Journal of Cellular Biochemistry 97:698–708 (2006)

ARTICLES

MAP Kinases p38 and JNK Are Activated by the Steroid Hormone 1α,25(OH)$_2$-Vitamin D$_3$ in the C2C12 Muscle Cell Line

Claudia G. Buitrago, Ana C. Ronda, Ana Russo de Boland, and Ricardo Boland*

Departamento de Biología, Bioquímica & Farmacia, Universidad Nacional del Sur, San Juan 670, 8000 Bahía Blanca, Argentina

Abstract In chick skeletal muscle cell primary cultures, we previously demonstrated that 1α,25(OH)$_2$-vitamin D$_3$ [1α,25(OH)$_2$D$_3$], the hormonally active form of vitamin D, increases the phosphorylation and activity of the extracellular signal-regulated mitogen-activated protein (MAP) kinase isoforms ERK1 and ERK2, their subsequent translocation to the nucleus and involvement in DNA synthesis stimulation. In this study, we show that other members of the MAP kinase superfamily are also activated by the hormone. Using the muscle cell line C2C12 we found that 1α,25(OH)$_2$D$_3$ within 1 min phosphorylates and increases the activity of p38 MAPK. The immediately upstream mitogen-activated protein kinase kinases 3/6 (MKK3/MKK6) were also phosphorylated by the hormone suggesting their participation in p38 activation. 1α,25(OH)$_2$D$_3$ was able to dephosphorylate/activate the ubiquitous cytosolic tyrosine kinase c-Src in C2C12 cells and studies with specific inhibitors imply that Src participates in hormone induced-p38 activation. Of relevance, 1α,25(OH)$_2$D$_3$ induced in the C2C12 line the stimulation of mitogen-activated protein kinase activating protein kinase 2 (MAPKAP-kinase 2) and subsequent phosphorylation of heat shock protein 27 (HSP27) in a p38 kinase activation-dependent manner. Treatment with the p38 inhibitor, SB203580, blocked p38 phosphorylation caused by the hormone and inhibited the phosphorylation of its downstrean substrates. 1α,25(OH)$_2$D$_3$ also promotes the phosphorylation of c-jun N-terminal protein kinases (JNK 1/2), the response is fast (0.5–1 min) and maximal phosphorylation of the enzyme is observed at physiological doses of 1α,25(OH)$_2$D$_3$ (1 nM). The relative contribution of ERK-1/2, p38, and JNK-1/2 and their interrelationships in hormonal regulation of muscle cell proliferation and differentiation remain to be established. J. Cell. Biochem. 97: 698–708, 2006.

Key words: 1α,25(OH)$_2$D$_3$; non-genomic actions; muscle; C2C12 cells; p38 MAPK; JNK; c-Src

1α,25-dihydroxyvitamin D$_3$ [1α,25(OH)$_2$D$_3$], the major biologically active metabolite of vitamin D$_3$, in addition to its well-recognized role in the regulation of mineral metabolism, cellular proliferation, and differentiation [Walters, 1992], modulates skeletal muscle contractility and growth [Smith and Stern, 1969; Boland, 1986, 2005; Boland et al., 1995].

Besides regulating gene expression via the specific intracellular vitamin D receptor (VDR) [Minghetti and Norman, 1988], 1α,25-(OH)$_2$D$_3$ also exerts in its target tissues fast non-transcriptional responses involving stimulation of transmembrane signal transduction pathways [de Boland and Nemere, 1992]. The initiation of the fast 1α,25(OH)$_2$D$_3$ signal may involve binding to a novel membrane receptor, whose existence received experimental support first in enterocytes [Nemere et al., 1994, 2004]. On the other hand, the VDR may also participate in the hormone-induced non-genomic responses [Buitrago et al., 2001a; Capiati et al., 2002]. Although there is much information about the rapid responses to this steroid hormone [Civitelli et al., 1990; Norman et al., 1992, 1999; Baran, 1994; Boland et al., 2002], the mechanism underlying 1α,25-(OH)$_2$D$_3$-non-genomic actions has not been at present completely elucidated.

Grant sponsor: Agencia Nacional de Promoción Científica y Tecnológica; Grant sponsor: Universidad Nacional del Sur (UNS), Argentina.

*Correspondence to: Dr. Ricardo Boland, Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur, San Juan 670, 8000 Bahía Blanca, Argentina.
E-mail: rboland@criba.edu.ar

Received 14 April 2005; Accepted 22 July 2005
DOI 10.1002/jcb.20639

Within the MAP kinase family four different subgroups have been described. These include: extracellular signal-regulated kinases (ERKs), c-Jun NH_2-terminal or stress-activated protein kinases (JNK/SAPK), big MAP kinase 1(BMK1), also called ERK5, and p38 MAP kinases (p38). The ERK, JNK, and p38 MAP kinase cascades consist of three protein kinases (MAPKKK, MAPKK, MAPK) that sequentially activate each other by phosphorylation [Widmann et al., 1999]. The ERKs have been extensively characterized as key components of the signal transduction pathways in growth and differentiation responses [Cobb et al., 1991; Sugden and Clerk, 1997]. Recent evidence indicates that modulation of various of the responses to $1\alpha,25\text{-}(OH)_2D_3$ depends on the fast activation of MAP kinase pathways. Thus, in proliferating cultured myoblasts, $1\alpha,25(OH)_2D_3$ rapidly (within 1 min) promotes tyrosine phosphorylation of ERK-1/2 [Morelli et al., 2000]. In skeletal muscle cells [Buitrago et al., 2001b; Morelli et al., 2001] and in keratinocytes [Gniadecki, 1998], the tyrosine kinase c-Src has been shown to participate in the activation of ERK-1/2 by $1\alpha,25(OH)_2D_3$. The differentiation process in HL-60 cells stimulated by the hormone is potentiated by p38 inhibitors with a concomitant up-regulation of the JNK pathway [Wang and Studzinski, 2001]. Induction of apoptosis by $1\alpha,25(OH)_2D_3$ analogs in myeloid cells has been correlated with ERK-1/2 inhibition and p38 activation [Park et al., 2000]. In view of the role that $1\alpha,25(OH)_2D_3$ plays in the regulation of muscle cell proliferation and differentiation [Capiati et al., 1999, 2000], in this work we study the effects of the steroid hormone on p38 and JNK MAP kinase cascades using the mouse C2C12 myoblast cell line as an in vitro model for skeletal muscle [Burattini et al., 2004].

MATERIALS AND METHODS

Chemicals

$1\alpha,25(OH)_2D_3$ was kindly provided by Dr. Jan-Paul van de Velde from Solvay Pharmaceuticals (Weesp, The Netherlands). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Sigma Chemical Co. (St. Louis, MO). Anti-p38 α and β antibodies and anti-phospho p38 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Nonradioactive p38 MAP kinase assay kit, anti-phospho MAPKAPK-2, anti-phospho Hsp27, anti-phospho MKK3/6, anti c-Src, anti c-Src (Tyr 527), and anti-c-Src (Tyr 416) antibodies were acquired in Cell Signaling Technology, Inc. (Beverly, MA). Secondary antibodies goat anti-rabbit and goat anti-mouse horse radish peroxidase-conjugated IgG and anisomycin were obtained from Santa Cruz Biotechnology. The Super Signal CL-HRP substrate system for enhanced chemiluminiscence (ECL) was from Perkin Elmer (Boston, MA). The C2C12 cell line (American Type Culture Collection, Manassas, VA) was kindly provided by Dr. E. Jaimovich (Universidad de Chile, Santiago, Chile). The 4-amino-5-(4-chloro-phenyl)-7-(t-butyl)pyrazolo [3,4-d]pyrimidine (PP2) inhibitor was from Calbiochem-Novabiochem Corp. (La Jolla, CA). All other reagents were of analytical grade.

