Journal of Cellular Biochemistry 79:274–281 (2000)

Activation of Src Kinase in Skeletal Muscle Cells by 1,25-$(OH)_2$-Vitamin D_3 Correlates With Tyrosine Phosphorylation of the Vitamin D Receptor (VDR) and VDR-Src Interaction

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Abstract The rapid effect of 1α,25$(OH)_2$-vitamin D_3 [1α,25$(OH)_2D_3$] on tyrosine kinase Src and its relationship to the vitamin D receptor (VDR) was investigated to further characterize the hormone signaling mechanism in chick muscle cells. Exposure of cultured myotubes to 1α,25$(OH)_2D_3$ caused a time-dependent increase in Src activity, which was evident at 1 min (one-fold) and reached a maximum at 5 min (15-fold). Immunoblotting with anti-phosphotyrosine antibody of immunoprecipitated Src showed that the hormone decreased Src tyrosine phosphorylation state with maximal effects at 5 min. Using a database for protein consensus motifs we found a putative tyrosine phosphorylation site (amino acids 164–170: KTFDTTY) within the primary sequence of the chick VDR. When the myotube VDR was immunoprecipitated it appeared onto SDS-PAGE gels as a single band of 58 kDa recognized by an anti-phosphotyrosine antibody. Prior treatment of cells with $_1$α,25$(OH)_2D_3$ significantly increased tyrosine phosphorylation of the VDR (two- to three-fold above basal levels). In agreement with Src being a SH2-domain containing protein involved in recognition of tyrosine-phosphorylated targets, immunoprecipitation with anti-Src antibody under native conditions followed by blotting with anti-VDR antibody, or using the antibodies in inverse order, showed that the VDR co-precipitates with Src, thus indicating the existence of a VDR/Src complex. Stimulation with the cognate VDR ligand significantly increased formation of the complex with respect to basal conditions. These results altogether provide the first evidence to date for 1α,25$(OH)_2D_3$ activation involving Src association to tyrosine phosphorylated VDR. J. Cell. Biochem. 79:274–281, 2000.

Key words: 1α,25$(OH)_2$-vitamin D_3; non-genomic actions; Src activation; VDR phosphorylation; tyrosine phosphorylation

The steroid hormone 1α,25-dihydroxy-vitamin-D_3 [1α,25$(OH)_2D_3$] modulates calcium homeostasis in skeletal muscle cells by both a genomic action (long-term responses) which elicits control of gene expression through interaction with a specific intracellular receptor (VDR: vitamin D receptor) [Boland et al.,1995], and a non-genomic (rapid responses) mechanism implying direct membrane effects of the sterol mediated by a complex array of signaling systems [De Boland and Boland, 1994; Morelli et al., 1993]. In chick skeletal muscle cells 1α,25$(OH)_2D_3$ rapidly modulates Ca^{2+} influx by G-protein mediated activation of both phospholipase C and adenylyl cyclase [Morelli et al., 1996; Vazquez et al., 1995]. The concomitant generation of diacylglycerol, IP_3, and cyclic AMP leads to activation of protein kinase C and protein kinase A [Vazquez et al., 1996, 1997a], release of Ca^{2+} from inner stores [Vazquez et al., 1997b] and activation of both L-type voltage-dependent and store-operated Ca^{2+} (SOC) channels [Vazquez et al., 1993, 1998].

Recent evidence indicates that modulation of various of the fast as well as long-term responses to 1α,25$(OH)_2D_3$ depends on the activation of pathways implying tyrosine phosphorylation of key signaling components. In human keratinocytes the hormone rapidly

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Received 25 February 2000; Accepted 12 April 2000

stimulates Src kinase activity and Src-mediated tyrosine phosphorylation of the Shc adapter protein, which in turn associates with Grb2 and Sos [Gniadecki, 1998]. Sterol-dependent Src activation has also been observed in rat colonocytes [Khare et al., 1997]. The nature and complexity of the non-genomic actions of 1α,25(OH)$_2$D$_3$, has led to the proposal that a putative cell surface receptor for the hormone is involved in the activation of signalling systems in muscle cells [Boland et al., 1995], as postulated for this and other steroids in different cell types[Nemere and Farach-Carson, 1998; Mellay et al., 1997]. However, the existence of such novel membrane receptor has been not conclusively demonstrated. Alternatively, other lines of evidence point to a role of the VDR itself in mediating some of the rapid, non-genomic effects of the hormone [Kim et al., 1996; Barsony et al., 1994]. In fact, Gniadecki [1996, 1998] has reported that in human keratinocytes the VDR becomes rapidly associated with tyrosine-phosphorylated Shc under conditions of sterol stimulation, without excluding the participation of another protein in the interaction between the VDR and Shc.

