Activation of RAF-1 through Ras and Protein Kinase Ca Mediates 1α,25(OH)₂-Vitamin D₃ Regulation of the Mitogen-activated Protein Kinase Pathway in Muscle Cells*

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We have previously shown that stimulation of proliferation of avian embryonic muscle cells (myoblasts) by 1α,25(OH)₂-vitamin D₃ (1α,25(OH)₂-D₃) is mediated by activation of the mitogen-activated protein kinase (MAPK; ERK1/2). To understand how 1α,25(OH)₂-D₃ up-regulates the MAPK cascade, we have investigated whether the hormone acts upstream through stimulation of Raf-1 and the signaling mechanism by which this effect might take place. Treatment of chick myoblasts with 1α,25(OH)₂-D₃ (1 nM) caused a fast increase of Raf-1 serine phosphorylation (1- and 3-fold over basal at 1 and 2 min, respectively), indicating activation of Raf-1 by the hormone. These effects were abolished by preincubation of cells with a specific Ras inhibitor peptide that involves Ras in 1α,25(OH)₂-D₃ stimulation of Raf-1. 1α,25(OH)₂-D₃ rapidly induced tyrosine de-phosphorylation of Ras-GTPase-activating protein, suggesting that inhibition of Ras-GTP hydrolysis is part of the mechanism by which 1α,25(OH)₂-D₃ activates Ras in myoblasts. The protein kinase C (PKC) inhibitors calphostin C, bisindolylmaleimide I, and Ro 318220 blocked 1α,25(OH)₂-D₃-induced Raf-1 serine phosphorylation, revealing that hormone stimulation of muscle cells with an antisense oligodeoxynucleotide against PKCe mRNA suppressed serine phosphorylation by 1α,25(OH)₂-D₃. The increase in MAPK activity and tyrosine phosphorylation caused by 1α,25(OH)₂-D₃ could be abolished by Ras inhibitor peptide, compound PD 98059, which prevents the activation of MEK by Raf-1, or incubation of cell lysates before 1α,25(OH)₂-D₃ exposure with an anti-Raf-1 antibody. In conclusion, these results demonstrate for the first time in a 1α,25(OH)₂-D₃ target cell that activation of Raf-1 via Ras and PKCα-dependent serine phosphorylation plays a central role in hormone stimulation of the MAPK-signaling pathway leading to muscle cell proliferation.

Raf-1 was discovered as the first member of a cytoplasmatic family of serine/threonine kinases and plays a crucial role in the activation of the classical cytoplasmatic-signaling cascade that is involved in the regulation of cellular proliferation, differentiation, and apoptosis (1). Activation of Raf-1 downstream of protein-tyrosine kinase receptors is mediated by the Ras GTP-binding proteins, which are required for stimulation of Raf-1 kinase activity (2, 3). Raf-1 then phosphorylates and activates the mitogen-activated protein kinase kinase, also known as MEK (4), initiating a protein kinase cascade that follows with the phosphorylation and activation of the extra-cellular signal-regulated mitogen-activated protein (MAP) kinase isozymes ERK1 and ERK2 (5). Upon activation, MAP kinase (MAPK) is translocated from the cytoplasm into the nucleus (6), where it phosphorylates transcription factors (7) and culminates in proliferation or differentiation of a variety of cell types.

With an estimated molecular mass of 74 kDa, Raf-1 possesses three conserved regions, CR1, CR2, and CR3, that are embedded in variable regions. The CR1 and CR2 domains are part of the regulatory N-terminal half of the Raf-1 protein, whereas CR3 forms the C-terminal kinase domain (1). The molecular mechanism of Raf-1 activation, however, is still unclear. Although Raf-1 binds directly to the effector domain of activated GTP-bound Ras proteins, this interaction does not appear to stimulate Raf-1 kinase activity (8). Rather, it seems that the role of the Ras-Raf interaction is to recruit Raf-1 to the plasma membrane, where it can be activated by membrane lipids or other protein kinases, the nature of which remains to be determined (9, 10). Phosphorylation of Raf on both tyrosine and serine/threonine residues is required for enzymatic activity (11). It has been shown that the Src protein-tyrosine kinase can activate Raf by phosphorylation on Tyr-340 and Tyr-341 (12, 13). However, mutant Raf proteins in which Tyr-340 and Tyr-341 have been changed to Asp can still be activated after membrane association, indicating that an additional mecha-nism(s) of Raf activation exists (13). Among the mechanisms involved, there is evidence for the operation of both protein kinase C (PKC)-dependent and PKC-independent pathways of Raf activation in response to agonists (14).