Cell Culture

The mouse skeletal myoblastic cell line C2C12 was seeded at an appropriate density (120,000 cells/cm^2) in Petri dishes (100 mm diameter) with DMEM supplemented with 10% FBS and antibiotic-antimycotic solution. The cells were cultured at 37°C under a humidified atmosphere (95% air/5% CO_2). Under these conditions, myoblasts divide within the first 48 h and at day 6th these cells become differentiated into myotubes expressing morphological characteristics of adult skeletal muscle fibers [Burattini et al., 2004]. Cells cultured for 2 days (proliferative stage) were used for treatments.

Immunoprecipitation

After $1\alpha,25(OH)_2D_3$ or vehicle (ethanol <0.01%; control) treatment, muscle cells were lysed (at 4°C) in lysis buffer: 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM sodium orthovanadate (OV), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 1% Triton X-100, and homogenized by sonication for 5 s, four times. Insoluble material was pelleted in a microcentrifuge at 12,000*g* for 15 min. The protein content of the clear lysates of supernatant was determined according to Lowry et al. [1951]. Aliquots (200 μg protein) were incubated overnight with gentle rocking at 4°C with immobilized phospho-p38 MAP kinase (Thr 180/Tyr 182) monoclonal antibody. The

immune complexes were washed two times with cold lysis buffer.

Measurement of p38 Activity

Lysates were prepared followed by immunoprecipitation of p38 MAP kinase as described above. After washes with lysis buffer, the pellet was washed twice and afterwards resuspended with kinase buffer (25 mM Tris-HCl pH 7.5, 5 mM β-glycerolphosphate, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate, 10 mM $MgCl_2$). The immune complexes were incubated at 30°C for 30 min in kinase buffer (50 μl/sample) containing 2 μg activating transcription factor 2 (ATF-2) fusion protein as an exogenous substrate for p38 MAPK and 200 μM ATP. To terminate the reaction, 25 μl of 3× SDS sample buffer [Laemmli, 1970] were added. Finally, the sample was boiled for 5 min and then centrifuged for 2 min.

SDS–PAGE and Immunoblotting

Kinase assay samples (or lysate proteins) dissolved in Laemmli [1970] sample buffer were separated on SDS–polyacrylamide (10%) gels, and electrotransferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked for 1 h at room temperature in TBST (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 0.1% Tween-20) containing 5% dry milk. Membranes were subjected to immunoblotting using anti-phospho ATF-2 (Thr71), anti-phospho p38 MAPK, and anti-phospho JNK antibodies. Next, the membranes were washed three times in TBST, incubated in TBST containing 1% dry milk with a 1:5,000 dilution of peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies for 1 h at room temperature and washed three additional times with TBST. The membranes were then visualized using an enhanced chemiluminiscent technique (ECL), according to the manufacturer's instructions. Images were obtained with a model GS-700 Imaging Densitomer from Bio-Rad (Hercules, CA) by scanning at 600 dpi and printing at the same resolution. Bands were quantified using the Molecular Analyst program (Bio-Rad).

To strip the membranes for reprobing with anti-p38 α or anti-JNK antibodies, the membranes were washed 10 min in TBST and then incubated in stripping buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS and 50 mM mercapthoethanol) for 30 min at 50°C. The membranes were again blocked and blotted as described above.

Statistical Analysis

Statistical significance of the data was evaluated using Student's *t*-test [Snedecor and Cochran, 1967]. Probability values below 0.05 ($P < 0.05$) were considered significant and probability values below 0.01 ($P < 0.01$) were considered highly significant. Results are expressed as mean ± standard deviation (SD) from the indicated set of experiments.

RESULTS AND DISCUSSION

In skeletal muscle cells, we have previously demonstrated that the steroid hormone $1\alpha,25(OH)_2D_3$ stimulates the c-Src/Ras/Raf-1/MEK/ERK-1/2 pathway which leads to muscle cell proliferation [Buitrago et al., 2001b, 2003; Morelli et al., 2001]. However, the effector cascades mediating $1\alpha,25(OH)_2D_3$ actions in skeletal muscle cells are not completely characterized.

The p38 MAP kinases have been involved in inflammation, cell growth, cell differentiation and death, and the cell cycle progression [Ono and Han, 2000]. Diverse extracellular stimuli including UV light, heat shock, ionizing radiation, high osmotic stress, shear stress, proinflammatory cytokines, epidermal growth factor (EGF), and hemopoietic growth factors with the exception of interleukin-4 have been shown to trigger the p38 MAP kinase pathway through phosphorylation of a TGY motif within the kinase activation loop [Cano and Mahadevan, 1995]. Activation of the p38 pathway results in a plethora of changes in transcription, protein synthesis, cell surface receptor expression, and cytoskeletal structure, ultimately affecting cell survival or leading to programmed cell death [Obata et al., 2000]. In addition, the p38 MAP kinase pathway has been suggested to play important roles in embryonic development and organogenesis [Ganiatsas et al., 1998; Mikkola et al., 1999]. At least three p38 isoforms (α, β, γ) are expressed in skeletal muscle [Ho et al., 2004].

With the aim to elucidate whether $1\alpha,25(OH)_2D_3$ is able to activate other MAP kinase cascades in muscle, we first investigated changes in the phosphorylation and activity of p38 MAP kinase in response to the steroid in C2C12 cells. To that end, cells were exposed for

1 min to 1 nM $1\alpha,25(OH)_2D_3$. To monitor p38 MAP kinase phosphorylation, whole cell lysates were subjected to SDS–PAGE and then immunoblotted with an antibody that reacts with the phosphorylated form of all p38 MAP kinase isoforms (α, β, γ, and δ). As shown in Figure 1A, control cells showed low or undetectable levels of activated p38, whereas $1\alpha,25(OH)_2D_3$ at a physiologically dose and in a fast way (1 min) caused a sevenfold increase in p38 phosphorylation. Tyrosine phosphorylation and activation of p38 can be affected by agents that bypass membrane-associated receptors such as the translation blocker protein anisomycin [Nahas et al., 1996]. Therefore, we used as a positive control muscle cells treated with 38 μM anisomycin for 20 min. p38 kinase activity, assayed in vitro with ATF-2 as substrate after immunoprecipitation of cells incubated with 1 nM $1\alpha,25(OH)_2D_3$ for 1 min or with anisomycin, paralleled the amounts of phosphorylated p38 (Fig. 1B). As shown in Figure 2A, the phosphorylation of p38 was time-dependent, already increased at 0.5 min, peaked at 1 min and decreased later at 2 and 3 min of exposure to $1\alpha,25(OH)_2D_3$. The hormone also induced, in a time-dependent fashion, the phosphorylation of MKK3/6, the kinases immediately upstream of p38 [Keesler et al., 1998], event that was temporally correlated to p38 activation.

