1,25 Dihydroxyvitamin D_3 Stimulates Phospholipase C-γ in Rat Colonocytes: Role of c-Src in PLC-γ Activation

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Abstract

Our laboratory has previously demonstrated that 1,25-dihydroxyvitamin D_3 (1,25$[OH]_2D_3$) rapidly stimulated polyphosphoinositide (PI) hydrolysis, raised intracellular Ca^{2+}, and activated two Ca^{2+}-dependent protein kinase C (PKC) isoforms, PKC-α and -β_{II} in the rat large intestine. We also showed that the direct addition of 1,25$(OH)_2D_3$ to isolated colonic membranes failed to stimulate PI hydrolysis, but required secosteroid treatment of intact colonocytes, suggesting the involvement of a soluble factor. Furthermore, this PI hydrolysis was restricted to the basal lateral plasma membrane of these cells. In the present studies, therefore, we examined whether polyphosphoinositide-phospholipase C-γ (PI-PLC-γ), a predominantly cytosolic isoform of PI-PLC, was involved in the hydrolysis of colonic membrane PI by 1,25$(OH)_2D_3$. This isoform has been shown to be activated and membrane-associated by tyrosine phosphorylation. We found that 1,25$(OH)_2D_3$ caused a significant increase in the biochemical activity, particulate association, and the tyrosine phosphorylation of PLC-γ, specifically in the basal lateral membranes. This secosteroid also induced a twofold increase in the activity of Src, a proximate activator of PLC-γ in other cells, with peaks at 1 and 9 min in association with Src tyrosine dephosphorylation. 1,25$(OH)_2D_3$ also increased the physical association of activated c-Src with PLC-γ. In addition, Src isolated from colonocytes treated with 1,25$(OH)_2D_3$, demonstrated an increased ability to phosphorylate exogenous PLC-γ in vitro. Inhibition of 1,25$(OH)_2D_3$-induced Src activation by PP1, a specific Src family protein tyrosine kinase inhibitor, blocked the ability of this secosteroid to stimulate the translocation and tyrosine phosphorylation of PLC-γ in the basolateral membrane (BLM). Src activation was lost in D deficiency, and was reversibly restored with the in vivo repletion of 1,25$(OH)_2D_3$. These studies demonstrate for the first time that 1,25$(OH)_2D_3$ stimulates PLC-γ as well as c-Src in rat colonocytes, and indicate that PLC-γ is a direct substrate of secosteroid-activated c-Src in these cells. (*J. Clin. Invest.* 1997. 99:1831–1841.) Key words: 1,25-dihydroxycholecalciferol • nonreceptor tyrosine kinase • basolateral membrane • vitamin D deficiency

Introduction

In addition to its well-established role in the regulation of mineral metabolism, it has become increasingly clear that 1,25-dihydroxyvitamin D_3 (1,25$[OH]_2D_3$), the major active metabolite of vitamin D_3, causes a number of other important biochemical and physiological cellular effects. We have previously shown, for example, that this secosteroid is mitogenic in rat colonocytes (1). In these cells, moreover, our laboratory has previously demonstrated that 1,25$(OH)_2D_3$, in a dose-dependent manner (10^{-9}–10^{-7} M), rapidly stimulated membrane polyphosphoinositide (PI)[1] breakdown, which in turn, raised intracellular calcium ($[Ca^{2+}]_i$), inhibited Na^+/H^+ exchange, and induced the translocation of protein kinase C (PKC) from the cytosolic to particulate fraction of these cells (1). These latter secosteroid-induced biochemical effects were lost in colonocytes from vitamin D-deficient rats, and were restored with the in vivo repletion of these rats with 1,25$(OH)_2D_3$ (2). In more recent studies, our laboratory has demonstrated that the aforementioned PI hydrolysis induced by 1,25$(OH)_2D_3$ in colonocytes from vitamin D-sufficient rats was restricted to the basal lateral plasma membrane of these cells (3), and required intact colonocytes rather than isolated membranes (4). Furthermore, this PI hydrolysis, which generated 1,2-diacylglycerol (DAG) and increased $[Ca^{2+}]_i$, was associated with activation of two Ca^{2+}-dependent isoforms of PKC present in these cells, PKC-α (5) and -β_{II} (6).

The requirement for intact colonocytes rather than isolated membranes for 1,25$(OH)_2D_3$ to elicit polyphosphoinositide hydrolysis (4) suggested that recruitment of a soluble factor may be necessary to elicit this response. These cells express several isoforms of phosphoinositide phospholipase C (PI-PLC), including PLC-γ (4), PLC-δ (4) and PLC-β_3 (unpublished observations), but only PLC-γ is predominantly soluble and would presumably require recruitment to the plasma membrane if it were involved in this PI hydrolysis. In other cell types, activation of PLC-γ, in addition to translocation from the soluble to the membrane fraction, also involves tyrosine phosphorylation of this PLC isoform. In this regard, in preliminary studies, our laboratory has noted that 1,25$(OH)_2D_3$ induced the tyrosine phosphorylation of a number of proteins in rat colonocytes (7), in agreement with recent findings of this secosteroid's actions in acute promyelocytic NB4 cells (8). In the present studies, we examined the effects of 1,25$(OH)_2D_3$

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1. *Abbreviations used in this paper:* BLM, basolateral membrane; PI, polyphosphoinositide; PKC, protein kinase C; PLC, phospholipase C.

on the tyrosine phosphorylation, subcellular distribution, and biochemical activity of PI-PLC-γ in intact colonocytes.

Since c-Src, a widely expressed nonreceptor tyrosine kinase, has been shown to be involved in agonist-induced PLC-γ activation in other intact cells (9), as well as to phosphorylate PLC-γ directly in a cell-free system (10), in the present study it was also of interest to determine whether $1,25(OH)_2D_3$ was capable of activating c-Src, and, if so, whether this secosteroid might induce the association of c-Src with PLC-γ. Furthermore, as noted above, the coupling of $1,25(OH)_2D_3$ to polyphosphoinositide hydrolysis and PKC activation in rat colonocytes has been shown to depend upon the vitamin D status of these animals. In particular, while in vitamin D deficiency, $1,25(OH)_2D_3$ could no longer stimulate these events, this coupling was restored with in vivo repletion of this secosteroid (2). We, therefore, also examined the effect of vitamin D status on the ability of $1,25(OH)_2D_3$ to activate c-Src. The results of these studies and a discussion of their significance, therefore, serve as the basis for the current report.

Methods

Materials. EDTA, urea, poly-L-proline, cyanogen bromide-activated Sepharose, leupeptin, and protein A and G agarose were purchased from Sigma Chemical Co. (St. Louis, MO). EGTA was purchased from Fluka AG (Buchs, Switzerland). Ammonium persulfate, TEMED, and 2-mercaptoethanol were obtained from Serva Biochemicals (Hauppauge, NY). 1α,25-dihydroxyvitamin D_3 was purchased from Steroids Limited (Chicago, IL). 1β,25-dihydroxyvitamin D_3 and 25-hydroxyvitamin D_3 were provided by Dr. M.R. Uskokovic (Hoffmann-La Roche, Nutley, NJ). Electrophoretic grade acrylamide, bisacrylamide, Tris, SDS, and prestained molecular weight markers were purchased from Bio-Rad Labs (Richmond, CA). Kodak (Rochester, NY) supplied the X-OMAT™ AR film. The protease inhibitors, 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) and aprotinin, were obtained from Calbiochem Corp. (San Diego, CA). Monoclonal PY-20 anti-phosphotyrosine antibodies, polyclonal anti–Src antibodies (SC-19) and anti–PLC-γ antibodies (SC-81) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Upstate Biotechnology Inc., (Lake Placid, NY) provided immunoprecipitating monoclonal anti–Src antibodies (05–184), monoclonal PLC-γ antibodies (05–163), Src peptide substrate derived from $p34^{cdc-2}$, and anti–phosphotyrosine 4G–10 antibodies. PVDF membranes (Immobilon-P) were purchased from Millipore Inc. (Bedford, MA). The enhanced chemiluminescence kit for Western blotting protein detection and the peroxidase-coupled sheep anti–mouse and donkey anti–rabbit antibodies were supplied by Amersham Corp. (Arlington Heights, IL). Phosphatidylcholine and phosphatidylethanolamine were purchased from Avanti Polar Lipids (Alabaster, AL), and phosphatidylinositol 4,5 bisphosphate, Staphylococcus aureus V8 proteinase, and *N*-octyl-β-D-glucoside were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Okadaic acid was purchased from LC Laboratories (Woburn, MA). [^{3}H]phosphatidylinositol 4,5 bisphosphate and [γ^{32}P]ATP were obtained from New England Nuclear (Boston, MA). Expired human platelets were provided by the Blood Bank of the University of Chicago. PP1, a selective protein tyrosine kinase inhibitor of the Src family, was provided by Pfizer Central Research (Groton, CT). Unless noted, all other reagents were obtained from Sigma Chemical Co. or Fisher Scientific Co. (Springfield, NJ) and were of the highest purity available.