The 13 members of the PKC family can be grouped into three major classes of Ca²⁺-dependent classical PKCs, Ca²⁺-independent, novel PKCs, and Ca²⁺- and lipid-independent, atypical PKCs. There is a fourth PKC subgroup consisting of PKCµ (15). Previous reports showed that classical and novel PKCs activate the MAP kinase pathway at the level of Raf-1, whereas atypical PKCs activate MEK by an independent mechanism (16, 17). One of the classical PKCs, PKCe, can directly activate
The steroid hormone 1α,25(OH)2D3 (1α,25(OH)2D3) triggers responses in muscle cells both through a nuclear receptor-mediated mechanism that promotes gene transcription (19) and a fast non-genomic mode of action independent of new RNA and protein synthesis (20, 21). In previous work we have demonstrated that the steroid hormone rapidly stimulates in skeletal muscle cells the phosphorylation and activity of the MAP kinase isofoms ERK1 and ERK2 and have implicated the MAPK cascade in hormone control of myoblast proliferation (22). Moreover, initial investigations on the mechanisms underlying 1α,25(OH)2D3 stimulation of the muscle cell (myoblast) MAPK pathway revealed that PKC and Ca2+ are two upstream activators mediating the hormone effect (23). In addition, it has been shown that 1α,25(OH)2D3 enhancement of myoblast proliferation correlates to increased PKCa expression, whereas decreased PKCa levels are observed during the subsequent activation of muscle cell differentiation by the hormone (24). Furthermore, inhibition of PKCa expression by using antisense oligonucleotide technology resulted in a significant decrease of culture cell density and DNA synthesis, clearly showing that this isoyme is involved in signaling cascades that promote muscle cell proliferation (25).

The upstream-signaling pathway that leads to activation of the Ras/Raf-1/MAPK (ERK1/2) cascade by 1α,25(OH)2D3 remains incompletely understood, and direct evidence on the participation of any of the PKC isoforms is lacking. In view of the information discussed above, we have investigated the role of Ras as well as PKCa in Raf-1 activation.

MATERIALS AND METHODS

Chemicals—1α,25(OH)2D3 was kindly provided by Hoffmann-La Roche. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, specific Ras inhibitory peptide (VPPPVPVPRR), and protein A-Sepharose were from Sigma. Lipofectin was from Invitrogen. Sense and antisense oligodeoxynucleotides were synthesized by the DNAGeny (Malvern, PA). Rabbit polyclonal anti-phosphoserine antibody was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-Raf-1 monoclonal antibody and anti-phospho-active MAP kinase antibody (reactive against p24 and p44 isoforms) were from Promega (Madison, WI). Anti-Ras-GTPase-activating protein (GAP) antibody and donkey anti-goat IgG antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibody goat anti-rabbit horseradish peroxidase-conjugated IgG and the Super Signal CL-HRP substrate system for enhanced chemiluminescence (ECL) were obtained from PerkinElmer Life Sciences. The compounds PD 98059, Ro 318220, PD 98059 (10 µM, 10 min) or Ras inhibitor peptide (25 µM, 10 min) and then exposed for 1 min with 1 µg 1α,25(OH)2D3 or vehicle (< 0.01% ethanol) at 37 °C. Lysates were prepared following immunoprecipitation of MAP kinase (p42 and p44) as described above. After 3 washes with immunoprecipitation buffer and 2 washes with kinase buffer (10 mM Tris-HCl, pH 7.2, 5 µg MgCl2, 1 mM MgCl2, 1 mM dithiothreitol, 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 20 µg/ml leupeptin, and 20 µg/ml aprotinin), the immune complexes were incubated at 37 °C for 10 min in kinase buffer (50 µl/sample) containing myelin basic protein as an exogenous substrate for MAP kinase (20 µg/ml assay), 25 µg ATP, and [γ-32P]ATP (3,000 Ci/mmol) was from PerkinElmer Life Sciences. The compounds PD 98059, Ro 318220, calphostin C, and bisindolylmaleimide I were from Calbiochem. All other reagents were of analytical grade.