JNKs are phosphorylated and activated by the dual-specificity enzyme mitogen-activated protein kinase kinase 4 (MEK4) which is in turn activated by mitogen-activated protein kinase kinase kinase 1 (MEKK1) [Widmann et al., 1999]. A variety of cellular stresses such as UV

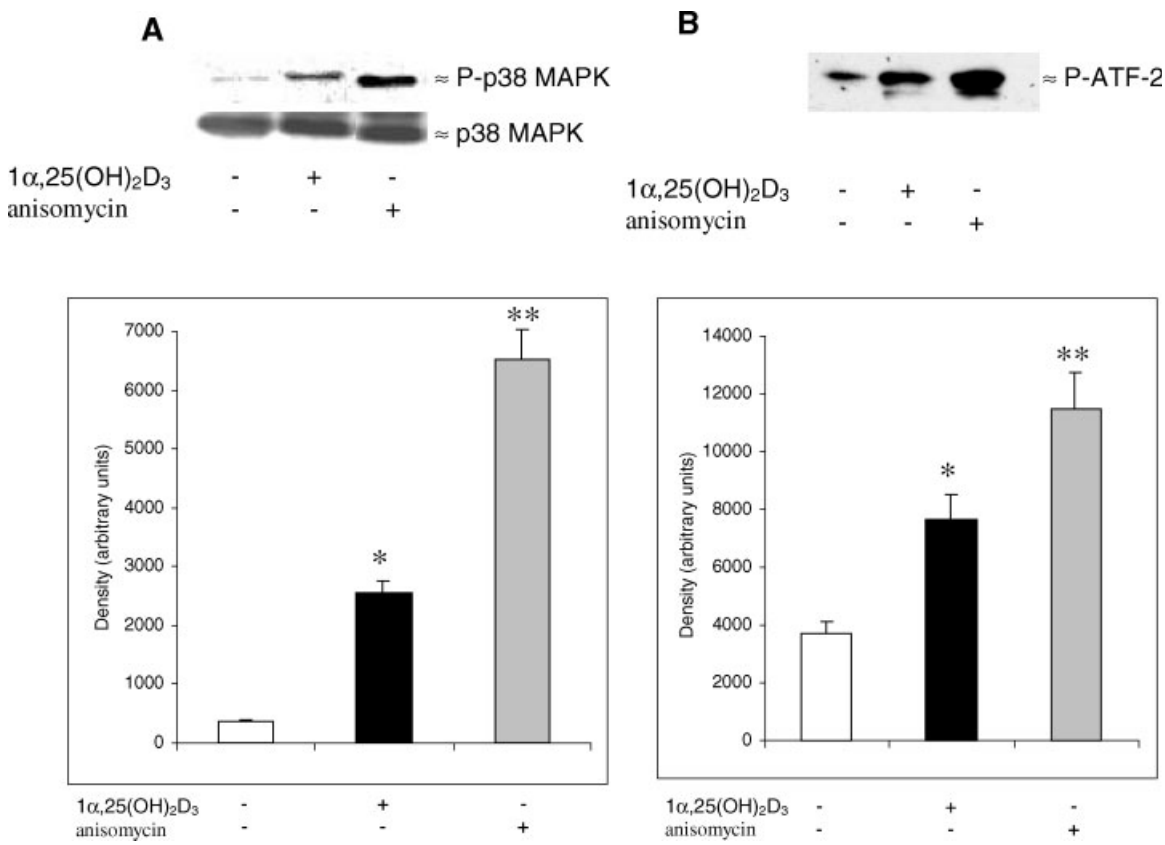

Fig. 1. $1\alpha,25(OH)_2D_3$ stimulates p38 MAPK phosphorylation and activity in C2C12 muscle cells. Cells were treated with 1 nM $1\alpha,25(OH)_2D_3$ for 1 min or with 38 μM anisomycin for 20 min (positive control). **A**: p38 phosphorylation: After cell lysis, Western blot analysis of lysates were carried out using specific anti-phospho p38 antibody. The blotted membranes were re-probed with anti-p38 antibody in order to ensure the equivalence of p38 content among the different experimental conditions. **B**: p38 activity: After cell lysis, kinase activity assays were carried out using ATF-2 as exogenous substrate as described in ''Materials and Methods.'' Representative immunoblots (**top panels**) and quantification by scanning volumetric densitometry of blots from three independent experiments (**lower panels**); average ± SD are given. $^{**}P < 0.01$; $^{*}P < 0.05$, with respect to the control.

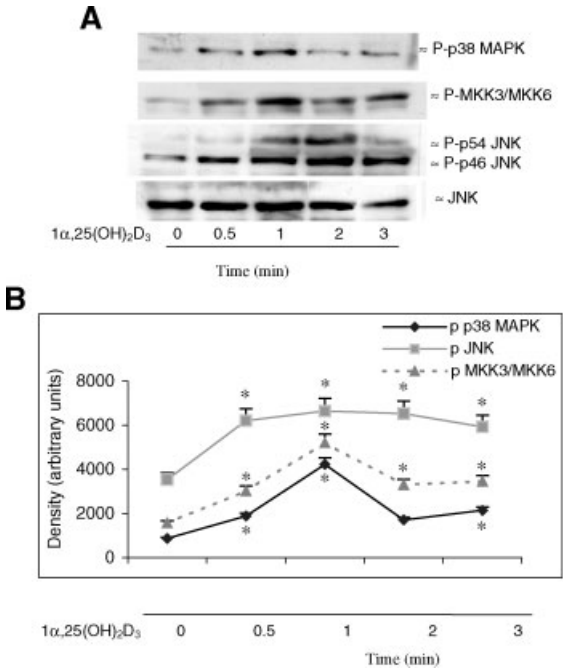

Fig. 2. Time profile of $1\alpha,25(OH)_2D_3$ effects on p38 MAPK, MKK3/MKK6 and JNK phosphorylation. C2C12 cells were treated with 1 nM $1\alpha,25(OH)_2D_3$ for different time intervals (0.5–3 min). After cell lysis, Western blot analysis of lysates were carried out using specific anti-phospho p38, anti-phospho MKK3/MKK6 and anti-phospho-JNK antibodies. **A**: Representative immunoblots showing the time course of phosphorylation for each enzyme. The membranes incubated with anti-phospho p38 antibody were stripped and reblotted with the other anti-phospho antibodies, followed by anti-JNK antibody to ensure the equivalence of protein content among the different experimental conditions (**bottom panel**). **B**: Quantification by scanning volumetric densitometry of blots from three independent experiments; average ± SD are given. $^*P < 0.05$ with respect to the corresponding control.

radiation, osmotic and heat shock, protein synthesis inhibitors, and pro-inflammatory cytokines stimulate JNK activity [Ip and Davis, 1998]. Once activated, JNKs can translocate to the nucleus [Cavigelli et al., 1995], and their immediate downstream targets include a variety of transcription factors, notably c-Jun [Kyriakis et al., 1994], ATF-2 [Gupta et al., 1995], and Elk-1 [Cavigelli et al., 1995]. JNKs are highly expressed in skeletal muscle and are greatly activated in response to muscle contraction, but little is known about the biological functions of JNK in muscle cells [Fujii et al., 2004]. Therefore, we also evaluated the effect of $1\alpha,25(OH)_2D_3$ on JNK. Its phosphorylation was measured in cell lysates by Western blot analysis using anti-phospho JNK antibody, as described under "Materials and Methods." $1\alpha,25(OH)_2D_3$ (1 nM) stimulation of C2C12 cells promotes JNK 1/2 (p54/p46) phosphorylation which reaches a maximum at 1 min (threefold), but differently to p38, the response remains sustained up to 3 min (Fig. 2B).