We have recently observed that in skeletal muscle cells tyrosine kinase phosphorylation of cellular proteins plays a key role in 1α,25(OH)$_2$D$_3$-dependent modulation of non-genomic responses, such as fast increases in cytosolic Ca^{2+} and mitogen-activated protein kinase (MAPK) stimulation [De Boland et al., 1998]. In the present study we investigated whether the cytosolic non-receptor tyrosine kinase Src is involved in 1α,25(OH)$_2$-vitamin D$_3$ signaling in these cells. Moreover, the existence of an association between VDR and Src was also evaluated.

MATERIALS AND METHODS

Chemicals

1α,25(OH)$_2$D$_3$ was kindly provided by Dr. Heinrich Bachmann (Hoffman-La Roche Ltd., Basel, Switzerland). Protein A-Sepharose CL4B, enolase, polyvinylidene difluoride (PVDF) membranes, Dulbecco's modified Eagle's medium, and fetal bovine serum were from Sigma Chemical Co. (St. Louis, MO). Rabbit polyclonal anti-phosphotyrosine and rat monoclonal anti-VDR (chick) antibodies were obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-Src mouse monoclonal antibody was from Promega (Madison, WI). Secondary, peroxidase-conjugated anti-rat antibody was from Affinity Bioreagents (Golden, CO). Anti-rabbit and anti-mouse peroxidase-labelled antibodies and the enhanced chemiluminescence (ECL) kit were obtained from Amersham Corp. (Arlington Heights, IL). [γ^{32}P]-ATP (3,000 Ci/mmol) was from New England Nuclear (Chicago, IL). All other reagents were of analytical grade.

Cell Culture

Undifferentiated, myogenic chick skeletal muscle cells (myoblasts) were isolated from the breast muscle of 13-day-old chick embryos (*Gallus gallus*) essentially as described before [Vazquez and de Boland, 1993], and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37°C under a humidified atmosphere (air/5% CO_2) until confluence (4–6 days after plating) before use. By this time, myoblasts become differentiated into myotubes with both biochemical and morphological characteristics of adult skeletal muscle fibers [Capiati et al., 1999].

Immunoprecipitation Under Denaturing Conditions

After 1α,25(OH) $_2$D$_3$ or vehicle (ethanol) treatment, muscle cells were lysed (30 min at 4°C) in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EGTA, 25 mM NaF, 0.2 mM sodium orthovanadate (OV), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml leupeptin, 2 μg/ml aprotinin, 0.25% sodium deoxycholate and 1% NP40. Lysates were clarified by centrifugation (14,000*g*, 10 min) and protein content of the supernatants was determined according to Lowry et al. [1951]. Samples (500–700 μg protein) were incubated overnight at 4°C with either anti-Src, anti-phosphotyrosine, or anti-VDR antibodies, followed by precipitation of the immunocomplexes with protein A-Sepharose. The precipitated complexes were washed five times with cold immunoprecipitation buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.2 mM PMSF, 0.2 mM OV, 1% Triton X-100, and 1% NP40).

Co-immunoprecipitation

Co-immunoprecipitation assays were performed under native conditions in order to pre-

serve protein-protein associations, and were conducted essentially as described [Shieh and Zhu, 1996; Xu et al., 1997] with minor modifications. Briefly, after hormone treatment, cells were lysed (15 min at 4°C) in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 3 mM KCl, 0.5 mM EDTA, 0.2 mM OV, 1 mM NaF, 1 mM PMSF, 6 μg/ml leupeptin, 8 μg/ml aprotinin, 1% Tween-20. Lysates were clarified by centrifugation (14,000*g*, 10 min) and immunoprecipitation on the supernatants was performed with the indicated antibodies as described above, except that precipitated immunocomplexes were washed five times with phosphate-buffered saline solution (PBS).