Cell Culture—Chick skeletal muscle cells were obtained from 13-day-old chick embryo breast muscles by stirring in Earle’s balanced salt solution containing 0.06% trypsin for 30 min essentially as previously described (26). The freed cells were collected by centrifugation, and the pellet was resuspended in DMEM supplemented with 10% fetal bovine serum and antibiotic-antimycotic solution. The suspension was dispensed by pipette, filtered through nylon mesh, and “preplated” on gelatin-coated Petri dishes to remove contaminating fibroblasts. The unadsorbed cells were seeded at an appropriate density (120,000 cells/cm2) in Petri dishes (100-mm diameter) and cultured at 37 °C under a humidified atmosphere (air 95%, 5% CO2). Under these conditions, myoblasts divide within the first 48 h and at day 4 become differentiated into myotubes expressing both biochemical and morphological characteristics of adult skeletal muscle fibers (27). Cells cultured for 2 days (proliferative stage) were used for treatments.

Immunoprecipitation—After 1α,25(OH)2D3 or vehicle (ethanol, < 0.01%) treatment, muscle cells were lysed (15 min at 4 °C) in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 25 mM NaF, 0.2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 2 µg/ml leupeptin, 0.25% N,N-diethylmaleimide, 1% Nonident P-40 and homogenized by sonication for 15 s. Insoluble material was pelleted in a microcentrifuge at 12,000 × g for 15 min. The protein content of the clear lysates was determined according to Lowry et al. (28). Aliquots (500–700 µg of protein) were incubated overnight at 4 °C with anti-Raf-1 or anti-Ras-GAP antibodies followed by precipitation of the complexes with protein A conjugated with Sepharose 4B. The immune complexes were washed 4 times with cold immunoprecipitation buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM sodium orthovanadate, 1% Triton X-100 and 1% Nonidet P-40) and a final wash in cold phosphate-buffered saline.

Cell Transfection—Transfection with oligodeoxynucleotides (ODNs) using Lipofectin was performed according to the manufacturer’s instructions. ODNs were incubated with Lipofectin in DMEM for 15 min at room temperature. Plates of subconfluent cells were washed to remove serum before the addition of ODN-Lipofectin mixtures, and incubation was performed for 4 h at 37 °C. The ODN solution was removed, DMEM was added, and the plates were placed into a metabolic incubator for an additional period of 20 h. Control treatments included DMEM and time-response studies for Lipofectin and ODNs were previously performed to establish optimum conditions for the effective blockade of PKCa expression (25). The following ODN sequences with phosphorothioate linkages throughout the entire ODN molecule were used: antisense-PKCα (AS), 5′-CATGGTCT- CCCCCAACACCC-3′, Y = T or C (antisense sequence against 20 GGGGGCAACGACCC-3′, R = A or G (25). Each antisense oligonucleotide was used at a final concentration of 5 µM. These antisense sequences showed no homology to any DNA in the GenBank except PKCa (human, rat, mouse, and rabbit species). Cell death in cultures under control and treatment (ODNs) conditions was measured by trypan blue staining.

Statistical Analysis—Statistical significance of the data was evaluated using Student’s t test (30), and probability values below 0.05 (p < 0.05) were considered significant. Results are expressed as the means ± S.D. from the indicated set of experiments.
part of the 11. To evaluate whether the serine-threonine kinase Raf-1 is activated on Ser 338 and Tyr-341 is a critical step in this process, we explored the effect of 1,25(OH)2D3 on Raf-1 serine phosphorylation. Chick skeletal muscle cells were incubated in the presence of 1,25(OH)2D3 (1 nM) or vehicle (ethanol <0.01%) for the indicated times. Immunoprecipitation of Raf-1 and immunoblotting with an anti-phosphoserine (anti-P-serine) antibody were carried out in cell lysates as detailed under "Materials and Methods." A, representative immunoblot. Quantification by scanning volumetric densitometry of blots from three independent experiments performed in duplicate; averages ± S.D. are given. *, p < 0.01, with respect to the control.