Animals. Male albino Sprague-Dawley rats, initially weighing 40–60 g, were obtained from Harlan Sprague Dawley, Inc. (Madison, WI) and maintained on a vitamin D-sufficient diet. Weanlings rats from partially vitamin D-deficient mothers were fed a vitamin D-deficient diet, and were maintained in a facility devoid of fluorescent light. Diets were obtained from ICN Biochemicals Inc. (Cleveland, OH). By design, for the vitamin D-deficient group, the dietary contents of calcium and phosphorous were raised to 1.8 and 0.8%, respectively, to prevent the development of calcium and phosphorous depletion and secondary hyperparathyroidism (2). The compositions of these diets have previously been described in detail (11). After 12–15 wk, half of the latter group of animals were treated for 10 d with 10 ng/100 g body wt/d of $1,25(OH)_2D_3$ by mini-osmotic pumps implanted subcutaneously (Alzet mini-osmotic pump, model 2002; Alza Corporation, Palo Alto, CA), whereas the others received vehicle (5% ethanol in propylene glycol) as previously described (2). At death, sera was obtained from rats, and $25(OH)D_3$ and $1,25(OH)_2D_3$ levels were measured as described (2). As previously observed (5), rats in the D-deficient group all had $25(OH)D_3$ levels < 5 ng/ml. The levels of $1,25(OH)_2D_3$ in the D-repleted group were all in the normal range (5).

Isolation and treatment of intact rat colonocytes. Nonfasted rats were killed, and their colons were excised and flushed with cold saline containing 1 mM dithiothreitol. Isolated intact colonocytes were prepared by a technique that combined chelation of divalent cations with mild mechanical dissociation as previously described (1). Cells (10^6 cells/ml) were incubated at 37°C in Krebs-Ringer glucose (KRG) buffer containing 145 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 10 mM Hepes, pH 7.2, 10 mM glucose, and 2 mM $CaCl_2$ for the indicated times and concentrations of $1,25(OH)_2D_3$ or vehicle (0.05% ethanol). Where indicated, cells were preincubated with dimethylsulfoxide alone (DMSO, 0.05%, final concentration), or with 100 μM PP1 for 15 min, and then treated with ethanol or $1,25(OH)_2D_3$.

Immunoprecipitation and Western blotting of PLC-γ. In preliminary experiments, tyrosine phosphorylation of PLC-γ was maximal in the presence of 30 nM of this secosteroid, with no further increase with concentrations as high as 100 nM $1,25(OH)_2D_3$. Isolated intact colonocytes were therefore treated with vehicle (ethanol) or 30 nM $1,25(OH)_2D$, unless otherwise indicated. Whole cell lysates were prepared in an extraction buffer containing 50 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 1% NP-40, 1 mM sodium vanadate, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 0.1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), 10 μg/ml leupeptin, 1 mM L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone, 1 mM L-1-chloro-3-[4-tosylamido]-7-amino-2-heptanone-HCl, and 2 μg/ml aprotinin. Phosphotyrosine-containing proteins were immunoprecipitated with agarose-conjugated 4G-10 antibodies (Upstate Biotechnology Inc.), and PLC-γ abundance in the 4G-10 immunoprecipitates was assessed by Western blotting, using monoclonal anti–PLC-γ antibodies (Upstate Biotechnology Inc.) detected by an enhanced chemiluminescence system and quantified by scanning densitometry as previously described (12). Proteins were measured by amido black staining of samples spotted on nitrocellulose with bovine serum albumin as the standard (13).

To examine the effect of $1,25(OH)_2D_3$ on PLC-γ in the antipodal plasma membranes, isolated colonocytes were stimulated with vehicle or with 30 nM secosteroid for 30 s at 37°C, and then brush border and basal lateral plasma membranes were prepared as previously described (14). Appropriate specific markers for basal lateral, brush border, and intracellular membranes were assayed, which confirmed very low levels of cross contamination in each of these antipodal preparations in agreement with previous studies (14). To quantify changes in PLC-γ abundance, membranes were solubilized in SDS-containing buffer, and proteins (40 μg) were probed by Western blotting for PLC-γ. To determine changes in PLC-γ tyrosine phosphorylation, the membranes were solubilized in the extraction buffer and an aliquot (150 μg protein) incubated with 10 μg of PY-20 anti–phosphotyrosine antibodies for 2 h. Immune complexes were collected by incubation for 1 h with 50 μl of a 50% mixture of protein A and protein G agarose and 5 μg goat anti–mouse antibodies (Kirkegaard & Perry Laboratories, Gaithersburg, MD), followed by centrifugation. Immunoprecipitates were washed three times in extraction buffer, and antiphosphotyrosine-immunoprecipitated PLC-γ was detected by Western blotting using PLC-γ antibodies as described for whole cell lysates.

Measurement of PLC-γ biochemical activity. Isolated colonocytes were stimulated for 30 s with ethanol or 30 nM $1,25(OH)_2D_3$ at 37°C, and then basal lateral plasma membranes were prepared (14). Membranes were solubilized at 4°C for 30 min in an extraction buffer containing 25 mM Tris-HCl, pH 7.4, 1% Triton X-100, 10% glycerol, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium vanadate, 20 μM AEBSF, and 10 μg/ml leupeptin. After centrifugation at 15,000 *g* for 10 min, PLC-γ was immunoprecipitated from the soluble fraction (250 μg protein) with 2 μg polyclonal anti–PLC-γ antibodies (SC-81) for 1 h followed by an overnight incubation with 40 μl (50% wt/vol) protein A agarose. The immunoprecipitates were washed three times by resuspension in PLC assay buffer containing 5 mM Tris-HCl, pH 7.0, 75 mM KCl, 0.416 mM $CaCl_2$, 0.4 mM EGTA, and 0.1 mM NaN_3. The free ionized Ca^{2+} was calculated to be ~ 25 μM (15). The phospholipase C activity of immunoprecipitated PLC-γ was assayed as described previously for the purified enzyme (16) at 37°C for 60 min in PLC buffer (100 μl final volume) containing 0.5 mM dithiothreitol and small unilammelar liposomes. The liposomes were prepared by sonication, and were composed of 30 μM [^{3}H] PIP_2 (700 cpm/μl), 50 μM phosphatidylcholine, 50 μM phosphatidylethanolamine and 10 μM profilin (final concentrations). Profilin was purified to homogeneity from human platelets by polyproline affinity chromatography (17), and was preincubated (final concentration 10 μM) with the sonicated liposomes.