The response of C2C12 cells to $1\alpha,25(OH)_2D_3$ is dose-dependent. As shown in Figure 3, the steroid exerts the greatest activation of p38 and JNK at a concentration of 1 nM. Exposure of muscle cells to UV light or anisomycin for 20 min, also induced phosphorylation of p38 and JNK (data not shown).

We have recently reported that the non-receptor tyrosine kinase c-Src mediates $1\alpha,25(OH)_2D_3$ activation of the MAP kinases isoforms ERK-1/2 in skeletal muscle cells [Buitrago et al., 2001b]. It has been well established that Src family tyrosine kinases are involved in signal transduction pathways regulating a broad spectrum of physiological responses, including cell cycle control, cell proliferation, differentiation, adhesion, migration, and survival [Thomas and Brugge, 1997]. Furthermore, pharmacological inhibition of

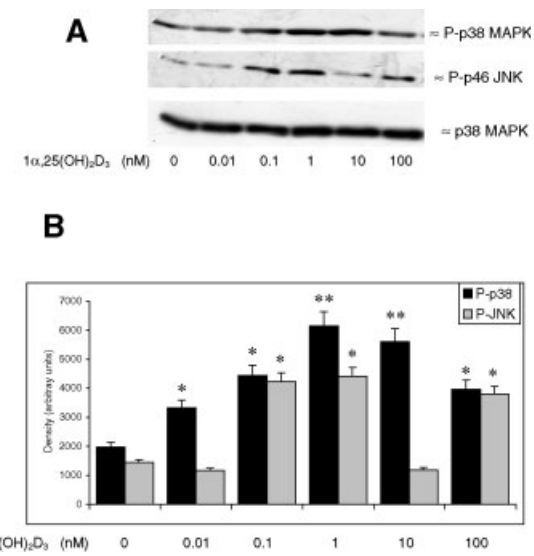

Fig. 3. Dose-response of $1\alpha,25(OH)_2D_3$ effects on p38 and JNK phosphorylation. C2C12 cells were treated for 1 min with 0.01–100 nM $1\alpha,25(OH)_2D_3$. After cell lysis, Western blot analysis of lysates were carried out using specific anti-phospho p38 and anti-phospho JNK antibodies. The blotted membranes were re-probed with anti-p38 antibody in order to ensure the equivalence of protein content among the different experimental conditions. **A**: Representative immunoblots. **B**: Quantification by scanning volumetric densitometry of blots from three independent experiments; average ± SD are given. $^{**}P < 0.01$; $^*P < 0.05$, with respect to the corresponding control.

TABLE I. $1\alpha,25(OH)_2D_3$-Stimulation of p38 MAP Kinase Phosphorylation Is Suppressed by the c-Src Inhibitor PP2

	p38 phosphorylation (% of control)
Control	100
Anisomycin	511.42 ± 29.34
$1\alpha,25(OH)_2D_3$	428.35 ± 14.28
PP2	100
PP2 + $1\alpha,25(OH)_2D_3$	115.57 ± 9.87

Muscle cells were treated with 1 nM $1\alpha,25(OH)_2D_3$ for 1 min with or without 10 μM PP2, a specific Src family kinases inhibitor. Cells incubated with 38 μM anisomycin for 30 min were used as positive control. After cell lysis, the lysate (30 μg protein) was fractionated by SDS–PAGE followed by immunoblot analysis using a specific anti-phospho p38 antibody as described under "Materials and Methods." Results are the average ± SD of three independent experiments.

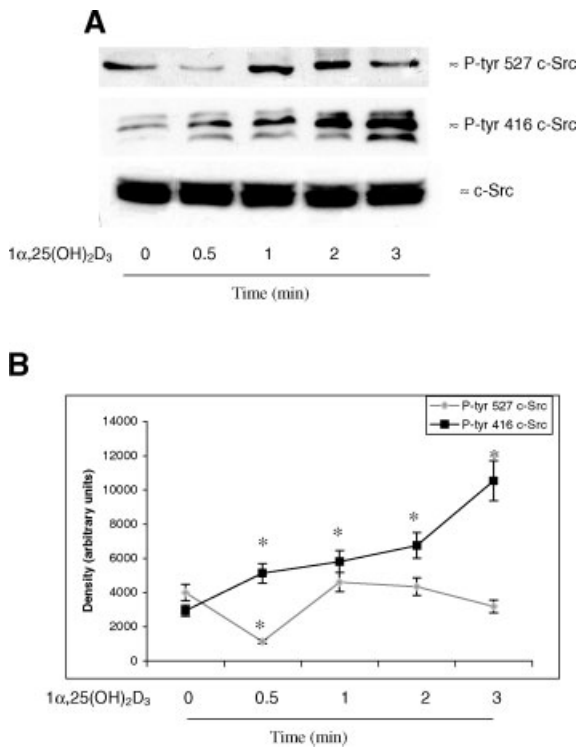

Fig. 4. Tyrosine phosphorylation state of c-Src activated by $1\alpha,25(OH)_2D_3$. C2C12 cells were exposed to 1 nM $1\alpha,25(OH)_2D_3$ for the indicated times. Cell lysates were obtained, Src was immunoprecipitated and its tyrosine phosphorylation state was assayed by immunoblotting with (**A**, **top**) anti-P-tyrosine 527 antibody, or (**A**, **bottom**) anti-P-tyrosine 416 antibody, as described in ''Materials and Methods.'' A representative immunoblot and densitometric analysis of changes in Src tyrosine phosphorylation from three immunoblots are shown. Blotted membranes shown were re-probed with anti-Src antibody in order to evaluate equivalence of Src kinase content among the different experimental conditions. **B**: Quantification by scanning volumetric densitometry of blots from three independent experiments; average ± SD are given. $^*P < 0.05$ with respect to the corresponding control.

Src-class kinases affects cellular components that regulate proliferation and differentiation in muscle, and repress proliferation and promotes differentiation of the C2C12 muscle cell line [Rosoff and Swope, 2002]. Therefore, to identify signaling pathways involved in hormone-induced p38 phosphorylation, we examined the possible involvement of Src family kinases. To this aim, the cells were preincubated in the presence of PP2, a potent inhibitor of the Src kinase family, before being exposed to $1\alpha,25(OH)_2D_3$. The data presented in Table I show that treatment with this tyrosine kinase inhibitor suppressed the transient phase of $1\alpha,25(OH)_2D_3$-dependent p38 phosphorylation, revealing a specific involvement of Src-like kinases, in the activation of the p38 MAP kinase cascade by $1\alpha,25(OH)_2D_3$. Depending on the stimulus and the cell type, p38 phosphorylation has been shown to be dependent [Mocsai et al., 2000; Cambien et al., 2001; Kabuyama et al., 2002; Ouwens et al., 2002] or independent [Yoshizumi et al., 2000; Gardner et al., 2003; Kyaw et al., 2004] of non receptor tyrosine kinases of the Src family.

