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Involvement of Src in the Vitamin D Signaling in Human Keratinocytes

*Robert Gniadecki**

DEPARTMENT OF DERMATOLOGICAL RESEARCH, LEO PHARMACEUTICAL PRODUCTS, BALLERUP, AND DEPARTMENT OF DERMATOLOGY, UNIVERSITY OF COPENHAGEN, BISPEBJERG HOSPITAL, COPENHAGEN, DENMARK

ABSTRACT. 1,25-dihydroxyvitamin D_3 (VD) is a modulator of growth and differentiation of many cell types, including keratinocytes. We have recently shown in cultured keratinocytes that VD induces tyrosine phosphorylation of proteins involved in signal transduction, such as Shc. In an attempt to identify VD-responsive tyrosine kinases, we studied the effects of VD on the activity of the nonreceptor tyrosine kinase Src. Although VD did not stimulate Src activity in keratinocytes cultured in standard media containing 0.15 mM calcium, preincubation of the cells with 1.8 mM Ca^{2+} caused a rapid activation of Src in response to VD (10^{-8}–10^{-7} M). Elevation of calcium concentration alone caused an increase in Src activity as well, but the peak of Src activity was delayed (60 min vs. 15 min) and approximately 2-fold lower in comparison with VD-treated cells. VD treatment also induced tyrosine dephosphorylation of Src and a formation of an Src-Shc-Grb2 complex. Taken together, these findings imply that Src is involved in VD signaling in keratinocytes. BIOCHEM PHARMACOL 55;4:499–503, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. cholecalciferols; nonreceptor protein kinases; Src; signal transduction; calcium; keratinocytes

The hormonal form of vitamin D_3 (1,25-dihydroxyvitamin D_3, VD)† plays an important role in the regulation of keratinocyte growth and differentiation. The capability of VD and the synthetic vitamin D analogues to suppress cell growth and induce differentiation has been well documented in cultured keratinocytes [1, 2] and exploited in the therapy of psoriasis [3, 4]. However, in keratinocytes induced to differentiate by incubation with high calcium concentrations or by suspension in the semisolid media, stimulation rather than inhibition of DNA synthesis by VD has been observed [5]. A similar stimulation of epidermal proliferation also occurs after the topical application of VD and a range of vitamin D analogues in hairless mice [6, 7].

The signaling pathways involved in the regulation of keratinocyte growth and differentiation by VD have not been fully elucidated. The traditional view of the vitamin D receptor acting as a transcription factor (the 'genonic' model) has been recently challenged by showing that some of the effects of VD are likely to be mediated by second messengers such as protein kinase C [8–14], inositol triphosphate and calcium [15–17], phosphatidylcholine metabolites [18], or cyclic nucleotides [19]. It has been argued that activation of the second messengers is independent of the transcription factor activity of the vitamin D receptor and therefore referred to as the 'nongenomic' events [20]. We have recently suggested that modulation of keratinocyte growth by VD depends on the activation of signaling pathways dependent on tyrosine phosphorylation [21]. Treatment with VD causes tyrosine phosphorylation of the adapter molecule Shc which interacts with Grb2 and mSos. These events may be responsible for activation of downstream signaling, such as the mitogen-activated protein kinase cascade [22]. Nonreceptor kinases of the Src family, Src, Lck, Fyn, or Lyn, phosphorylate a range of signaling molecules on tyrosine residues and are responsible for generation and propagation of cellular signals [23, 24]. To test the possibility that Src may be involved in VD signaling, we have investigated whether this hormone activates Src and its association with Shc and Grb2.

MATERIALS AND METHODS

Cell Culture and Treatment

Cryopreserved human neonatal keratinocytes were purchased from Clonetics and cultured in the keratinocyte growth medium which contained 0.15 mM $CaCl_2$, 100 ng/mL recombinant human epidermal growth factor (EGF) and 0.1% bovine pituitary extract (Clonetics). Cultures were maintained at 37° in a humidified atmosphere of 5% CO_2 and passaged at 80–90% confluence after trypsinisation with 0.2 mL/cm^2 0.05% trypsin with 0.02% EDTA solution. Second and third passage cells were used for the studies. Because EGF treatment stimulates Src activity in keratinocytes, the cultures were starved in the medium lacking EGF for 3 days before experiments. In some cases, the concentration of calcium was adjusted to 1.8 mM from

* Corresponding author: Robert Gniadecki, MD, PhD, Department of Dermatology D92, Bispebjerg Hospital, Bispebjerg Bakke 23, 2400 Copenhagen NV, Denmark. TEL. +45 35 31 60 05; FAX: +45 35 31 60 10.