Immunoprecipitation and Western blotting of c-Src. Following treatment with vehicle (ethanol) or $1,25(OH)_2D_3$ for the indicated times and concentrations, cells were rapidly chilled by the addition of 10 vol of ice cold calcium-free KRG buffer containing 2 mM EGTA. Colonocytes were collected and lysed in a buffer containing 50 mM Tris-HCl, pH 7.0, 5% Nonidet P-40, 1 mM EDTA, 10% glycerol, 0.1 mM L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone, 0.1 mM L-chloro-3-[4-tosylamido]-7-amino-2-heptanone-HCl, 10 μg/ml leupeptin, 1 mM sodium vanadate, 100 μM AEBSF, 10 μg/ml aprotinin, 50 mM β-glycerophosphate, 10 mM *p*-nitrophenylphosphate and 10 nM okadaic acid. After centrifugation at 10,000 *g* for 5 min, the soluble fraction (250 μg protein, 1 mg/ml) was incubated with 2 μg of anti–Src monoclonal antibodies (Upstate Biotechnology Inc., 05–184) for 2 h, and was then collected with 50 μl protein G agarose and washed 3× in lysis buffer. For measurement of c-Src protein expression and tyrosine phosphorylation in the immunoprecipitates, samples were solubilized in a modified Laemmli buffer under nonreducing conditions to prevent interference by immunoglobulin heavy chain chemiluminescence. Polyclonal (SC-19; Santa Cruz Biotechnology), or monoclonal (mAB327; provided by Dr. Joan Brugge, ARIAD Pharmaceuticals, Cambridge, MA) anti–Src antibodies were used to identify c-Src by Western blotting with comparable results. To investigate changes in Src tyrosine phosphorylation, colonocytes were treated with vehicle or $1,25(OH)_2D_3$ and c-Src subsequently immunoprecipitated from lysates (50–70 μg protein). The immunoprecipitates were separated by SDS-PAGE under nonreducing conditions on a 10% resolving gel, and were probed by Western blotting with a mixture of monoclonal anti–phosphotyrosine antibodies, PY-20 and 4G-10.

c-Src kinase assay. Src immunoprecipitates were assayed for kinase activity at 30°C for 20 min by autophosphorylation in the presence of [γ^{32}P]ATP, or by ^{32}P-incorporation into a peptide substrate derived from $p34^{cdc-2}$, cdc-2 (6–20) NH_2 (18), in a kinase buffer containing 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, and either 2 μM ATP (1 μCi/assay) alone (Src autophosphorylation), or with 50 μM ATP (2 μCi/assay) and 250 μM $p34^{cdc-2}$ peptide substrate. In autophosphorylation experiments, the reactions were terminated by the addition of SDS buffer that did not contain reducing agents to prevent immunoglobulin heavy chain interference. Samples were separated on a 10% resolving gel, and Src bands were identified by autoradiography and quantified by scanning densitometry. In experiments employing the synthetic peptide substrate, the reactions were terminated by spotting the supernatant on P81 phosphocellulose strips (which were washed three times in 75 mM phosphoric acid), and the radioactivity was quantified by Cherenkov counting. Parallel immunoprecipitates were probed for c-Src abundance to ensure comparable kinase mass in samples from control, and $1,25(OH)_2D_3$-treated cells. To examine the ability of $1,25(OH)_2D_3$ to directly activate c-Src, multilamellar liposomes were prepared composed of phosphatidylcholine (750 μM) alone, or containing vitamin D_3 analogues. Colonic c-Src was immunoprecipitated from unstimulated rat colonocytes, and was preincubated in the Src kinase buffer (35 μl) with multilamellar liposomes (10 μl) for 10 min at 30°C. Src kinase activity was measured using the $p34^{cdc-2}$-derived peptide as previously described (18).

In vitro phosphorylation of exogenous PLC-γ by colonic Src. Colonocytes were stimulated with vehicle or 30 nM $1,25(OH)_2D_3$ for 1 min, and Src was immunoprecipitated as described above for Src kinase activity. PLC-γ was immunoprecipitated from a soluble fraction of rat brain. Immunoprecipitates of colonic c-Src and rat brain PLC-γ were mixed, and PLC-γ was phosphorylated under conditions described above for c-Src autophosphorylation. The immunoprecipitated proteins were resolved by SDS-PAGE, and an autoradiogram was prepared. ^{32}P-labeled PLC-γ was identified from the autoradiogram and excised from the gel. The radioactivity was quantified by Cherenkov counting, and was expressed as percentage above control.

Coimmunoprecipitation of c-Src and PLC-γ. To investigate the association of c-Src with PLC-γ in rat colonocytes, we followed conditions previously described (19), which successfully preserved the interaction of these proteins in rat 3Y1 fibroblasts. Specifically, isolated rat colonocytes were treated with 30 nM $1,25(OH)_2D_3$, or with vehicle for 30 s, and cells were broken by sonication in a lysis buffer containing 50 mM HEPES-NaOH, pH 7.4, 1 mM EDTA, 1 mM sodium vanadate, 0.2 mM AEBSF, 10 μg/ml aprotinin, 20 mM β-glycerophos-

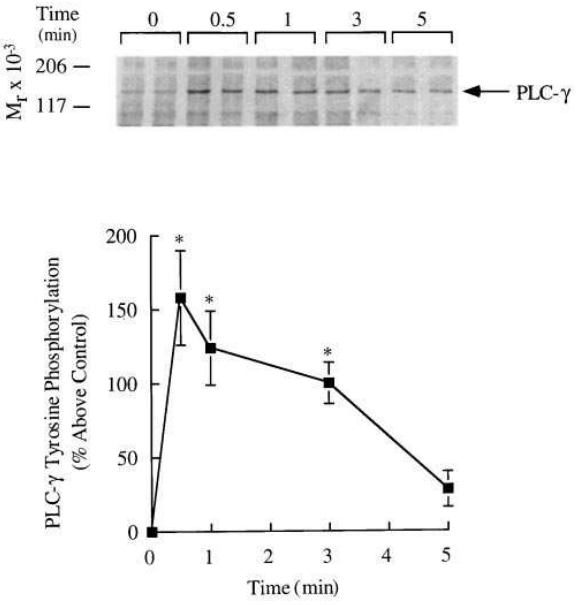


Figure 1. $1,25(OH)_2D_3$ stimulates PLC-γ tyrosine phosphorylation in isolated colonocytes. Cells were isolated and incubated with ethanol or 30 nM $1,25(OH)_2D_3$ for the indicated times, and then lysed. Tyrosine-phosphorylated proteins were immunoprecipitated with agarose-conjugated 4G-10 antibodies that were then probed for PLC-γ abundance by Western blotting as described in Methods. The 0 time point is an ethanol alone (0.05%) control, and treatment with ethanol for up to 5 min caused no changes in PLC-γ tyrosine phosphorylation. (*A*) Representative xerogram of three independent experiments, each in duplicate. (*B*) Densitometric analysis of PLC-γ tyrosine phosphorylation determined by quantitative Western blotting and expressed as the percentage±SEM above control. $P < 0.05$, compared with ethanol-treated cells ($n = 3$ independent experiments each in duplicate).

phate, 0.1 M NaCl and 10% glycerol. After centrifugation at 100,000 *g* for 30 min, the particulate fraction was extracted in 1 ml lysis buffer supplemented with 2% *N*-octyl-β-D-glucoside, and the centrifugation was repeated. PLC-γ was immunoprecipitated from the solubilized membrane fraction (500 μg protein), and the immunoprecipitates were probed by Western blotting for c-Src. Since c-Src was not detectable by Western blotting in the PLC-γ immunoprecipitates, a more sensitive assay involving c-Src autophosphorylation in the presence of [γ^{32}P]ATP was employed. As a control, c-Src was immunoprecipitated with Src antibodies and ^{32}P-labeled by autophosphorylation in the presence of [γ^{32}P]ATP as described above. The ^{32}P-labeled Src band and a comigrating ^{32}P-labeled band in the PLC-γ immunoprecipitates were each excised from the gel, and subjected to peptide mapping as described by Cleveland et al. (20).

Immunohistochemical localization of c-Src in rat colons from normal and vitamin D-deficient rats. Colonic samples from normal and vitamin D-deficient rats were harvested and fixed in 10% buffered formalin overnight. Fixed samples were paraffin-embedded, and 5-μm sections were prepared. Sections were deparaffinized, incubated in 0.2 M glycine for 30 min, and then blocked in 5% normal goat serum containing 3% bovine serum albumin for 30 min. Sections were incubated in either 2 μg/ml polyclonal anti-Src (Santa Cruz Biotechnology), or in 2 μg/ml control nonimmune rabbit Ig (Vector Laboratories, Inc., Burlingame, CA) for 30 min at room temperature followed by a subsequent incubation with biotinylated antirabbit IgG. Endogenous peroxidase activities were blocked with a 15-min exposure to 1% hydrogen peroxide. Sections were incubated with ABC reagent (Vector Laboratories, Inc.), and then signal-developed using 3,3′ diaminobenzidine as substrate. Sections were counterstained with hematoxylin, mounted in Merckoglas (EM Science, West Germany), and photographed using an Olympus BH-2 microscope at 400× magnification.