RESULTS AND DISCUSSION

To understand how the steroid hormone 1α,25(OH)2D3 controls the MAP kinase cascade in skeletal muscle cells, it is essential to identify the molecules that participate in the cellular sequence of events involved in the signaling pathway of this steroid hormone. As in other cell types, Raf-1 and MEK belong to the MAP kinase cascade that leads to muscle cell proliferation (31, 32). As a major step in this direction, we report here for the first time that 1α,25(OH)2D3 stimulation of Raf-1 involves at least in part rapid activation of Raf-1 and provides information on the mechanism of action by which this hormone-regulated event takes place.

The complex process of Raf activation is still incompletely understood. Existing data suggest that activation of Raf-1 engages multiple factors and steps (9, 10), and phosphorylation of Raf-1 on Ser 338 and Tyr-341 is a critical step in this process (11). To evaluate whether the serine-threonine kinase Raf-1 is part of the 1α,25(OH)2D3-signaling mechanism in chick muscle cells, we first investigated the effect of the steroid hormone on Raf-1 serine phosphorylation. To that end, muscle cells were exposed to 1 nM 1α,25(OH)2D3 (0.5–5 min), and cell lysates were immunoprecipitated with a highly specific anti-Raf-1 monoclonal antibody followed by Western blotting with anti-phosphoserine antibody. As shown in Fig. 1, 1α,25(OH)2D3 caused a time-dependent increase in Raf-1 phosphorylation in muscle cells. The stimulation of Raf-1 serine phosphorylation could be detected already at 30 s, increased 1-fold over basal at 60 s, and reached a maximum after 2 min of hormone exposure (3-fold).

It has been reported that the small G protein Ras is the direct upstream activator of Raf, which, in its GTP-bound activated form binds to the Ras binding domain (Raf-RBD) and recruits the inactive cytoplasmic Raf to the plasma membrane for activation (33). This binding induces a conformational change of Raf-1 that yields an opened structure suitable for phosphorylation by membrane-associated kinases. To study Ras-dependent changes in Raf activation induced by 1α,25(OH)2D3, muscle cells were preincubated for 2 h with a specific Ras inhibitor peptide for 2 min. After cell lysis, comparable aliquots were immunoprecipitated with an anti-Raf-1 antibody followed by Western blotting with anti-phosphoserine antibody as described under "Materials and Methods." A, representative immunoblot. Quantification by scanning volumetric densitometry of blots from three independent experiments performed in duplicate; averages ± S.D. are given. *, p < 0.01, with respect to basal; **, p < 0.05 with respect to 1α,25(OH)2D3 stimulation.
1α,25(OH)2D3 showed a rapid and transient tyrosine dephosphorylation of Ras-GAP, which was maximal at 0.5 min (80%) and returned to basal level upon 10 min of hormone exposure. This result suggests that inhibition of Ras-GTP hydrolysis is part of the mechanism by which 1α,25(OH)2D3 activates Ras in skeletal muscle cells.

PKC, discovered as a serine/threonine kinase (39), mediates intracellular responses to a variety of agonists including 1α,25(OH)2D3 (40). The direct phosphorylation of Raf-1 by PKC isoforms has been suggested as an activation mechanism of PKC on the Raf-1/ERK1/2-signaling pathway (16, 18). In previous investigations we have demonstrated that stimulation of the extracellular signal-regulated mitogen-activated protein kinases ERK1 and ERK2 by 1α,25(OH)2D3 is mediated by PKC and Ca2+ (23). These data prompted us to study whether PKC participates in Raf-1 serine phosphorylation induced by the hormone. To that end, muscle cells were pretreated with the PKC inhibitors calphostin C or bisindolylmaleimide I and stimulated with 1α,25(OH)2D3 (1 nM, 2 min) followed by immunoprecipitation of cell lysates with anti-Raf-1 antibody and then immunoblotting with anti-phosphoserine antibody. As shown in Fig. 4, Raf-1 serine phosphorylation was suppressed by either PKC inhibitor. Moreover, Ro 318220, a PKC specific inhibitor that blocks all PKC isoforms completely, abolished Raf-1 serine phosphorylation (Fig. 5) and further confirmed that hormone activation of Raf-1 is a PKC-dependent event. It has been shown that PKCα can directly phosphorylate and activate Raf-1 in other cell types (18). PKCα belongs to the family of conventional protein kinases that are Ca2+-dependent.