Furthermore in C2C12 cells, the steroid hormone induces c-Src activation through changes in tyrosine phosphorylation of the enzyme (Fig. 4). We demonstrate that the first event in the activation of c-Src is the dephosphorylation of tyrosine 527 (that happens after

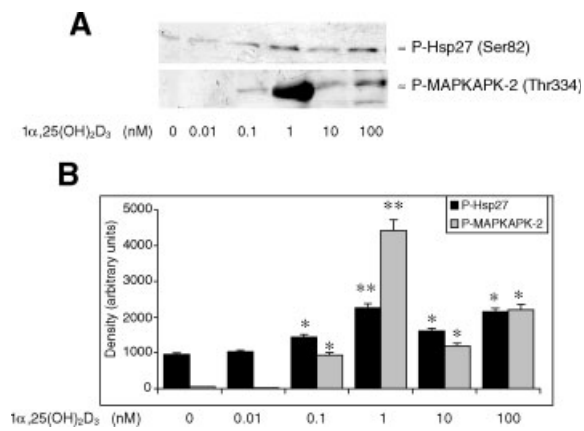

Fig. 5. $1\alpha,25(OH)_2D_3$ induces MAPKAPK-2 and Hsp27 phosphorylation in a dose-dependent fashion. C2C12 cells were treated with 0.01–100 nM $1\alpha,25(OH)_2D_3$ for 1 min. After cell lysis, Western blot analysis of lysates were carried out using specific anti-phospho Hsp27 and anti-phospho MAPKAPK-2 antibodies. **A**: Representative immunoblots. **B**: Quantification by scanning volumetric densitometry of blots from three independent experiments; average ± SD are given. $^{**}P < 0.01$; $^*P < 0.05$, with respect to the corresponding control.

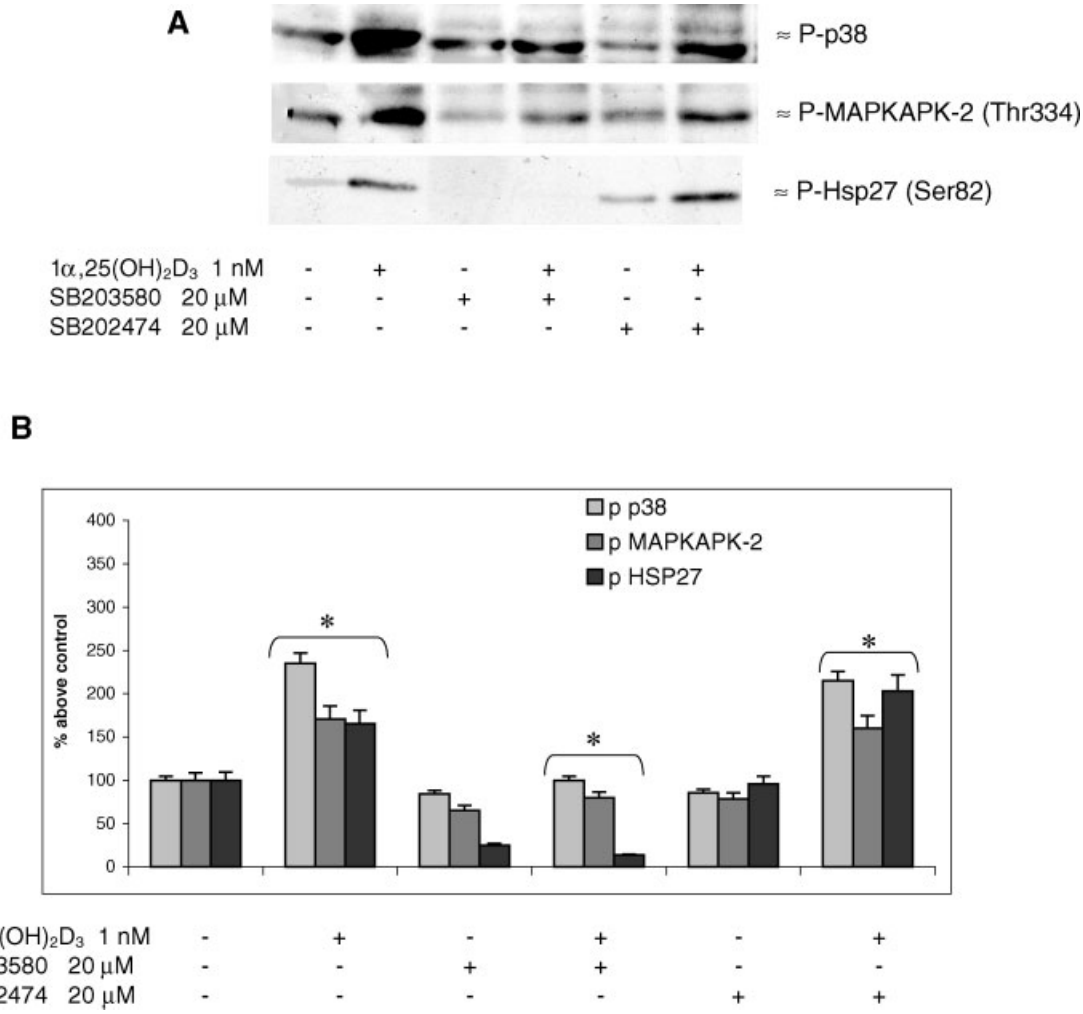

Fig. 6. p38 inhibition abolishes $1\alpha,25(OH)_2D_3$-induced MAPKAPK-2 and Hsp27 phosphorylation. C2C12 cells were preincubated with SB203580 (20 μM), a selective inhibitor of p38 or with the inactive analog SB202474 (20 μM) prior to challenge with $1\alpha,25(OH)_2D_3$ (1 nM, 1 min). After cell lysis, Western blot analysis of lysates were carried out using specific anti-phospho Hsp27 and anti-phospho MAPKAPK-2 antibodies. **A**: Representative immunoblots. **B**: Quantification by scanning volumetric densitometry of blots from three independent experiments; average ± SD are given. $^*P < 0.05$, for 1 nM $1\alpha,25(OH)_2D_3$ versus control (vehicle); $^*P < 0.05$, for SB203580 + $1\alpha,25(OH)_2D_3$ versus 1 nM $1\alpha,25(OH)_2D_3$; $^*P < 0.05$, for SB 202474 + 1 nM $1\alpha,25(OH)_2D_3$ versus SB 202474.

few seconds of hormonal treatment), followed by a second event of activation with phosphorylation at tyrosine 416, in agreement with the well-established mechanism for c-Src activation [Thomas and Brugge, 1997].

MAPKAPK-2 was the first identified p38 substrate [Freshney et al., 1994]. Actually it is considered a known target of p38 together with MAPKAPK-3 [Ono and Han, 2000]. The stress-induced activation of MAPKAPK-2 results in the phosphorylation of small heat-shock proteins (HSPs) [Larochelle and Suter, 1995] including HSP27 [Ono and Han, 2000]. HSPs are considered to be molecular chaperones which play an universal role in maintaining cellular homeostasis [Liu and Steinacker, 2001]. It is well recognized that HSP27 is expressed in skeletal muscle cells [Kato et al., 1991; Inaguma et al., 1995] including those of the C2C12 line [Ito et al., 2001]. This protein has an important role through its association with the actin microfilament system [Fischer et al., 2002] and cytoskeletal remodeling of muscle cells [An et al., 2004]. In view of these data, we studied changes in MAPKAPK-2 and HSP27 phosphorylation in C2C12 cells. To that end, cells were exposed to different concentrations (0.01–100 nM) of $1\alpha,25(OH)_2D_3$ followed by