Statistical analysis. Results were expressed as Means±SEM. Data were analyzed using ANOVA (Dunnett's test), with values of $P < 0.05$ compared with control considered significant (21).

Results

1,25(OH)$_2$D$_3$ treatment of colonocytes stimulates PLC-γ tyrosine phosphorylation, and increases the association and activity of PLC-γ in the basal lateral membranes. The coupling of 1,25(OH)$_2$D$_3$ to polyphosphoinositide (PI) hydrolysis in intact cells (1), but not isolated membranes (4), suggested the possible involvement of a soluble isoform of PI-PLC in this phenomenon. Since PI-PLC-γ is predominantly soluble and regulated by tyrosine phosphorylation (22), we initially investigated the effects of 1,25(OH)$_2$D$_3$ on the tyrosine phosphorylation of this isoform. As shown in Fig. 1, we found that treatment of colonocytes with 30 nM 1,25(OH)$_2$D$_3$ (the maximally activating concentration) significantly increased PLC-γ tyrosine phosphorylation above the vehicle-treated (ethanol) control ($P < 0.05$). This effect was transient, with a detectable increase as early as 30 s, and a return to basal tyrosine phosphorylation levels by 5 min, in agreement with a similar time course for polyphosphoinositide hydrolysis in rat colonocytes that we have previously described (1). Earlier studies from our laboratory, moreover, have identified polyphosphoinositide hydrolysis in the basal lateral membranes (BLM), but not the brush border membranes (BBM) of rat colonocytes, indicating selective activation of a PI-specific PLC in the BLM (3). It was, therefore, of interest to investigate changes in the distribution and tyrosine phosphorylation of PLC-γ induced by 1,25(OH)$_2$D$_3$ in the antipodal plasma membranes of rat colonocytes. Compared with ethanol treatment, 30 nM 1,25(OH)$_2$D$_3$ caused a rapid translocation of PLC-γ to the basal lateral, but not the brush border membranes, with an increase of 240±20% in the BLM association of this isoform (Fig. 2), and an increase of 168±19% in the tyrosine phosphorylation of this BLM-associated PLC-γ (Fig. 3 *A*). Furthermore, as shown in Fig. 3 *B*, 1,25(OH)$_2$D$_3$ treatment of intact cells, compared with controls, stimulated PLC-γ biochemical activity by 375±42% in the basal lateral plasma membranes of these cells. These results indicate that the γ isoform of PLC contributes to the observed increase in 1,25(OH)$_2$D$_3$-stimulated polyphosphoinositide hydrolysis in the basal lateral membranes of rat colonocytes.

1,25(OH)$_2$D$_3$ stimulates c-Src activity in isolated rat colonocytes. As PLC-γ activation requires tyrosine phosphorylation (22) and involves c-Src in other cell types (9), we investigated the effect of 1,25(OH)$_2$D$_3$ on this kinase in rat colonocytes. Using c-Src specific monoclonal antibodies, we found that Src in these cells was predominantly membrane-associated, and could be extracted in 5% NP-40 and quantitatively immunoprecipitated with these antibodies (data not shown). As shown in Fig. 4, 1,25(OH)$_2$D$_3$ caused a time- and concentration-dependent increase in Src kinase activity in intact colonocytes, as measured by phosphorylation of a Src substrate, a synthetic peptide derived from p34^{cdc-2}. The time course of Src activation was biphasic, with peaks at 1 and 9 min following secosteroid treatment, increasing kinase activity 80–100% above control (Fig. 4 *A*). 1,25(OH)$_2$D$_3$ increased Src activity at secosteroid concentrations as low as 10 nM, and caused maximal activation at 30 nM, with no further increase in kinase activity with concentrations as high as 100 nM (Fig. 4 *B*). As described in Meth-

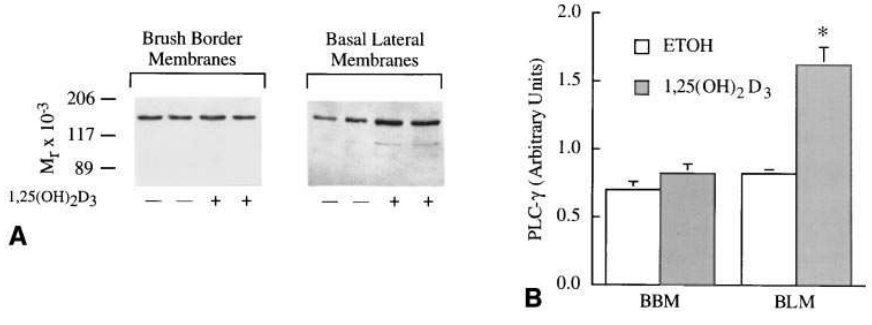


Figure 2. 1,25(OH)$_2$D$_3$ increases the association and tyrosine phosphorylation of PLC-γ to the basal lateral, but not the brush border, membrane. Colonocytes were isolated and incubated with ethanol (−), or with 30 nM 1,25(OH)$_2$D$_3$ (+) for 30 s, and then brush border (BBM) or basal lateral membranes (BLM) were prepared. BLM and BBM were solubilized in SDS-containing buffer, and the abundance of PLC-γ was measured by Western blotting as described in the Methods section. (*A*) Representative Western blot of three independent experiments, each in duplicate, demonstrating the effect of 1,25(OH)$_2$D$_3$ on PLC-γ in antipodal plasma membranes. (*B*) Quantitative changes in PLC-γ abundance in the BLM and BBM. Ethanol-treated cells, (□); 1,25(OH)$_2$D$_3$-treated cells, (■). *$P < 0.05$, when compared to ethanol-treated cells ($n = 3$ independent experiments in duplicate).

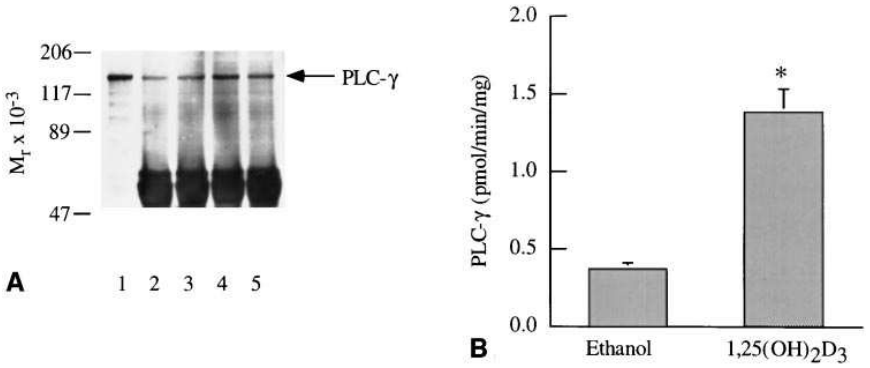


Figure 3. $1,25(OH)_2D_3$ stimulates PLC-γ tyrosine phosphorylation and enzyme activity in the basal lateral membrane. Isolated colonocytes were treated with either ethanol or 30 nM $1,25(OH)_2D_3$ for 30 s, and then BLM was prepared. The membranes were solubilized, and tyrosine-phosphorylated proteins were immunoprecipitated with PY-20 antibodies. In the antiphosphotyrosine immunoprecipitates, PLC-γ mass was determined by PLC-γ Western blotting, and enzyme activity was measured as described in Methods. (*A*) Representative xerogram of the effect of $1,25(OH)_2D_3$ on PLC-γ tyrosine phosphorylation in the BLM. Lane *1*: rat brain homogenate to confirm PLC-γ migration. Lanes *2* and *3*: vehicle-treated; Lanes *4* and *5*: $1,25(OH)_2D_3$-treated. (*B*) PLC-γ enzyme activity in the BLM. $^*P < 0.05$, compared with control ($n = 3$ independent experiments, each in duplicate).

ods, parallel immunoprecipitations confirmed that comparable amounts of Src were present in samples from control and $1,25(OH)_2D_3$-treated cells (data not shown). To confirm this biphasic time course of activation, we also examined the time dependence of $1,25(OH)_2D_3$-stimulated Src autophosphorylation, and found a similar pattern (Fig. 5). We then investigated the secosteroid-induced changes in tyrosine phosphorylation of c-Src, as assessed by antiphosphotyrosine Western blotting. $1,25(OH)_2D_3$ caused a decrease in Src tyrosine phosphorylation at 1 and 9 min, compared with controls (Fig. 6), a result which was consistent with an established major mechanism of Src activation (23–25).