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showing the amount of PKC
bottom panel
in Raf-1 serine phosphorylation;
experiments performed in duplicate; averages
scanning volumetric densitometry of top blot from four independent
against 20 nucleotides upstream of the AUG codon of PKC

stimulation in the presence of inhibitors.

Results are the average of three independent experiments performed in
duplicate; averages ± S.D. are given. *, p <
0.01 (*) and p < 0.05 (**), with respect to the corresponding control.

PKCα mRNA to suppress expression of its encoded protein. Under
these conditions, phosphorylation of Raf-1 induced by
1α,25(OH)2D3 was fully abolished (Fig. 6A, upper panel). Suppression
of PKCα expression was verified by immunoblotting of
cell lysates with anti-PKCα antibody (Fig. 6A, bottom panel). Although we cannot rule out the possibility that other PKC
isoforms may also contribute to Raf-1 serine phosphorylation,
our results clearly show that PKCα is also a component of the
mitogenic pathway leading to Raf-1 activation in response to
1α,25(OH)2D3 stimulation.

We finally evaluated the effects of Ras, Raf-1, and MEK
inhibition on MAP kinase activity and tyrosine phosphoryla-
tion changes induced by 1α,25(OH)2D3. To measure MAP
activity, the enzyme from cell lysates exposed for 1 min to the
hormone (1 nM) was immuno-precipitated with an anti-(phos-
pho)-active MAPK antibody, which recognizes both the p42 and
p44 active isoforms, and then incubated with [γ-32P]ATP and
myelin basic protein as exogenous substrate. Results are the average of three independent experiments performed in
triplect ± S.D. *, p < 0.01, with respect to basal and 1α,25(OH)2D3 stimulation in the presence of inhibitors.

Fig. 6. Effects of an antisense oligodeoxynucleotide against
PKCα mRNA on 1α,25(OH)2D3-induced Raf-1 serine phosphorylation.
Muscle cells were transfected with a sense (ODN-S) or an anti-
sense (ODN-AS) oligodeoxynucleotide against PKCα mRNA or Lipofec-
tin (LPP) to have a basal expression control. 48 h later the cells were
exposed to 1 nM 1α,25(OH)2D3 for 2 min. Raf-1 was immunoprecipitated
from cell lysates and then immunoblotted with anti-phosphoserine (an-
ti-P-serine) antibody. A, top panel, representative blot showing changes
in Raf-1 serine phosphorylation; bottom panel, representative blot
showing the amount of PKCα expressed in the cells. B, quantification by
scanning volumetric densitometry of top blot from four independent
experiments performed in duplicate; averages ± S.D. are given. p <
0.01 (*) and p < 0.05 (**), with respect to the corresponding control.

Materials and Methods.

Fig. 7. Stimulation of MAP kinase activity by 1α,25(OH)2D3 is
mediated by Ras and MEK. Cells were incubated for 1 min with
1α,25(OH)2D3 (1 nM) in the absence or presence of a MEK-specific
inhibitor (PD 98059, 10 μM) or a Ras inhibitory peptide (25 μM).
Immunoprecipitation of MAP kinase and assay of its activity using
[γ-32P]ATP and myelin basic protein as exogenous substrate were car-
ried out in cell lysates as described under "Materials and Methods." Results are the average of three independent experiments performed in
triplicate ± S.D. *, p < 0.01, with respect to basal and 1α,25(OH)2D3 stimulation in the presence of inhibitors.