† *Abbreviations:* EGF, epidermal growth factor; MAPK, mitogen-activated protein kinase; VD, 1,25-dihydroxyvitamin D_3.

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a 0.1 M stock $CaCl_2$ in water. VD (Chemical Research Department, Leo Pharmaceutical Products) was dissolved in isopropanol and added for the indicated periods of time to culture medium at the final VD concentration of 10^{-9} or 10^{-7} M. In the control experiments, the isopropanol vehicle was used alone. The final concentration of isopropanol was kept constant at 0.1% (v/v).

Immunoprecipitation

Immunoprecipitations were performed under denatured or native conditions, essentially as described [21]. Briefly, cell extracts containing in total 0.5 mg (denatured conditions) or 3 mg (native conditions) protein were immunoprecipitated with 3 μg of the monoclonal antiphosphotyrosine PY20 antibody, monoclonal anti-Shc antibody (both from Transduction Laboratories) or the polyclonal sheep antibody against Src (Affiniti) for 18 hr at 4°. Three micrograms of appropriate second antibodies (rabbit anti-mouse or rabbit anti-sheep, Dako) were added for 1 hr at 4° and complexes were adsorbed to protein A-Sepharose (Pharmacia). Washed complexes were analyzed with Western blotting [25] by probing the nitrocellulose membranes with anti-Src, anti-Shc, or anti-Grb2 (Transduction Laboratories) antibodies. Peroxidase-labeled anti-mouse or anti-sheep antisera (both from Dako) were used as the second antibodies. The enhanced chemiluminescent method (Amersham International) was used to develop the blots.

Immunocomplex Kinase Assay

Cells were lysed under native conditions for 60 min at 4°. Aliquots of supernatant containing 1 mg of protein were immunoprecipitated with 3 μg of the anti-Src polyclonal antibody. The precipitated complexes were washed 3 times in the lysis buffer and the kinase buffer as described [21] and incubated at 37° for 30 min in the kinase buffer containing 25 μM ATP, 2.5 μCi of [^{32}P]ATP (Amersham International) and 1 mg/mL of enolase (Sigma) as the exogenous substrate for Src [26]. Phosphorylated product separated from the free isotope on phosphocellulose filters (Pierce) was counted in a scintillation counter.

Statistics

Data are presented as means (n = 3) with standard deviations. The two-way analysis of variance (ANOVA) was used to determine differences in Src activation curves between different groups. $P \leq 0.05$ was considered significant.

RESULTS

VD Stimulates Src Activity

The activity of Src tyrosine kinase was stimulated by 100 ng/mL EGF ($P < 0.001$, ANOVA, Fig. 1) and thus for subsequent experiments the cells were preincubated in the

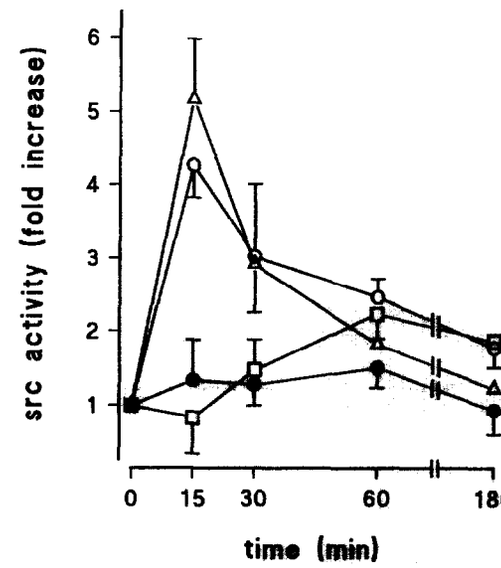

FIG. 1. Effects of VD on Src activity in cultured keratinocytes. Normal human keratinocytes were incubated for 3 days in the EGF-deficient keratinocyte growth medium containing 0.15 mM Ca^{2+}. Keratinocytes were stimulated with 10^{-8} M VD (*closed circles*), 10^{-8} M VD and 1.8 mM $CaCl_2$ (*open circles*), 1.8 mM $CaCl_2$ (*squares*), or 100 ng/mL EGF (*triangles*) for indicated periods of time. Cell extracts were precipitated under native conditions with the anti-Src sheep polyclonal antibody, and the tyrosine kinase activity in the precipitates was assayed using enolase as the exogenous substrate and [^{32}P]ATP as the source of phosphate. The radioactive product was separated on phosphocellulose and its activity counted in a scintillation counter. The results are presented as the fold change in radioactivity in comparison to the 0 min control. Mean values (n = 3) with SD are shown.