Western blot analysis with anti phospho-HSP27 and anti phospho-MAPKAPK-2 antibodies. As shown in Figure 5, $1\alpha,25(OH)_2D_3$ increased the phosphorylation of MAPKAPK-2 and HSP27 in this skeletal muscle cell line. The response was dose-dependent with maximal phosphorylation (+2.5-fold and +5-fold, respectively) achieved at a physiological concentration (1 nM). It has been reported that the α and β isoforms of p38 are responsible for the activation of HSPs 25 and 27 and MAPKAP-2 [Stein et al., 1997]. Thus, our results suggest that p38 α and/or p38 β phosphorylate the downstream substrates HSP27 and MAPKAPK-2 in C2C12 cells stimulated with $1\alpha,25(OH)_2D_3$. Furthermore, $1\alpha,25(OH)_2D_3$-dependent phosphorylation of p38 and its downstream substrates HSP27 and MAPKAPK-2 was blocked by pretreatment with SB203580, a selective inhibitor of p38 [Lee et al., 1994; Young et al., 1997], whereas SB202474, an inactive analog of SB203580 [Lee et al., 1994], had little effect (Fig. 6). Treatment with SB203580 alone also decreased the basal activity of p38 substrates as compared with the control cells. The p38 MAPK family includes the p38 α, β, γ, and δ isoforms [Ono and Han, 2000], but only limited information is available regarding the function of each individual isoform. Among the p38 subfamily of MAP kinases, p38 α and p38 β can be specifically inhibited by SB203580 through its binding in the ATP pocket [Gum et al., 1998], whereas p38 γ and p38 δ are not sensitive to this compound [Goedert et al., 1997]. Therefore, it appears that $1\alpha,25(OH)_2D_3$ induced the phosphorylation of p38 α and/or p38 β in C2C12 cells.

In conclusion, the results of this study show that the steroid hormone $1\alpha,25(OH)_2D_3$

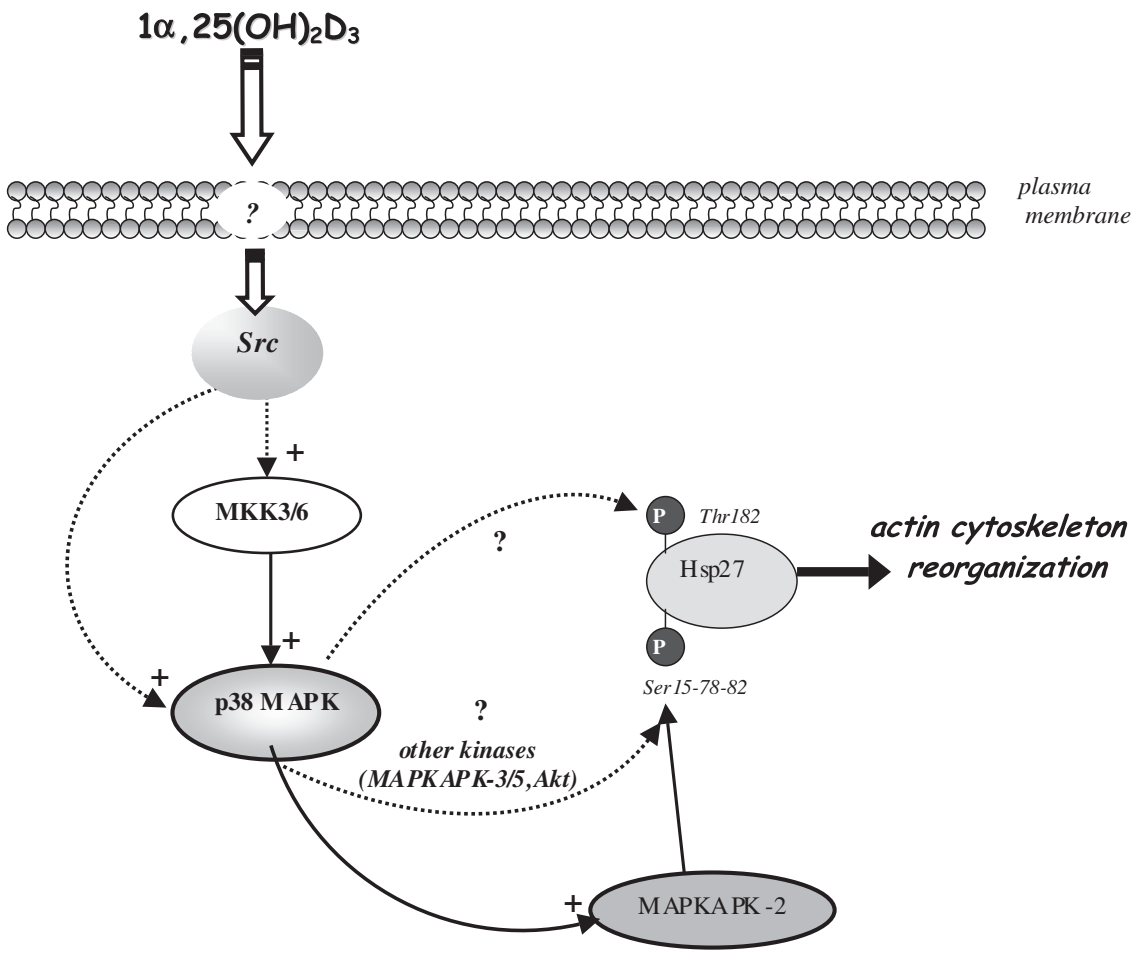

Fig. 7. Signal transduction pathways involved in the sequential activation of p38 MAPK and Hsp27 by $1\alpha,25(OH)_2D_3$ in C2C12 muscle cells. Thin solid arrows indicate signal transduction events supported by experimental evidence obtained in these studies. The thick solid arrow refers to the well-established intervention of HSP27 in cytoskeletal reorganization. Dotted arrows denote the operation of alternative pathways not demonstrated yet.

activates p38 MAP kinase, its downstream susbstrates MAPKAPK-2 and HSP27 in the skeletal muscle line C2C12 and that the cytosolic tyrosine kinase c-Src is an upstream mediator of hormone-dependent-p38 MAP kinase activation. This sequence of events may hypothetically lead to muscle cell differentiation through reorganization of actin cytoskeleton (Fig. 7). The relative roles of c-jun NH2-terminal kinase and ERK-1/2 which are also activated by $1\alpha,25(OH)_2D_3$ [this study and Morelli et al., 2000, respectively], and their interelationships with p38-mediated cascades, need to be investigated. Altogether, knowledge on the molecular aspects of regulation of MAP kinases by $1\alpha,25(OH)_2D_3$ in muscle cells has clinical and potential therapeutic implications in vitamin D-dependent myopathies characterized by atrophy and weakness of the proximal musculature.

ACKNOWLEDGMENTS

C. Buitrago, A.R. de Boland, and R. Boland are members of the Investigator Career of the Argentine National Research Council (CONICET). A.C. Ronda is recipient of a graduate research fellowship from CONICET.

REFERENCES

An SS, Fabry B, Mellema M, Bursac P, Gerthoffer WT, Kayyali US, Gaestel M, Shore SS, Fredberg JJ. 2004. Role of heat shock protein 27 in cytoskeletal remodeling of the airway smooth muscle cell. J Appl Physiol 96:1701–1713.

Baran DT. 1994. Nongenomic actions of the steroid hormone 1 alpha,25-dihydroxyvitamin D_3. J Cell Biochem 56:303–306.

Boland RL. 1986. Role of vitamin D in skeletal muscle function. Endocr Rev 7:434–448.

Boland R. 2005. Vitamin D and muscle. In: Feldman D, Pike W, Glorieux F, editors. Vitamin D, 2nd edn.. Amsterdam, The Netherlands: Elsevier, Inc. pp 883–897.