Western Blot Analysis

Immunoprecipitated proteins were resolved on SDS-polyacrylamide (8–10%) gels according to Laemmli [1970], and electrotransferred to PVDF membranes. After blocking in TBST (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1% Tween-20) containing 1% non-fat dry milk, membranes were incubated overnight with the appropriate dilution of the indicated antibody. Non-interacting antibody was washed out, and membranes were incubated with the corresponding secondary antibody. Immunoreactive proteins were then visualized by ECL according to manufacturer's intructions. 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).

Stripping of PVDF membranes for reprobing with anti-Src antibody (see experiments in Fig. 2C) was done by washing the membranes 10 min in TBST and then incubating them in stripping buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS and 50 mM mercaptoethanol) for 30 min at 50°C. Membranes were blocked again and blotted with anti-Src antibody as described above.

Src Kinase Activity

Src was immunoprecipitated (denaturing conditions) as indicated above from conveniently treated cell lysates and incubated at 30°C for 10 min in buffer containing 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM OV, 50 μM [γ_{32}-P]ATP (2 μCi/assay) and enolase (2.5 μg/assay) as Src substrate, essentially as described by Kapus et al. [1999]. Reactions were stopped by adsorbing phosphorylated enolase onto phosphocellulose filters (Whatman P-81). Papers were immediatly washed in ice-cold 75 mM H_3PO_4 for four times and counted in a liquid scintillation counter.

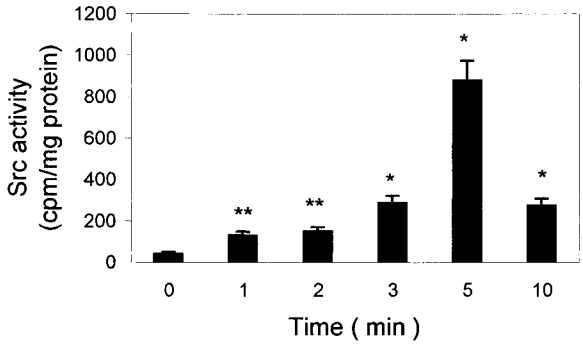

Fig. 1. Time course of 1α,25(OH)$_2$D$_3$-stimulation of Src kinase activity. Chick muscle cells were incubated in the presence of 1α,25(OH)$_2$D$_3$ (1 nM) or vehicle (ethanol <0.1%) for the indicated times. Immunoprecipitation of Src and assay of Src tyrosine kinase activity using [γ^{32}P]ATP and enolase as exogenous substrate were carried out in cell lysates as detailed in Materials and Methods. Results are the average of three independent experiments performed in triplicate ± s.d. $^*P < 0.001$; $^{**}P < 0.01$.

Statistical Analysis

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

RESULTS AND DISCUSSION

To evaluate whether the tyrosine kinase Src is part of the 1α,25(OH) $_2$D$_3$ signaling mechanism in chick muscle cells, we first investigated the effect of the steroid on Src kinase activity. To that end, the enzyme from lysates of cells exposed for different times to the hormone was immunoprecipitated with a highly specific anti-Src monoclonal antibody and then [γ-^{32}P]ATP and enolase, acting as exogenous Src substrate, were added. 1α,25(OH)$_2$D$_3$ caused a time-dependent increase in Src kinase activity in muscle cells (Fig. 1). Phosphorylation of the Src substrate could be detected at 1 min (one-fold increase over basal), reached a