EGF-free media. Calcium switch (elevation of Ca^{2+} concentration from the standard 0.15 mM to 1.8 mM) caused a slow, moderate but significant ($P < 0.01$) increase in Src activity which peaked after approximately 60 min. Addition of VD at 10^{-8} M (Fig. 1) or 10^{-7} M (not shown) to keratinocytes grown at low calcium concentrations did not significantly affect Src activity. In contrast, when VD was added to the keratinocytes pretreated for 2 hr with 1.8 mM Ca^{2+}, a significant ($P < 0.001$) and rapid stimulation of tyrosine kinase activity was measured in Src-containing precipitates (Fig. 1). There was no difference in the magnitude of Src activation after 10^{-8} M and 10^{-7} VD. 10^{-9} M VD did not have any effect on Src activity at any calcium concentrations.

VD Induces Tyrosine Dephosphorylation of Src

The increase in the activity of Src could depend on the increase in the amount of Src protein or the activation of existing Src molecules [27]. The former possibility was excluded in view of the fact that the effect of VD was relatively rapid (minutes) and there was no observable increase in Src protein content in Western blotting upon VD exposure (10^{-8} M–10^{-7} M) for the periods up to 120 min (not shown). To analyze the tyrosine phosphorylation

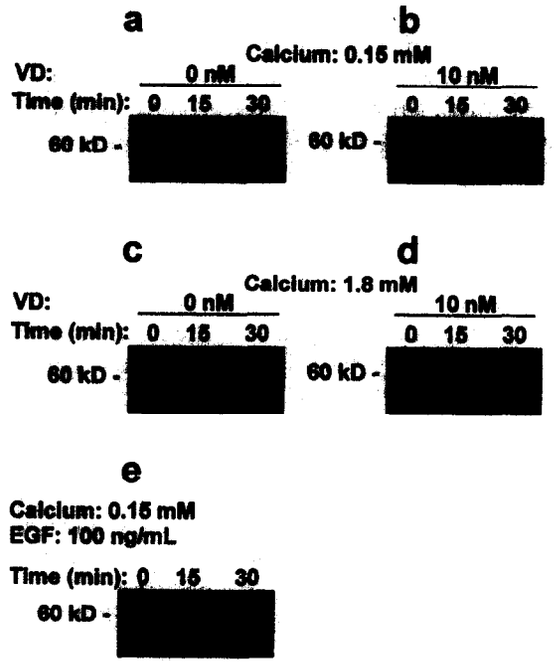

FIG. 2. Effects of VD on the tyrosine phosphorylation of Src Cells were cultured as in the legend for Fig. 1 and stimulated for the indicated periods of time (0, 15, 30 min) with: *a*) the EGF-deficient keratinocyte growth medium with 0.15 mM Ca^{2+}; *b*) the same medium containing 10^{-8} M VD; *c*) the EGF-deficient keratinocyte growth medium with 1.8 mM Ca^{2+}; *d*) the same medium containing 10^{-8} M VD; and *e*) the keratinocyte growth medium containing 100 ng/mL EGF and 0.15 mM Ca^{2+}. Cell lysates were immunoprecipitated with the antiphosphotyrosine antibody under denatured conditions and probed with the anti-Src antibody. Positions of the tyrosine-phosphorylated Src protein bands (60 kDa) are indicated. The result of a representative experiment is shown; all blots were developed under identical conditions.