Boland R, De Boland AR, Marinissen MJ, Santillán G, Vazquez G, Zanello S. 1995. Avian muscle cells as targets for the secosteroid hormone 1,25-dihydroxy-vitamin D3. Mol Cell Endocrinol 114:1–8.

Boland R, de Boland AR, Buitrago C, Morelli S, Santillan G, Vazquez G, Capiati D, Baldi C. 2002. Non-genomic stimulation of tyrosine phosphorylation cascades by $1,25(OH)_2D_3$ by VDR-dependent and -independent mechanisms in muscle cells. Steroids 67:477–482.

Buitrago C, Vazquez G, de Boland AR, Boland R. 2001a. The vitamin D receptor mediates rapid changes in muscle protein tyrosine phosphorylation induced by $1,25(OH)_2D_3$. Biochem Biophys Res Commun 289:1150–1156.

Buitrago C, Boland R, de Boland AR. 2001b. The tyrosine kinase c-Src is required for $1,25(OH)_2$-vitamin D_3 signalling to the nucleus in muscle cells. Biochim Biophys Acta 1541:179–187.

Buitrago C, Pardo VG, De Boland AR, Boland R. 2003. Activation of RAF-1 through Ras and protein kinase C alpha mediates 1alpha,$25(OH)_2$-vitamin D_3 regulation of the mitogen-activated protein kinase pathway in muscle cells. J Biol Chem 278:2199–2205.

Burattini S, Ferri P, Battistelli M, Curci R, Luchetti F, Falcieri E. 2004. C2C12 murine myoblasts as a model of skeletal muscle development: Morpho-functional characterization. Eur J Histochem 48:223–233.

Cambien B, Pomeranz M, Millet MA, Rossi B, Schmid-Alliana A. 2001. Signal transduction involved in MCP-1-mediated monocytic transendothelial migration. Blood 97:359–366.

Cano E, Mahadevan LC. 1995. Parallel signal processing among mammalian MAPKs. Trends Biochem Sci 20: 117–122.

Capiati DA, Tellez-Inon MT, Boland RL. 1999. Participation of protein kinase C alpha in 1,25-dihydroxyvitaminD_3 regulation of chick myoblast proliferation and differentiation. Mol Cell Endocrinol 153:39–45.

Capiati DA, Vazquez G, Tellez Inon MT, Boland RL. 2000. Antisense oligonucleotides targeted against protein kinase C alpha inhibit proliferation of cultured avian myoblasts. Cell Prolif 33:307–315.

Capiati D, Benassati S, Boland R. 2002. $1,25(OH)_2$-vitamin D_3 induces translocation of the vitamin D receptor (VDR) to the plasma membrane in skeletal muscle cells. J Cell Biochem 86:128–135.

Cavigelli M, Dolfi F, Claret X, Karin M. 1995. Induction of c-fos expression through JNK-mediated TCF/Elk-1 phosphorylation. EMBO J 14:5957–5964.

Civitelli R, Kim YS, Gunsten SL, Fujimori A, Huskey M, Avioli LV, Hruska KA. 1990. Nongenomic activation of the calcium message system by vitamin D metabolites in osteoblast-like cells. Endocrinology 127:2253–2262.

Cobb M, Boulton T, Robbins D. 1991. Extracellular signal-regulated kinases: ERKs in progress. Cell Regul 2:965–978.

de Boland AR, Nemere I. 1992. Rapid actions of vitamin D compounds. J Cell Biochem 49:32–36.

Fischer D, Matten J, Reimann J, Bonnemann C, Schroder R. 2002. Expression, localization and functional divergence of alpha B-crystallin and heat shock protein 27 in core myopathies and neurogenic atrophy. Acta Neuropathol (Berl) 104:297–304.

Freshney N, Rawlinson L, Guesdon F, Jones E, Cowley S, Hsuan J, Saklatvala J. 1994. Interleukin-1 activates a novel protein kinase cascade that results in the phosphorylation of Hsp27. Cell 78:1039–1049.

Fujii N, Boppart M, Dufresne SD, Crowley PF, Jozsi AC, Sakamoto K, Yu H, Aschenbach WG, Kim S, Miyazaki H, Rui L, White MF, Hirshman MF, Goodyear LJ. 2004. Overexpression or ablation of JNK in skeletal muscle has No effect on glycogen synthase activity. Am J Physiol Cell Physiol 287:C200–C208.

Ganiatsas S, Kwee L, Fujiwara Y, Perkins A, Ikeda T, Labow MA, Zon LI. 1998. SEK1 deficiency reveals mitogen-activated protein kinase cascade crossregulation and leads to abnormal hepatogenesis. Proc Natl Acad Sci USA 95:6881–6886.

Gardner O, Dewar B, Earp H, Samet J, Graves L. 2003. Dependence of peroxisome proliferator-activated receptor ligand-induced mitogen-activated protein kinase signaling on epidermal growth factor receptor transactivation. J Biol Chem 278:46261–46269.

Gniadecki R. 1998. Nongenomic signaling by vitamin D: A new face of Src. Biochem. Pharmacol 56:1273–1277.

Goedert M, Cuenda A, Craxton M, Jakes R, Cohen P. 1997. Activation of the novel stress-activated protein kinase SAPK4 by cytokines and cellular stresses is mediated by SKK3 (MKK6); comparison of its substrate specificity with that of other SAP kinases. EMBO J 16: 3563–3571.

Gum R, McLaughlin M, Kumar S, Wang Z, Bower M, Lee J, Adams JL, Livi G, Goldsmith E, Young P. 1998. Acquisition of sensitivity of stress-activated protein kinases to the p38 inhibitor, SB 203580, by alteration of one or more amino acids within the ATP binding pocket. J Biol Chem 273:15605–15610.

Gupta S, Campbell D, De'rijard B, Davies RJ. 1995. Transcription factor ATF2 regulation by the JNK signal transduction pathway. Science 267:389–393.

Ho R, Alcazar O, Fujii N, Hirshman M, Goodyear L. 2004. p38 gamma MAPK regulation of glucose transporter expression and glucose uptake in L6 myotubes and mouse skeletal muscle. Am J Physiol Regul Integr Comp Physiol 286:R342–349.

Inaguma Y, Hasegawa K, Goto S, Ito H, Kato K. 1995. Induction of the synthesis of hsp27 and aB crystallin in tissues of heat-stressed rats and its suppression by ethanol or a1-adrenergic antagonist. J Biochem 117: 1238–1243.

Ip Y, Davis R. 1998. Signal transduction by the c-Jun N-terminal kinase (JNK) from inflammation to development. Curr Opin Cell Biol 10:205–219.

Ito H, Kamei K, Iwamoto I, Inaguma Y, Kato K. 2001. Regulation of the levels of small heat-shock proteins during differentiation of C2C12 cells. Exp Cell Res 266:213–221.

Kabuyama Y, Homma M, Kurosaki T, Homma Y. 2002. Early signaling events induced by 280-nm UV irradiation. Eur J Biochem 269:664–670.

Kato K, Shinohara H, Kurobe N, Inaguma Y, Shimizu K, Ohshima K. 1991. Tissue distribution and developmentalprofiles of immunoreactive aB crystallin in the rat determined with a sensitive immunoassay system. Biochim. Biophys Acta 1074:201–208.

