et al*, 1999; Pandey *et al*, 1999). Retinoic acid induces the expression of PI3 kinase γ resulting in increased ERK2 activation in the myelomonocytic cell-line U937 (Baier *et al*, 1999). Furthermore, it has been shown that PI3 kinase activity is essential for the all-*trans*-retinoid acid-induced differentiation of HL-60 cells (Bertagnolo *et al*, 1999), and that the PI3 kinase is involved in the 1α,25(OH)$_2$D$_3$-induced differentiation of both myeloid cells and HL-60 cells (Marcinkowska *et al*, 1998; Hmama *et al*, 1999). Similarly, we found that the specific PI3 kinase inhibitors, Wortmannin and LY294002 (Powis *et al*, 1994; Vlahos *et al*, 1994), both completely blocked the 1α,25(OH)$_2$D$_3$-induced phosphorylation of ERK1/2 and JNK1 in human keratinocytes, indicating that ERK1/2 and JNK1 are activated by 1α,25(OH)$_2$D$_3$ through a PI3 kinase-dependent mechanism. Several downstream effectors of activated PI3 kinase have been identified, including the Akt/protein kinase B (Vanhaesebroeck and Alessi, 2000); however, surprisingly we found no activation of protein kinase B in cultured human keratinocytes incubated with 1α,25(OH)$_2$D$_3$ (data not shown).

Annexin II has previously been demonstrated immunohistochemically in the epidermal layer in neonatal foreskin (Ma and Ozers, 1996). Later it has been suggested that annexin II may serve as a surface membrane receptor for the rapid actions of 1α,25(OH)$_2$D$_3$ (Baran *et al*, 2000b). In this study preincubation of cultured keratinocytes with a polyclonal antibody directed against annexin II reduced the 1α,25(OH)2D3-induced phosphorylation of ERK1/2 and JNK1. Our data, therefore, support the idea that the 1α,25(OH)$_2$D$_3$-induced activation of the MAPK signal transduction pathway is mediated by the annexin II membrane receptor. Annexin II may be the membrane receptor activating the signal transduction pathway by which 1α,25(OH)$_2$D$_3$ induces keratinocyte differentiation. By immunohistochemical staining Ma and Ozers (1996) determined that annexin II is localized as a band at the cell periphery in all nucleated epidermal cells, suggesting that annexin II is present close to the cell membrane in undifferentiated keratinocytes.

Conflicting data exist on whether PI3 kinase is upstream of Ras or vice versa, depending on the cell type and the circumstances studied (Pandey *et al*, 1999; Wennstrom and Downward, 1999; Dajee *et al*, 2002). This hierarchy has not been investigated in this study. Based on our results, however, we propose a model of the signal transduction pathway induced by 1α,25(OH)$_2$D$_3$ (**Fig 10**). In this model, 1α,25(OH)$_2$D$_3$ binds to the membrane receptor annexin II, which leads to a PI3 kinase and/or Ras-dependent activation of the MEK/MAPK pathway. ERK1/2 and JNK1 are rapidly activated by phosphorylation leading to increased synthesis of the c-Fos, Fra1, and c-Jun proteins in conjunction with enhanced AP-1 DNA binding activity and increased transcription of specific genes involved in cell differentiation.

This work was supported by the Institute for Experimental Clinical Research, University of Aarhus, the Aage Bang Foundation, the Novo Nordisk Foundation, the Leo Research Foundation, the Danish Biotechnology Program, the Medical Research Council, Denmark, and the Danish Psoriasis Foundation.

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