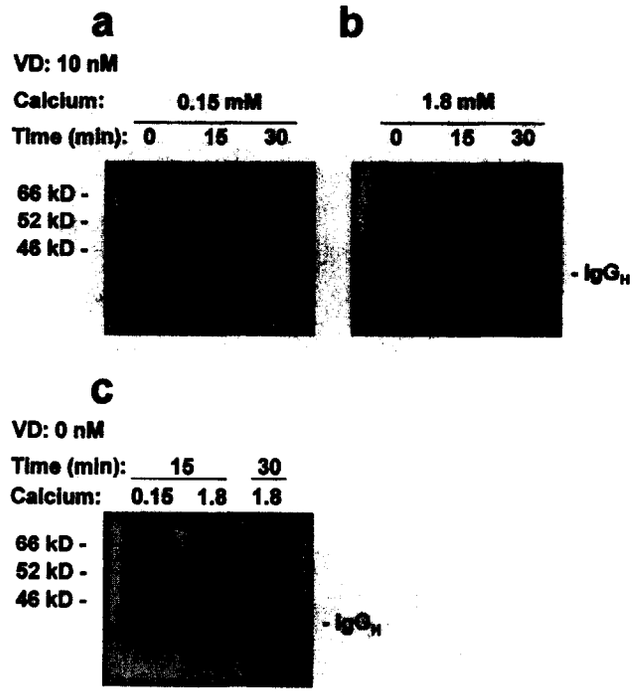

FIG. 3. Formation of complexes between Src and Shc. Cells were cultured as in the legend for Fig. 1 and stimulated for the indicated periods of time (0, 15, 30 min) with the EGF-deficient keratinocyte growth medium containing 10^{-8} M VD and: *a*) 0.15 mM Ca^{2+} or *b*) 1.8 mM Ca^{2+}. A control experiment where cells were stimulated for 15 or 30 min with the keratinocyte growth medium containing 0.15 or 1.8 mM $CaCl_2$ without VD is shown in (*c*). Cell lysates were immunoprecipitated with the anti-Src antibody under native conditions and probed with the anti-Shc antibody. Positions of three Shc species ($p46^{Shc}$, $p52^{Shc}$, and $p66^{Shc}$) are indicated. IgG_H shows the position of the immunoglobulin heavy chain.

of Src, we immunoprecipitated cell extracts under denaturing conditions with the antiphosphotyrosine antibody and probed the blots with the anti-Src antibody. As shown in Fig. 2, treatment with 10^{-8} M VD caused tyrosine dephosphorylation of Src. This effect was most pronounced for the cells precultured with 1.8 mM Ca^{2+}, although a slow Src dephosphorylation was also seen in the cells cultured with low concentrations of calcium.

Complex Formation between Src, Shc and Grb2

Tyrosine phosphorylated Shc serves as an adapter molecule transmitting the signal from activated Src [22]. To analyze whether VD stimulated the formation of complexes between Src and Shc, we immunoprecipitated cell extracts with anti-Src antibody at native conditions and probed the blots with the anti-Shc antiserum (Fig. 3). In the cells preincubated with 1.8 mM Ca^{2+}, treatment with 10^{-8} VD for 15–30 min induced complex formation between Src and $p66^{Shc}$, $p52^{Shc}$, and $p46^{Shc}$. At 0.15 mM Ca^{2+}, treatment with 10^{-8} M VD caused a formation of Src-Shc complexes at 15 min, but those complexes could not be detected 30 min after VD treatment. Control experiments were done with the reverse order of added antibodies: cell lysates were precipitated with anti-Shc and blotted with the anti-Src antibody. As shown in Fig. 4, treatment with 10^{-8} M VD clearly increased the amount of Src in the anti-Shc precipitates from the extracts of the cells precultured with 1.8 mM Ca^{2+}, whereas only a weak Src protein band was seen in the lysates from the cells incubated at 0.15 mM Ca^{2+}. The Grb2 protein was present in the anti-Shc precipitates at 1.8 mM Ca^{2+}, but was undetectable at 0.15 mM Ca^{2+} (Fig. 4).

DISCUSSION

Nonreceptor tyrosine kinases are believed to act as signal transduction molecules which link up the signals originating from various receptors with appropriate downstream effector pathways, such as the mitogen-activated protein kinase pathway [23, 24, 28, 29]. Both the receptor tyrosine kinases (such as the EGF receptor) and the receptors lacking an intrinsic tyrosine kinase activity (e.g. G-protein-coupled receptors or antigen receptors on T and B lymphocytes) are capable of activating the enzymatic activity of Src or other members of the Src kinase family (Fyn, Lck,