While dephosphorylation of a regulatory carboxy-terminal tyrosine is a major mechanism of Src regulation, conformational changes in Src have also been implicated to induce its activation (26). In this regard, $1,25(OH)_2D_3$, when incorporated into liposomes, has also been shown to directly activate PKC, a serine/threonine kinase (27). We therefore studied the ability of $1,25(OH)_2D_3$ to directly activate c-Src which had been isolated by immunoprecipitation from unstimulated colonocytes. Compared to liposomes containing phosphatidylcholine (PC) alone, PC liposomes with 1α,25 dihydroxyvitamin D_3 (30 nM) increased Src kinase activity 66±18% over controls (Fig. 7). In contrast, there was no kinase stimulation with the physiologically inactive β epimer, 1β,25-dihydroxyvitamin D_3 [30 nM], or with 25-hydroxyvitamin D_3 [300 nM] (Fig. 7), consistent with the established differences in their biological potencies, and supporting the potential biological relevance of these observations. These results suggest that, in addition to stimulating tyrosine dephosphorylation, $1,25(OH)_2D_3$ may also directly activate c-Src.

$1,25(OH)_2D_3$ increases the physical association of activated c-Src with PLC-γ. To address the possible direct activation of PLC-γ by Src in rat colonocytes, we examined the physical association of these proteins. As noted in Methods, since Src could not be detected in the PLC-γ immunoprecipitates by Western blotting with Src antibodies, we used a more sensitive assay to identify Src by assessing its autophosphorylation. Intact cells were treated with ethanol, or with $1,25(OH)_2D_3$ and PLC-γ subsequently immunoprecipitated and incubated in the presence of [γ^{32}P]ATP. When PLC-γ immunoprecipitates were resolved by SDS-PAGE, we observed that, compared to vehicle-treated cells, $1,25(OH)_2D_3$ induced an increase in ^{32}P-labeling of a protein comigrating with Src (Fig. 8 *A*). As shown in Fig. 8 *B*, this band was confirmed to be Src by comparison of its Cleveland map with that of authentic colonic Src (28). We next examined the ability of exogenous PLC-γ to serve as a substrate for colonic Src. Src, immunoprecipitated from

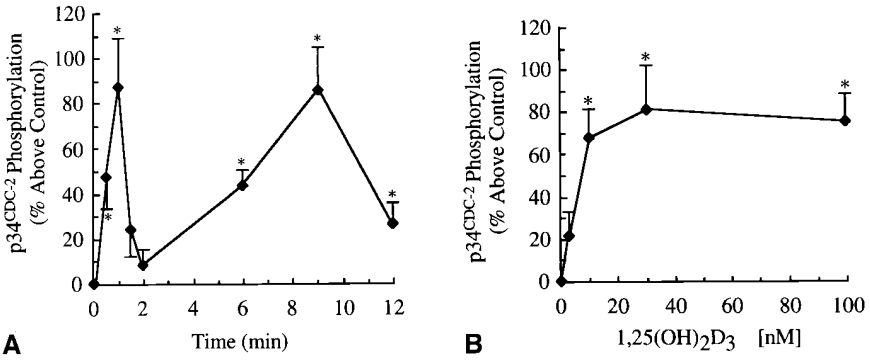


Figure 4. $1,25(OH)_2D_3$ stimulates colonic c-Src in a time- and concentration-dependent manner. Isolated cells were incubated with $1,25(OH)_2D_3$ or ethanol, and then lysed in extraction buffer containing 5% NP-40. c-Src was subsequently isolated by immunoprecipitation and kinase activity was determined by ^{32}P-incorporation into a peptide derived from $p34^{cdc-2}$ as described in Methods. (*A*) Time course for c-Src activation. Cells were incubated with 30 nM of $1,25(OH)_2D_3$ for the indicated times. (*B*) $1,25(OH)_2D_3$ dose-dependence for Src activation. Cells were incubated with the indicated concentrations of secosteroid for 1 min, and kinase activity was subsequently determined ($n = 3$ independent experiments each in duplicate). $^*P < 0.05$ when compared to vehicle-treated cells.

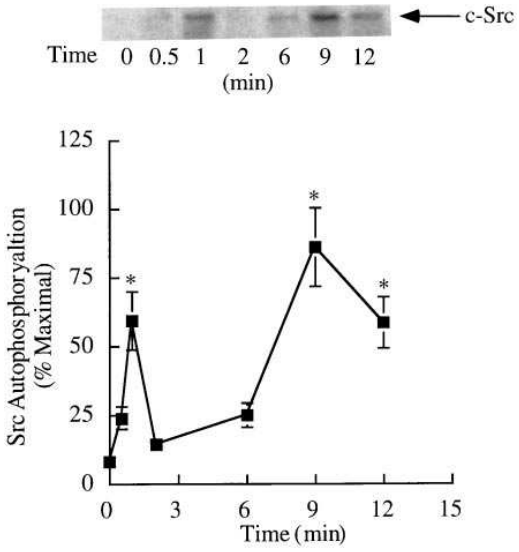


Figure 5. $1,25(OH)_2D_3$ stimulates a time-dependent increase in colonic c-Src autophosphorylation. Colonocytes were incubated with 30 nM $1,25(OH)_2D_3$ or ethanol for the indicated times, and c-Src was isolated by immunoprecipitation. Src was autophosphorylated in the presence of [γ^{32}P]ATP, was resolved by SDS-PAGE, and an autoradiogram was prepared. ^{32}P-Src labeling was quantified as described in Methods. $^*P < 0.005$ when compared to controls ($n = 3$ independent experiments). Inset: representative autoradiogram of the time course of $1,25(OH)_2D_3$-induced c-Src autophosphorylation.

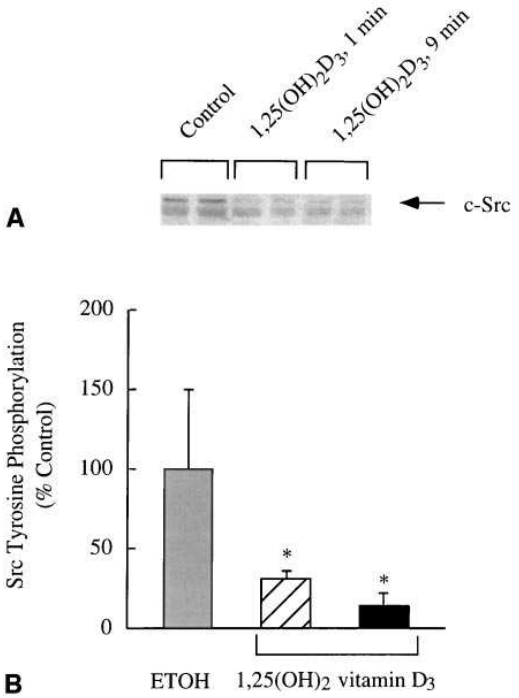


Figure 6. $1,25(OH)_2D_3$ increased c-Src activity is associated with decreased Src tyrosine phosphorylation. Src was immunoprecipitated from colonocytes treated with ethanol for 1 min or 30 nM $1,25(OH)_2D_3$ for the indicated times. Src tyrosine phosphorylation was assessed by Western blotting probed with a mixture of anti–phosphotyrosine antibodies as described in Methods. Src tyrosine phosphorylation was comparable for ethanol treatment for 9 min compared to that for 1-min ethanol-treated cells. (*A*) Representative xerogram of three independent experiments. (*B*) Quantitative changes in c-Src tyrosine phosphorylation. $^*P < 0.05$, compared with controls. Ethanol-treated, gray bar; $1,25(OH)_2D_3$-treated for 1 min, hatched bar; $1,25(OH)_2D_3$-treated for 9 min, black bar ($n = 3$ independent experiments in duplicate).