maximum after 5 min of hormone exposure (12-fold) and markedly declined at 10 min. No time-dependent changes in Src activity were observed under basal conditions (data not shown). It is well established that phosphorylation of a conserved tyrosine residue (Y527) at the carboxy-terminal tail of the Src molecule negatively modulates its kinase activity by a mechanism which apparently implies intramolecular interaction of the carboxy-terminal phosphorylated tyrosine with the SH2 domain of Src [Piwnica-Worms et al., 1987; Brown and Cooper 1996]. Therefore, a possible way by which 1α,25(OH)$_2$D$_3$ stimulated Src kinase activity could be, at least in part, by altering the state of tyrosine phosphorylation of the enzyme. We then investigated steroid-induced changes in tyrosine phosphorylation of Src, by immunoblotting with anti-phosphotyrosine antibody the Src immunoprecipitated in the experiments above. As shown in Figure 2 (A,B), the hormone effectively induced a decrease in Src tyrosine phosphorylation, with maximal desphosphorylation (−91% respect to control) achieved at 5 min, thus paralleling maximal 1α,25(OH)$_2$D$_3$ stimulation of Src kinase-mediated enolase phosphorylation. Immunoblotting with anti-Src antibody confirmed that equivalent amounts of Src were present in samples from control and 1α,25(OH) $_2$D$_3$-treated cells (Fig. 2C).

It has been reported that the human keratinocyte VDR associates with tyrosine-phosphorylated Shc upon fast treatment of cells with 1α,25(OH)$_2$D$_3$, although neither SH2 or phosphotyrosine-binding domains, known to be involved in Shc binding to target proteins, could be found within the VDR primary sequence [Gniadecki, 1996]. However, by using the Prosite database for protein consensus motifs [Hofmann et al., 1999] we detected within the sequence of chick VDR [Lu et al., 1997] a putative tyrosine phosphorylation site corresponding to amino acids 164–170 (KTFDTTY), a region located near the C-terminal end of the receptor DNA binding domain. Similar putative tyrosine phosphorylation sites were also found in human, rat, and mouse VDR sequences (Vazquez and Boland, unpublished observations). In order to evaluate if the VDR really represents a tyrosine phosphorylated protein, it was immunoprecipitated from cell lysates obtained from either 1α,25(OH)$_2$D$_3$-treated or untreated chick myotubes with anti-VDR antibody under denaturing conditions, resolved in SDS-PAGE gels and then immunoblotted with anti-phosphotyrosine antibody. The vitamin D receptor appeared as a single band of 58 kDa which was recognized by the anti-phosphotyrosine antibody (Fig. 3A). Upon sterol treatment of muscle cells, tyrosine phosphorylation of the VDR significantly increased with respect to basal levels, e.g., 2–2.5 fold at 1 min, remaining elevated (1.7-fold) after 10 min of hormone exposure (Fig. 3B). Similar results were obtained when control assays were carried out adding the antibodies in reverse order: cell lysates were precipitated with anti-phosphotyrosine and blotted with the anti-VDR antibody (not shown). Comparing total VDR immunoprecipitated with anti-VDR antibody with VDR immunoprecipitated with anti-phosphotyrosine antibody, it could be estimated, on the basis of densitometric measurements, that about 10–15% of total VDR is tyrosine phosphorylated under basal conditions (Fig. 3, "0" time-point in A). As only one consensus motif for tyrosine-phosphorylation was found within the avian VDR sequence (see above), we hypothesize that the increased amount of cellular tyrosine-phosphorylated VDR observed upon 1,25-dihydroxyvitamin D$_3$ treatment occurs at the expense of an increase in the number of VDR molecules that become tyrosine-phosphorylated. Although a total of seven tyrosine residues exist within Gallus gallus VDR sequence, only one of them (Y170) fits the requirements of a consensus motif for tyrosine phosphorylation; however, the lack of consensus environment in the remaining six tyrosines does not allow us to rule out the possibility that, under the present conditions, more than one tyrosine residue becomes phosphorylated within a VDR molecule.

In either case, the present finding represents the first evidence to date for tyrosine phosphorylation of the VDR. All steroid hormone receptors are phosphorylated and undergo ligand-induced hyperphosphorylation [Bodwell et al., 1998]. However, most of the phosphorylated residues identified to date are serines in the N-terminal motif, and tyrosine phosphorylation has only been documented for the estrogen receptor [Kuiper and Brinkmann, 1994]. Phosphorylation of the human VDR on serine residues has been shown to play a role in 1α,25(OH)$_2$D$_3$-dependent transcriptional activation [Hsieh et al., 1991; Jurutka et al., 1993].