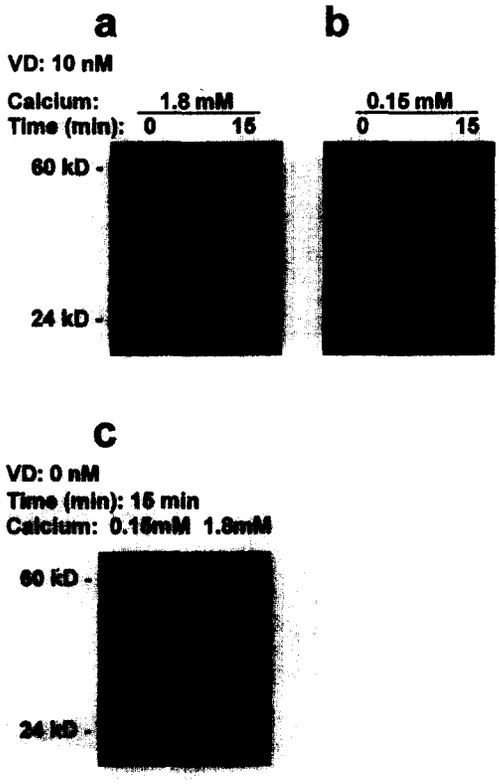

FIG. 4. Formation of complexes between Shc and Src or Grb2. Cells were cultured as in the legend for Fig. 1 and stimulated for 0 min or 15 min with the EGF-deficient keratinocyte growth medium with 10^{-8} M VD and: *a*) 1.8 mM Ca^{2+} or *b*) 0.15 mM Ca^{2+}. Panel (*c*) shows the results obtained from the cells stimulated for 15 min with the keratinocyte growth medium containing 0.15 mM or 1.8 mM Ca^{2+}. Cell lysates were immunoprecipitated with the anti-Shc antibody under native conditions and probed with the anti-Src antibody and anti-Grb2 antibody. Positions of the Src protein bands (60 kDa) and the Grb2 protein (24 kDa) are indicated.

Lyn) [23, 24]. Our results strongly suggest that VD, despite being a steroid hormone, also activates Src in normal human keratinocytes.

Very recently and independently of our results, Khare *et al.* [30] have shown that VD activates Src in another cell type, the colonocyte. An increase in Src activity was seen after 9 min of VD treatment, which is comparable with the 15 min observed in keratinocytes in this study. Moreover, Khare *et al.* observed another peak of Src activity that occurred in colonocytes 1 min after VD application. The question whether a similar pattern of Src activation occurs in keratinocytes was not resolved in this study, because in our hands Src activity was highly variable 1–5 min after VD exposure; therefore, conclusive measurements of the enzymatic activity of this kinase were not possible.

The VD-induced activation of Src was modulated by the calcium concentration in the culture media. The reason why the activation of Src took place only in the cells preincubated at high (1.8 mM) calcium concentration is not yet clear. However, a synergism between the effects of calcium and VD is not unique for Src and has been observed in other circumstances. For example, elevation of calcium concentration potentiates the effects of VD on keratinocyte differentiation [31] and activation of Raf-mitogen-activated protein kinase cascade [21]. One of the possible explanations for this synergism is that calcium treatment increases the proportion of mature keratinocytes which differ from their immature counterparts in their response to VD [5, 32].

The tyrosine kinase activity of Src is negatively regulated by 527-tyrosine phosphorylation mediated by the Src tyrosine kinase Csk [33]. However, tyrosine phosphorylation of Tyr-416 is stimulatory due to stabilisation of the Src catalytic domain in the active conformation (reviewed in [27]). In physiological situations, Src activation occurs mostly via dephosphorylation of Tyr-527. VD caused the tyrosine dephosphorylation of Src, which was more rapid in the cells pretreated with 1.8 mM Ca^{2+}. Although we did not attempt to determine the phosphorylation state of Tyr-416 and Tyr-527 separately, it is likely that the activatory dephosphorylation in the latter position took place. A similar pattern of Src dephosphorylation has also been described by Khare *et al.* [30] in colonocytes. It must be noted, however, that the dephosphorylation process was not the only mechanism of Src activation by VD in keratinocytes, because the profound dephosphorylation of the molecule 30 min after VD exposure was not accompanied by an increase in Src enzymatic activity in the cells cultured at 0.15 mM Ca^{2+}.

Knowledge as to the role of Src in keratinocyte biology is very limited, but there is evidence that nonreceptor protein kinases from the Src family are involved in the regulation of keratinocyte differentiation. For example, activation of Src and Fyn accompanied by the inactivation of Yes was observed during calcium, ionophore- or phorbol ester-induced keratinocyte differentiation [26, 34–36]. However, the question of the biological significance of the observed VD-induced Src activation could not be definitely resolved in this study. Indirect evidence supporting the possibility of the role of Src in VD signal transduction has been obtained by analysing the interactions between Src and other signal transducing molecules. It has been shown here that Src forms complexes with Shc and Grb2 in the VD-treated cells. Analogous to the case of Src activation, calcium pretreatment enhanced the effects of VD: the amount of Src, Shc, and Grb2 interacting with one another was higher and the Src-Shc-Grb2 complexes were more stable in the cells cultured in high calcium environment than in the cells incubated in standard media. It remains to be investigated whether these complexes are functional and capable of downstream signal propagation.

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