Effects of an antisense oligodeoxynucleotide against
PKCα mRNA on 1α,25(OH)2D3-induced Raf-1 activation, we used antisense
technology to block PKCα protein expression. Cultured muscle
cells were transfected with an antisense oligodeoxynucleotide
against 20 nucleotides upstream of the AUG codon of PKCα

Fig. 8. 1α,25(OH)2D3, stimulation of MAP kinase tyrosine phos-
phorylation (P) is suppressed by antibody inhibition of Raf-1.
After cell lysis, comparable aliquots of lysate protein (500 μg) were
incubated with anti-Raf antibody (2 μg) or anti-goat IgG antibody (2 μg,
as a control of the basal levels of MAP kinase) on an ice bath for 10 min.
Then the lysates were exposed to 1α,25(OH)2D3 (1 nM) or vehicle etha-
nol (43%) for 1 min. Proteins were resolved by SDS-PAGE followed
by immunoblotting with anti-(phospho)-active MAP kinase as described
under "Materials and Methods." A, representative immunoblot. B, quantification by scanning volumetric densitometry of blots from three
independent experiments performed in duplicate; averages ± S.D. are
given. *, p < 0.01 with respect to basal and 1α,25(OH)2D3 stimulation
in the presence of anti-Raf antibody.
hormone-induced MAPK tyrosine phosphorylation was also abolished (Fig. 8). Furthermore, suppression of PKCα expression in cells transfected with the antisense oligodeoxynucleotide against PKCα mRNA abolished by 70% the phosphorylation of MAPK induced by 1α,25(OH)₂D₃ (Fig. 9). These results stress the relevance of PKCα, Ras, Raf-1, and MEK in the 1α,25(OH)₂D₃-signaling pathway, which results in MAPK kinase stimulation in muscle cells.

With regard to the initiation of the 1α,25(OH)₂D₃ signal that leads to activation of the MAP kinase pathway, new lines of evidence indicate that steroid hormone intracellular receptors mediate rapid, non-transcriptional stimulation of MAPK via interaction with upstream components of the cascade (46). More specifically, we recently reported that activation of MAPK by 1α,25(OH)₂D₃ in muscle cells is preceded by rapid tyrosine phosphorylation of the vitamin D receptor followed by its association with Src (47). Subsequent interaction with the Src-Grb2-Sos-Ras complex may then occur as observed in keratinocytes (48). However, the involvement of a novel 1α,25(OH)₂D₃ membrane receptor, whose existence in various hormone target cell types has received experimental support (41, 42, 49), cannot be excluded. In summary, our results demonstrate for the first time in a 1α,25(OH)₂D₃ target cell that activation of Raf-1 via Ras (through inhibition of Ras-GAP activity by tyrosine dephosphorylation) and PKCα-dependent serine phosphorylation play a central role in hormone stimulation of the MAPK-signaling pathway (depicted in the schematic diagram of Fig. 10).

REFERENCES


Fig. 9. Effects of an antisense oligodeoxynucleotide against PKC α mRNA on 1α,25(OH)₂D₃-induced MAPK phosphorylation. Muscle cells were transfected with a sense (ODN-S) or an antisense (ODN-AS) oligodeoxynucleotide against PKCα mRNA. 48 h later the cells were exposed to 1 nm 1α,25(OH)₂D₃ for 2 min and then lysed. Proteins were resolved by SDS-PAGE followed by immunoblotting with an anti-(phospho)-active MAP kinase as described under “Materials and Methods.” Top panel, representative blot showing changes in phospho-MAPK. Bottom panel, quantification by scanning volumetric densitometry of the top blot from three independent experiments performed in duplicate; averages ± S.D. are given, p < 0.01 (**), and p < 0.05 (*), with respect to the corresponding control.

Fig. 10. Schematic diagram indicating the chain of events leading to 1α,25(OH)₂D₃ activation of MAP kinase in skeletal muscle cells. By acting at the plasma membrane, 1α,25(OH)₂D₃ inhibits Ras-GAP activity by tyrosine dephosphorylation (YP), which elicits Ras activation. The hormone also stimulates PKCα activation. Activated Ras along with PKCα lead to serine phosphorylation and stimulation of Raf-1 followed by MEK and MAP kinase activation.
1α,25(OH)₂D₃ Regulation of MAPK in Muscle