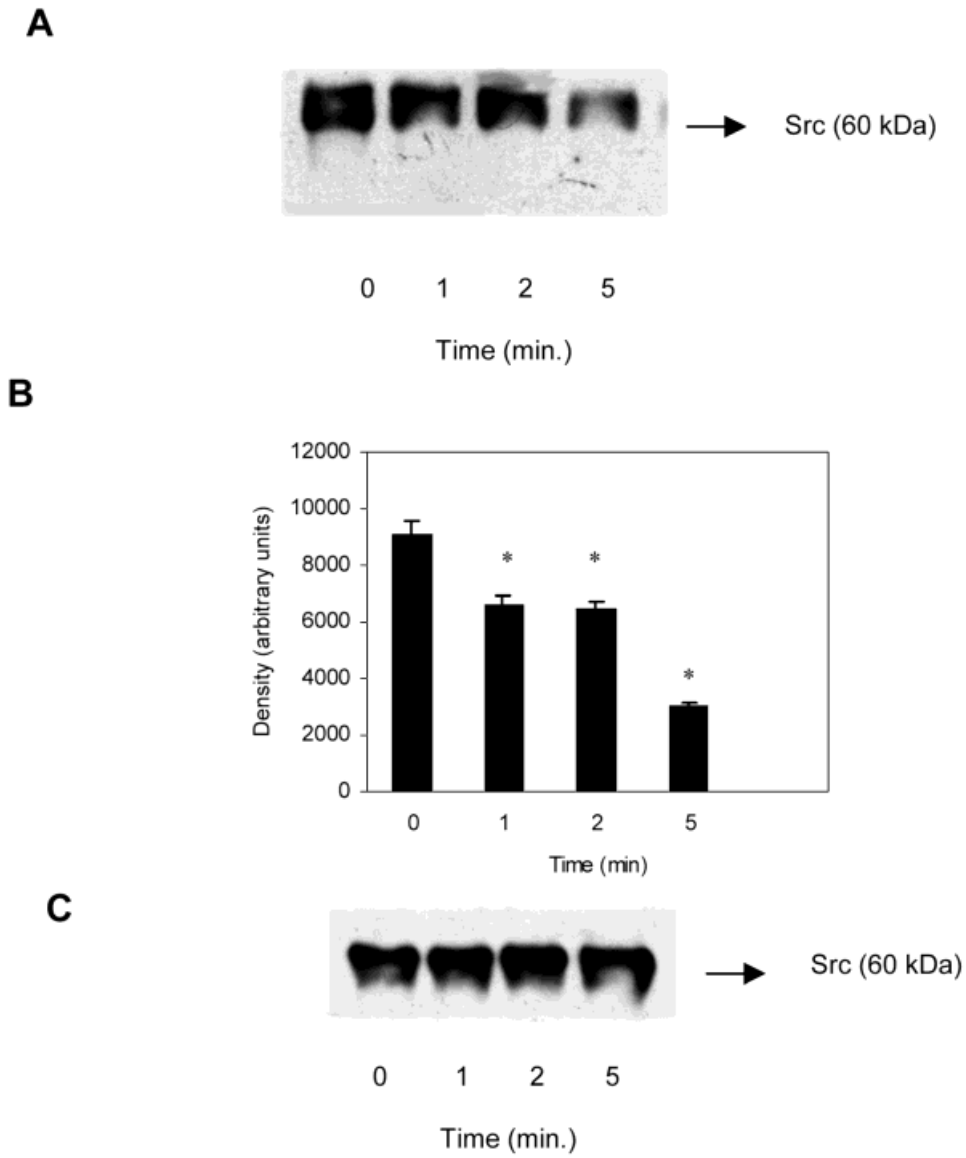

Fig. 2. Tyrosine-phosphorylated state of Src under $1\alpha,25(OH)_2D_3$ treatment. Muscle cells were exposed to 1 nM $1\alpha,25(OH)_2D_3$ or vehicle (ethanol <0.1%) for the indicated times. Cell lysates were obtained, Src was immunoprecipitated and its tyrosine phosphorylation state was assayed by immunoblotting with anti-phosphotyrosine antibody as described. **A**: Representative immunoblot from three independent "experiments "0" time-point was obtained in the presence of vehicle; no time-dependent changes were observed in vehicle-treated cells. **B**: Densitometric analysis of changes in Src tyrosine phosphorylation. Shown are the mean ± sd from three immunoblots; $*P < 0.001$. **C**: Blotted membrane shown in A, was re-probed with anti-Src antibody as indicated in Materials and Methods in order to evaluate equivalence of Src kinase content among the different experimental conditions.