$1,25(OH)_2D_3$-treated colonocytes, increased PLC-γ phosphorylation in vitro more than 50% when compared to PLC-γ phosphorylation by Src immunoprecipitated from unstimulated cells (data not shown). These studies demonstrated that $1,25(OH)_2D_3$ increased the physical association of activated Src with PLC-γ in intact colonocytes, and that PLC-γ is a direct substrate for colonic c-Src in a cell-free system.

Inhibition of Src tyrosine kinase limits tyrosine phosphorylation and translocation of PLC-γ to the BLM. We next investigated the effect of a specific inhibitor of the Src family of tyrosine kinases, PP1 (29), on $1,25(OH)_2D_3$-induced changes in PLC-γ in the BLM. Preincubation of intact colonocytes with 100 μM PP1 for 15 min caused a 57.2±7.4% inhibition in $1,25(OH)_2D_3$-induced c-Src activation (Fig. 9). This Src inhibition was associated with a 50% decrease in PLC-γ translocation (Fig. 10 *A*), and inhibition of PLC-γ tyrosine phosphorylation in the BLM induced by $1,25(OH)_2D_3$ (Fig. 10 *B*).

Vitamin D status modulates the coupling of c-Src activation by 1,25(OH)$_2$D$_3$. The ability of $1,25(OH)_2D_3$ to stimulate polyphosphoinositide hydrolysis and to activate PKC are lost in colonocytes from vitamin D-deficient rats (2). To examine the effect of vitamin D status on c-Src, we carried out experiments comparing the Src expression and kinase activity in colonocytes from D-deficient and D-repleted rats. Neither Src total expression (Fig. 11, *inset*), nor basal activity (data not shown) was altered by vitamin D status. As in the case of PKC-α (5), however, compared with vitamin D-sufficient rats, vitamin D deficiency was associated with a failure of $1,25[OH]_2D_3$ to stimulate Src kinase activity (Fig. 11). Furthermore, 1,25 dihydroxyvitamin D_3 responsiveness was partially, but significantly, restored by the in vivo repletion for 10 d with 10 ng/100 g

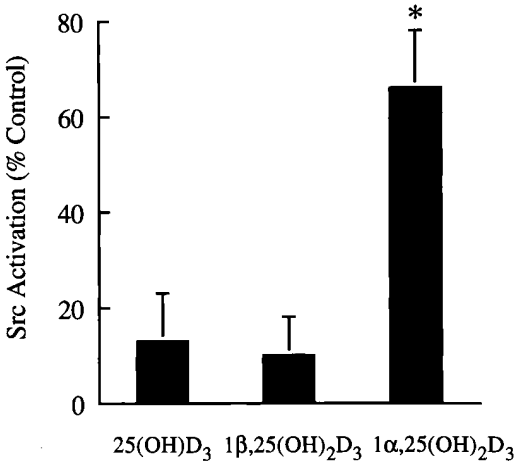
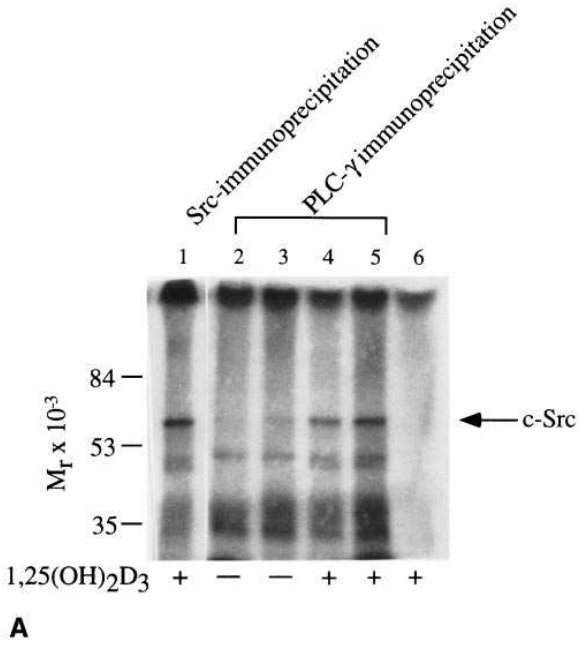


Figure 7. $1\alpha,25(OH)_2D_3$, but not $1\beta,25(OH)_2D_3$ or $25(OH)D_3$ incorporated into phosphatidylcholine liposomes stimulates c-Src. Src was immunoprecipitated from unstimulated colonocytes, and then incubated with multilamellar phosphatidylcholine (PC) liposomes alone (control), or PC liposomes containing 300 nM $25(OH)D_3$, 30 nM $1\beta,25(OH)_2D_3$, or 30 nM $1\alpha,25(OH)_2D_3$. Src activity was then assayed as described in Methods, and expressed as % above control. $^*P < 0.01$, compared with controls ($n = 3$ independent experiments).



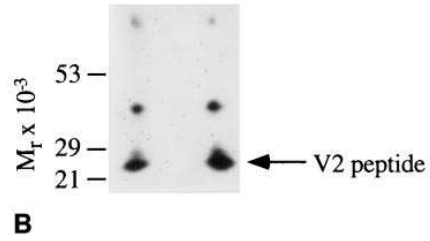


Figure 8. 1,25(OH)$_2$D$_3$ increases the association of activated c-Src with PLC-γ. (*A*) Isolated colonocytes were incubated with ethanol (−) or 30 nM 1,25(OH)$_2$D$_3$ (+) for 30 s, and a soluble fraction was prepared as described in Methods. Src (Lane *1*) or PLC-γ was immunoprecipitated with immune (Lanes *2–5*) or nonimmune sera (Lane *6*), and incubated for 15 min at 30°C in the presence of 2 μM [γ^{32}P]ATP (10 μCi), and then resolved by SDS-PAGE under nonreducing conditions. A band comigrating with autophosphorylated c-Src (Lane *1*) was increased in the PLC-γ immunoprecipitates from 1,25(OH)$_2$D$_3$-treated cells (Lanes *4* and *5*). (*B*) Cleveland map of autophosphorylated c-Src (*left*) and the comigrating band with M_r 60,000 in the PLC-γ immunoprecipitate (*right*). The V2 peptide is the V8 protease fragment containing Tyrosine 416, the Src autophosphorylation site (28).

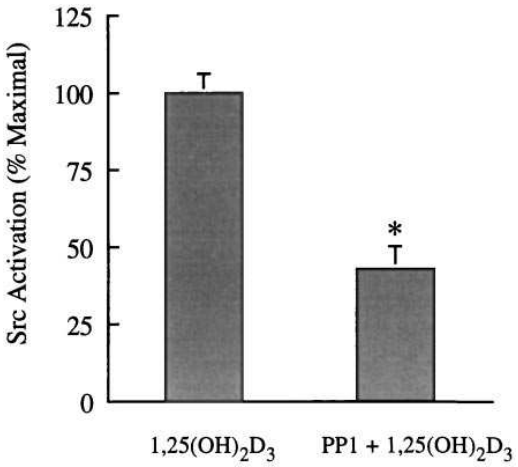


Figure 9. PP1, the Src tyrosine kinase inhibitor, limits the ability of 1,25(OH)$_2$D$_3$ to stimulate c-Src in intact colonocytes. Cells were preincubated with buffer containing DMSO (0.05% final concentration) alone, or PP1 (100 μM) for 15 min. Cells were then stimulated with 30 nM 1,25(OH)$_2$D$_3$ for 1 min, and were c-Src isolated and assayed as described in Methods. Results are expressed as the percentage of Src activation by 1,25(OH)$_2$D$_3$ alone. $P < 0.05$, compared with 1,25(OH)$_2$D$_3$-treated alone. ($n = 2$ independent experiments, each in duplicate).

body wt/d of 1,25(OH)$_2$D$_3$ administered subcutaneously by mini osmotic pumps (Fig. 11). The preservation of c-Src expression in D-deficiency, as assessed by Western blotting, was confirmed by comparable immunohistochemical staining for c-Src in colonic sections from D-deficient and control rats (Fig. 12). Furthermore, in these sections c-Src expression was noted to increase from the crypt base to the colonic lumen (Fig. 12).