Keesler G, Bray J, Hunt J, Jonhson D, Gleason T, Yao Z, Wang S, Parker C, Yamane H, Cole C, Lichenstein H. 1998. Purification and activation of recombinant p38 isoforms alpha, beta, gamma, and delta. Protein Expr Purif 14:221–228.

Kyaw M, Yoshizumi M, Tsuchiya K, Kagami S, Izawa Y, Fujita Y, Ali N, Kanematsu Y, Toida K, Ishimura K, Tamaki T. 2004. Src and Cas are essentially but differentially involved in angiotensin II-stimulated migration of vascular smooth muscle cells via extracellular signal-regulated kinase 1/2 and c-Jun NH_2-terminal kinase activation. Mol Pharmacol 65:832–841.

Kyriakis J, Banerjee P, Nikolakaki E, Dai T, Rubie E, Ahmad M, Avruch J, Woodgett J. 1994. The stress-activated protein kinase subfamily of c-Jun kinases. Nature 369:156–160.

Laemmli U. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Larochelle S, Suter B. 1995. The *Drosophila melanogaster* homolog of the mammalian MAPK-activated protein kinase-2 (MAPKAPK-2) lacks a proline-rich N-terminus. Gene 163:209–214.

Lee J, Laydon J, McDonnell P, Gallagher T, Kumar S, Green D, McNulty D, Blumenthal M, Heys J, Landvatter S, Strickler J, McLaughlin M, Siemens I, Fisher S, Livi G, White J, Adams J, Young P. 1994. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. Nature 372:739–745.

Liu Y, Steinacker JM. 2001. Changes in skeletal muscle heat shock proteins: Pathological significance. Front Biosci 6:D12–D25.

Lowry O, Rosebrough N, Farr A, Randall R. 1951. Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275.

Mikkola I, Bruun J, Bjorkoy G, Holm T, Johansen T. 1999. Phosphorylation of the transactivation domain of Pax6 by extracellular signal-regulated kinase and p38 mitogen-activated protein kinase. J Biol Chem 274:15115–15126.

Minghetti P, Norman A. 1988. $1,25(OH)_2$-vitamin D_3 receptors: Gene regulation and genetic circuitry. FASEB J 2:3043–3053.

Mocsai A, Jakus Z, Vantus T, Berton G, Lowell CA, Ligeti E. 2000. Kinase pathways in chemoattractant-induced degranulation of neutrophils: The role of p38 mitogen-activated protein kinase activated by Src family kinases. J Immunol 164:4321–4331.

Morelli S, Buitrago C, Vazquez G, De Boland AR, Boland R. 2000. Involvement of tyrosine kinase activity in $1,25(OH)_2$-vitamin D_3 signal transduction in skeletal muscle cells. J Biol Chem 275:36021–36028.

Morelli S, Buitrago C, Boland R, De Boland AR. 2001. The stimulation of MAP kinase by $1,25(OH)_2$-vitamin D_3 in skeletal muscle cells is mediated by protein kinase C and calcium. Mol Cell Endocrinol 173:41–52.

Nahas N, Molski T, Fernández G, Shaaf R. 1996. Tyrosine phosphorylation and activation of a new mitogen-activated protein (MAP)-kinase cascade in human neutrophils stimulated with various agonists. Biochem J 318:247–253.

Nemere I, Dormanen M, Hammond M, Okamura W, Norman A. 1994. Identification of a specific binding protein for 1 alpha,25-dihydroxyvitamin D_3 in basal-lateral membranes of chick intestinal epithelium and relationship to transcaltachia. J Biol Chem 269:23750–23756.

Nemere I, Farach-Carson M, Rohe B, Sterling T, Norman A, Boyan B, Safford S. 2004. Ribozyme knockdown funcionally links a $1,25(OH)_2D_3$ membrane binding protein (1,25D3-MARRS) and phosphate uptake in intestinal cells. PNAS 101:7392–7397.

Norman A, Nemere I, Zhou L, Bishop J, Lowe K, Maiyar A, Collins E, Taoka T, Sergeev I, Farach-Carson M. 1992. $1,25(OH)_2$-vitamin D_3, a steroid hormone that produces biologic effects via both genomic and nongenomic pathways. J Steroid Biochem Mol Biol 41:231–240.

Norman A, Song X, Zanello L, Bula C, Okamura W. 1999. Rapid and genomic biological responses are mediated by different shapes of the agonist steroid hormone, 1alpha,$25(OH)_2$-vitamin D_3. Steroids 64:120–128.

Obata T, Brown G, Yaffe M. 2000. MAP kinase pathways activated by stress: The p38 MAPK pathway. Crit Care Med 28:N67–N77.

Ono K, Han J. 2000. The p38 signal transduction pathway: Activation and function. Cell Signal 12:1–13.

Ouwens D, de Ruiter N, van der Zon G, Carter A, Schouten J, van der Burgt C, Kooistra K, Bos J, Maassen J, van Dam H. 2002. Growth factors can activate ATF2 via a two-step mechanism: phosphorylation of Thr71 through the Ras-MEK-ERK pathway and of Thr69 through RalGDS-Src-p38. EMBO J 21:3782–3793.

Park W, Seol J, Kim E, Hyun J, Jung C, Lee C, Binderup L, Koeffler H, Kim B, Lee Y. 2000. Induction of apoptosis by vitamin D_3 analogue EB1089 in NCI-H929 myeloma cells via activation of caspase 3 and p38 MAP kinase. Br J Haematol 109:576–583.

Rosoff W, Swope S. 2002. Role for cellular Src kinase in myoblast proliferation. J Cell Physiol 193:328–339.

Smith R, Stern G. 1969. Muscular weakness in osteomalacia and hyperparathyroidism. J Neurol Sci 8:511–520.

Snedecor G, Cochran G. 1967. Statistical methods. Ames, IA: Iowa State University Press.

Stein B, Yang M, Young D, Janknecht R, Hunter T, Murray B, Barbosa M. 1997. p38-2, a novel mitogen-activated protein kinase with distinct properties. J Biol Chem 272:19509–19517.

Sugden P, Clerk A. 1997. Regulation of the ERK subgroup of MAP kinase cascades through G protein-coupled receptors. Cell Signal 9:337–351.

Thomas S, Brugge J. 1997. Cellular functions regulated by Src family kinases. Annu Rev Cell Dev Bio 13:513–609.

Walters M. 1992. Newly identified actions of the vitamin D endocrine system. Endocr Rev 13:719–764.

Wang X, Studzinski G. 2001. Inhibition of p38MAP kinase potentiates the JNK/SAPK pathway and AP-1 activity in monocytic but not in macrophage or granulocytic differentiation of HL60 cells. J Cell Biochem 82:68–77.

Widmann C, Gibson S, Jarpe MB, Johnson GL. 1999. Mitogen-activated protein kinase: Conservation of a three-kinase module from yeast to human. Physiol Rev 79:143–180.

Yoshizumi M, Abe J, Haendeler J, Huang Q, Berk B. 2000. Src and Cas mediate JNK activation but not ERK1/2 and p38 kinases by reactive oxygen species. J Biol Chem 275:11706–11712.

Young P, McLaughlin M, Kumar S, Kassis S, Doyle M, McNulty D, Gallagher T, Fisher S, McDonnell P, Carr S, Huddleston M, Seibel G, Porter T, Livi G, Adams J, Lee J. 1997. Pyridinyl imidazole inhibitors of p38 mitogen-activated protein kinase bind in the ATP site. J Biol Chem 272:12116–12121.