Functionally, the fact that VDR behaves as a phosphotyrosine protein, opens the possibility that this receptor protein could be interacting with other phosphotyrosine binding proteins, such as Src. Src has a single SH2 domain which specifically recognizes the phosphorylated state of tyrosine residues [Grucza et al., 1999]. To analyze whether VDR was able to form a complex with Src, and if $1\alpha,25(OH)_2D_3$ stimulated the formation of such a complex, lysates from $1\alpha,25(OH)_2D_3$-treated or untreated skeletal muscle cells were immunoprecipitated with anti-VDR antibody under native conditions, in order to preserve protein-protein associations, and then the blots were probed with anti-Src antibody. As shown in Figure 4A, Src

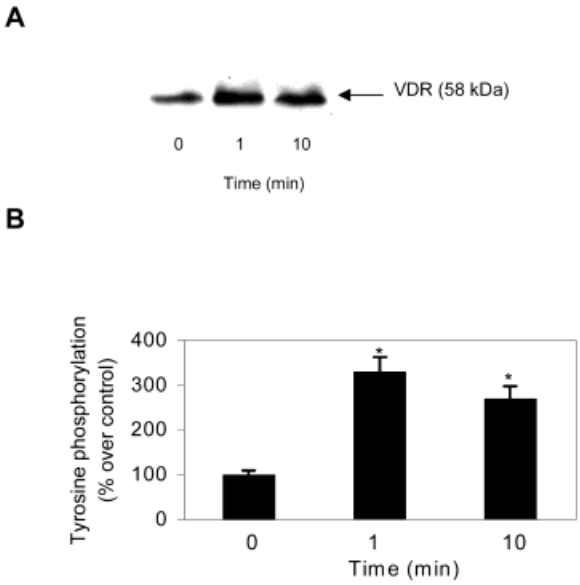

Fig. 3. Tyrosine phosphorylation of VDR. Muscle cells were exposed to 1 nM 1α,25(OH)$_2$D$_3$ or vehicle (ethanol <0.1%) for the indicated times. Cell lysates were obtained, VDR was immunoprecipitated with anti-VDR monoclonal antibody under denaturing conditions, resolved onto 10% SDS-PAGE gels and then immunoblotted with anti-phosphotyrosine antibody as described. **A**: A representative immunoblot from four separate experiments is shown. "0" time-point was obtained in the presence of vehicle; no time-dependent changes were observed in vehicle-treated cells. **B**: Quantitative changes in VDR tyrosine phosphorylation. Means ± sd from the densitometric analysis of four immunoblots are given; $*P < 0.001$.

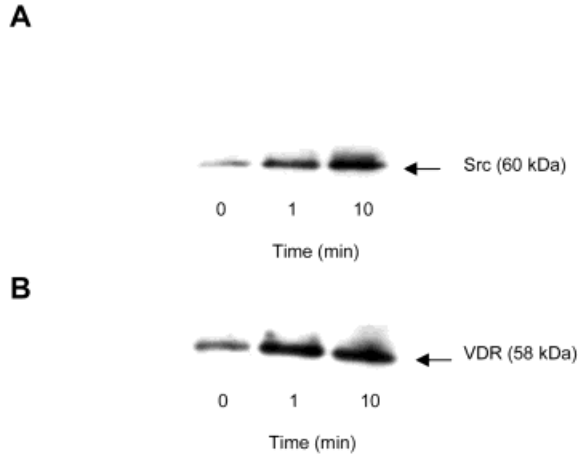

Fig. 4. Association of VDR with the non-receptor tyrosine kinase Src. **A**: Muscle cells were exposed to 1 nM 1α,25(OH)$_2$D$_3$ or vehicle (ethanol < 0.1%) for the indicated times. Cell lysates were obtained, VDR was immunoprecipitated with anti-VDR monoclonal antibody under native conditions, resolved onto 10% SDS-PAGE gels and then immunoblotted with anti-Src monoclonal antibody as described. **B**: As in A, but immunoprecipitation was performed with anti-Src monoclonal antibody and immunoblotting with the anti-VDR antibody as described. "0" time-point was obtained in the presence of vehicle; in either A or B, no time-dependent changes were observed in vehicle-treated cells.