Discussion

The present studies have demonstrated for the first time that 1,25(OH)$_2$D$_3$ stimulated PI-PLC-γ and c-Src in rat colonocytes. To our knowledge, the activation of these ubiquitous and important signal transduction elements by this secosteroid has not previously been reported in any cell type to date. In the present study we have also demonstrated that 1,25(OH)$_2$D$_3$ significantly stimulated the translocation and increased the tyrosine phosphorylation and the biochemical activity of PLC-γ in the basal lateral, but not the brush border, membranes. These findings indicate that PI-PLC-γ, at least in part, is responsible for the hydrolysis of basal lateral membrane polyphosphoinositides induced by 1,25(OH)$_2$D$_3$ that we have previously observed in these cells (1). It remains uncertain whether PLC-β$_3$ and/or -δ, the other PI-PLC isoforms present in these cells, are also involved in this phenomenon. In our prior studies using isolated colonic membranes, 1,25(OH)$_2$D$_3$ failed to directly stimulate PI hydrolysis (4), making the involvement of these predominantly membrane-associated isoforms of PI-PLC in this phenomenon less likely. In human keratinocytes, however, 1,25(OH)$_2$D$_3$ increased both the mRNA and protein abundance of PLC-β$_1$ (30). Given these latter observations, and the inherent complexities of studying regulated PLC activity in reconstituted in vitro experiments, this issue will require further investigation.

In the present studies, as noted above, we have also demonstrated that 1,25(OH)$_2$D$_3$ caused a time- and concentration-dependent increase in the activity of the nonreceptor tyrosine kinase c-Src. The biphasic time course of activation, as assessed with a Src substrate, was confirmed by a similar time course for Src autophosphorylation. The decreases in Src tyrosine phosphorylation induced by 1,25(OH)$_2$D$_3$ at 1 and 9 min, that corresponded to peaks in Src activity, are consistent with carboxy-terminal Src tyrosine dephosphorylation, a known major mechanism of Src regulation (23–25). While we have not mapped the specific phosphorylation site(s), as short term viability of primary colonocytes precludes high specific labeling of

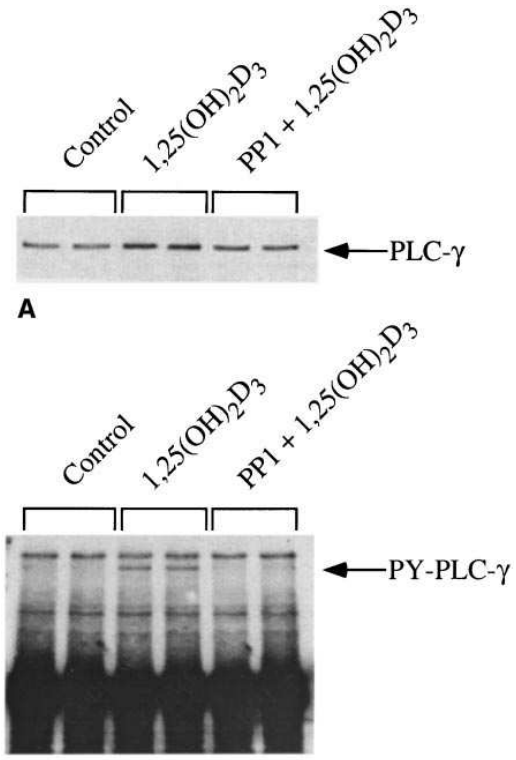


Figure 10. PP1, the Src tyrosine kinase inhibitor, decreases the translocation and blocks the tyrosine phosphorylation of PLC-γ in the BLM induced by $1,25(OH)_2D_3$. Colonocytes were preincubated with buffer containing DMSO (0.05% final concentration) alone (control), or with PP1 (100 μM) for 15 min. Cells were subsequently treated for 30 s with ethanol or 30 nM $1,25(OH)_2D_3$. BLM were prepared, an aliquot (20 μg protein) was denatured in SDS-containing buffer, and a separate aliquot (200 μg protein) was used for antiphosphotyrosine immunoprecipitation with PY-20 antibodies. The solubilized membranes and PY-20 immunoprecipitates were probed by Western blotting with anti–PLC-γ antibodies as described in Methods. (*A*) Total PLC-γ abundance in the BLM; (*B*) PLC-γ abundance in antiphosphotyrosine immunoprecipitates (PY-PLC-γ) from BLM. PLC-γ was identified by comigration with brain PLC-γ in a parallel lane with M_r 145,000 (not shown). The band above PLC-γ is a nonspecific band present in all BLM samples. Results are representative of two independent experiments in duplicate.

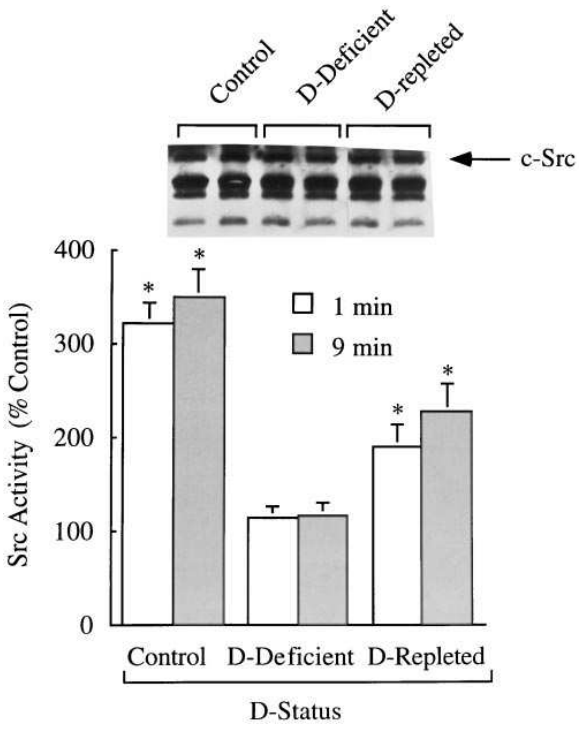


Figure 11. Vitamin D status modulates the signal coupling of $1,25(OH)_2D_3$ to c-Src. Colonocytes from D-sufficient (*Control*), D-deficient and D-repleted animals were isolated and stimulated for 1 min (□) or 9 min (■) with ethanol or 30 nM $1,25(OH)_2D_3$. c-Src was immunoprecipitated from total cell lysates and the kinase activity was assayed with the $p34^{cdc-2}$ peptide as described in Methods. Results are expressed as percentage±SEM of vehicle-treated D-sufficient Controls. $*P < 0.05$, compared with control ($n = 2$ independent experiments, in duplicate). *Inset:* Western blot of Src immunoprecipitated from colonic lysates from two untreated D-sufficient (*Control*), D-deficient and D-repleted rats, showing comparable Src expression in all three conditions.

c-Src with [^{32}P]orthophosphoric acid, a dephosphorylation of Src at tyrosine 527 is likely involved in its activation (25, 31). The additional finding that immunoprecipitated Src was stimulated by $1\alpha,25(OH)_2D_3$ incorporated into liposomes, suggests that this secosteroid may also directly activate the kinase, perhaps by an allosteric mechanism similar to that observed for PKC (27), and proposed as a mechanism for Src activation by the EGF receptor, independent of Src dephosphorylation (26). While the present studies in rat colonocytes indicate that Src activation induced by $1,25(OH)_2D_3$ involves, at least in part, tyrosine dephosphorylation, the specific role of allosteric alterations in this activation will require further study.

The present studies also indicate that c-Src may be intimately involved in the $1,25(OH)_2D_3$-induced stimulation of PLC-γ in rat colonocytes. Our findings that $1,25(OH)_2D_3$ increased the association of activated c-Src coprecipitating with PLC-γ, and enhanced the ability of Src, isolated from secosteroid-treated cells, to phosphorylate exogenous PLC-γ, suggest that this PLC isoform may be a direct substrate of c-Src in intact rat colonocytes. This conclusion is further strengthened by the observation that in intact cells, inhibition of $1,25(OH)_2D_3$-induced c-Src activation was associated with a block in the ability of this secosteroid to cause PLC-γ tyrosine phosphorylation and translocation to the BLM. The ability of electroinjected Src antibodies to block agonist-induced PLC activity in platelets (32), and to prevent the tyrosine phosphorylation and activation of PLC-γ in smooth muscle cells (9), further supports Src involvement in this PI signaling cascade. The results in the present study are also in agreement with those previously reporting in vitro phosphorylation of PLC-γ by Src (10), and with agonist-induced activation and coprecipitation of this kinase with PLC-γ (19, 32). The failure of $1,25(OH)_2D_3$ to stimulate c-Src in vitamin D-deficiency also supports the involvement of Src in secosteroid-induced PI hydrolysis, as this hydrolysis was also lost in D-deficiency, and both were restored by the in vivo repletion with this secosteroid. The derangements in signaling do not, however, appear to involve

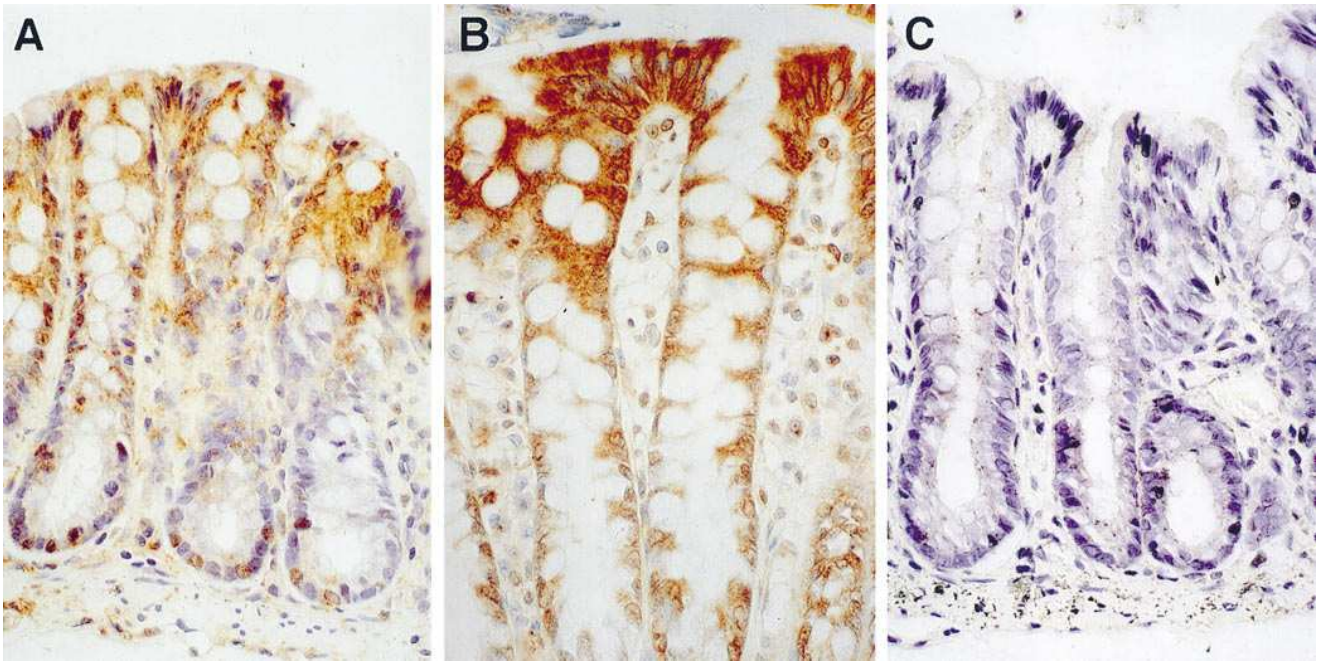


Figure 12. Immunohistochemical staining of c-Src in rat colon from normal and vitamin D-deficient rats. Colonic samples were prepared as described in Methods. All samples are shown at X400. (*A*) Normal rat colon reacted with 2 μg/ml c-Src antibody. (*B*) Colon from a vitamin D-deficient rat reacted with 2 μg/ml c-Src antibody. (*C*) An adjacent section of normal rat colon reacted with 2 μg/ml nonimmune rabbit Ig (control). Overall, immunoreactivity of c-Src is comparable in the normal and vitamin D-deficient colonic samples. As illustrated in *A* and *B*, both normal and vitamin D-deficient samples exhibited similar gradients of c-Src expression with increasing immunoreactivity towards the luminal surface of the colon.

changes in the expression of the PKC isoforms (5), or c-Src (Figs. 11 and 12), since these were not altered in vitamin D deficiency. The D status, moreover, did not change the gradient of Src expression along the crypt axis, as assessed by immunohistochemistry. Whether the block in $1,25(OH)_2D_3$ stimulation involves a single critical mechanism, for example, inactivation or loss of a putative plasma membrane receptor for vitamin D (33, 34), or multiple different pathways, remains to be determined.

Recent studies have demonstrated that supplementation with calcium, or with $1,25(OH)_2D_3$ or its analogues, is chemoprotective in several animal models of chemical colonic carcinogenesis (11, 35, 36). In contrast, in carcinogen treated D-deficient animals, calcium is no longer protective (11), and concomitantly in D-deficiency there is a loss of activation of Src (present study) and PKC (2) by this secosteroid. Whereas, as noted above, the changes in the underlying signal transduction events responsible for this lack of D-responsiveness in D-deficiency are yet to be defined, loss of these secosteroid-induced kinase activations may be involved in the failure of calcium supplementation to protect vitamin D-deficient rats against tumor formation in this rat model of colonic carcinogenesis (11). Elucidation of the mechanisms involved in these derangements may, therefore, provide valuable insights into the chemopreventive mechanisms of $1,25(OH)_2D_3$ in this model (36). In addition, this understanding may have more global import since epidemiologic studies have suggested that this secosteroid may be chemoprotective in human colon cancer as well (37).

In addition to its established role in mitogenesis (38, 39), Src appears to be involved in nonproliferative events, as suggested by its very high expression in several nondividing tissues, including platelets (40) and neuronal cells (41). In PC12 cells, Src was required for nerve growth factor-induced differentiation with neurite outgrowth (42). Src has also previously been implicated in endothelial cell migration (43). Studies in Madin-Darby canine kidney cells, another epithelial-derived cell line, have shown that changes in c-Src expression are associated with alterations in the shape of both individual cells and multicellular structures, without induction of mitogenesis (44). More recently, targeted gene deletion of a specific substrate of Src, the focal adhesion tyrosine kinase (45), which is an important component of the integrin-activated signaling complex, was shown to result in loss of cell motility (46). In view of these aforementioned observations, and the intriguing findings in the present study that Src immunohistochemical expression increases with migration up the colonic crypts, it would appear reasonable to suggest that Src may also be involved in cell migration and/or differentiation in postmitotic colonocytes.

In summary, we have demonstrated that $1,25(OH)_2D_3$ activates c-Src by phosphorylation-dependent, and perhaps phosphorylation-independent mechanisms in intact rat colonocytes. While vitamin D deficiency does not alter Src total expression or distribution along the crypt axis, this condition is associated with the reversible loss of Src activation by $1,25(OH)_2D_3$. Furthermore, we have identified PLC-γ as one of the vitamin D-responsive PI-PLC isoforms, as $1,25(OH)_2D_3$ induced the activation, tyrosine phosphorylation, and translocation of PLC-γ to the basal lateral membrane. Finally, taken together, the observations that: (*a*) $1,25(OH)_2D_3$ stimulated the association of

c-Src with PLC-γ in intact cells; (*b*) Colonic Src, activated by treatment of intact cells with $1,25(OH)_2D_3$, directly phosphorylated PLC-γ in a cell free system; and (*c*) Src kinase inhibition with PP1 limited the translocation and tyrosine phosphorylation of PLC-γ induced by this secosteroid in the BLM, all strongly support the hypothesis that PLC-γ is a direct substrate for this ubiquitous nonreceptor tyrosine kinase.

Acknowledgments

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