co-precipitated with VDR thus indicating the existence of a VDR/Src complex. Similar results were obtained when the antibodies were used in reverse order (Fig. 4B). No co-precipitation of Src and VDR was observed when denaturing conditions were used to lyse the cells (not shown). Although association among these two proteins was clearly detectable under basal conditions, in agreement with the previous detection of VDR tyrosine phosphorylation in the absence of added exogenous 1α,25(OH)$_2$D$_3$, stimulation with the hormone significantly increased formation of the complex (three- to four-fold, $P < 0.001$). These results also represent a novel finding for muscle as well as other hormone target cells, which throw light on previous studies reporting that in keratinocytes the sterol induces tyrosine phosphorylation of Shc through activation of Src kinase, in parallel to the association of Shc to the VDR [Gniadecki, 1996, 1998]. In view of our observations and the known sequence of reactions in the tyrosine kinase cascade it is then likely that the primary event consists in a 1α,25(OH)$_2$D$_3$-dependent interaction between the VDR and Src followed by signaling to Shc, Grb2, and Sos which may form part of the same complex.

We speculated above that a possible mechanism by which 1α,25(OH)$_2$D$_3$ stimulates Src activity in muscle cells could be by inducing tyrosine dephosphorylation of the enzyme. However, the observation that the VDR is tyrosine phosphorylated and that its association with Src significantly increases in response to the hormone opens the possibility that binding of 1α,25(OH)$_2$D$_3$ to its cognate receptor could induce a conformational change on this protein which is then sensed by the receptor-associated Src. There is evidence indicating that in rat colonocytes 1α,25(OH)$_2$D$_3$ activates Src by both phosphorylation-dependent and phosphorylation-independent mechanisms [Khare et al., 1997]. A similar mechanism for Src activation by the EGF receptor, independently of Src dephosphorylation, has been proposed by Stover et al. [1995]. Moreover, as postulated for rat colonocytes [Khare et al., 1997], the possibility that 1α,25(OH)$_2$D$_3$ may also directly activate the kinase should be not completely excluded, al-

though such mode of action in muscle cells is not supported by the fact that the $1\alpha,25(OH)_2D_3$-induced elevation in Src activity was paralleled by the increases in tyrosine phosphorylation of the VDR and VDR-Src complex formation.

Knowledge on the role and biological significance of Src activity within the $1\alpha,25(OH)_2D_3$ non-genomic signaling pathway in muscle cells is still limited, but it was recently observed that activation of Src results in stimulation of the MAPK cascade and regulation of proliferation and differentiation of myoblasts in culture (Buitrago and De Boland, unpublished observations). In previous reports from our laboratory, we described that in chick skeletal muscle cells $1\alpha,25(OH)_2D_3$ rapidly modulates Ca^{2+} influx through activation of both L-type voltage-dependent and store-operated Ca^{2+} (SOC) channels [Vazquez et al., 1997b, 1998], by a mechanism which in part involves tyrosine kinase activity, particularly in the regulation of the SOC component of the cation influx pathway. As evidence exists for Src-dependent modulation of SOC influx in other cell types [Babnigg et al., 1997], further studies are required to address the role of $1\alpha,25(OH)_2D_3$-induced Src activation in the sterol-dependent SOC influx in muscle cells. Finally, the fact that $1\alpha,25(OH)_2D_3$ promotes complex formation between VDR and Src, not only expands the signaling capabilities of the sterol by linking its rapid actions with the intricate and versatile Src-related cascade, but also puts the attention onto the role of VDR itself in mediating some, if not all, of the non-genomic actions of $1\alpha,25(OH)_2D_3$.

ACKNOWLEDGMENTS

This research was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and the Comisión de Investigaciones Científicas de la Pcia. de Buenos Aires, Argentina.

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