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Evidence that v-Src-Induced Phospholipase D Activity Is Mediated by a G Protein

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v-Src-induced increases in diglyceride are derived from phosphatidylcholine via a type D phospholipase (PLD) and a phosphatidic acid phosphatase. v-Src-induced PLD activity, as measured by PLD-catalyzed transphosphatidylation of phosphatidylcholine to phosphatidylethanol, is inhibited by GDPβS, which inhibits G-protein-mediated intracellular signals. Similarly, v-Src-induced increases in diglyceride are also blocked by GDPβS. In contrast to the PLD activity induced by v-Src, PLD activity induced by the protein kinase C agonist, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), was insensitive to GDPβS. Consistent with the involvement of a G protein in the activation of PLD activity by v-Src, GTPγS, a nonhydrolyzable analog of GTP that potentiates G-protein-mediated signals, strongly enhanced PLD activity in v-Src-transformed cells relative to that in parental BALB/c 3T3 cells. The effect of GTPγS on PLD activity in v-Src-transformed cells was observed only when cells were prelabeled with [^{3}H]myristate, which is incorporated exclusively into phosphatidylcholine, the substrate for the v-Src-induced PLD. There was no difference in the effect of GTPγS-induced PLD activity on v-Src-transformed and BALB/c 3T3 cells when the cells were prelabeled with [^{3}H]arachidonate, which is not incorporated into phospholipids that are substrates for the v-Src-induced PLD. Similarly, GDPβS inhibited PLD activity in v-Src-transformed cells much more strongly than in BALB/c 3T3 cells when [^{3}H]myristate was used to prelabel the cells. The GTP-dependent activation of PLD by v-Src was dependent upon the presence of ATP but was unaffected by either cholera or pertussis toxin. These data suggest that v-Src induces PLD activity through a phosphorylation event and is mediated by a cholera and pertussis toxin-insensitive G protein.

Cellular transformation involves the disruption of intracellular signaling mechanisms (see reference 12 for a review). Protein-tyrosine kinase activity is frequently an early event in the transduction of intracellular signals and has been extensively implicated in transformation tumorigenesis (12). The oncogenic protein-tyrosine kinase, v-Src, transforms cells because of a constitutively active kinase (22). As a result of this constitutively active kinase activity, v-Src activates multiple intracellular signaling mechanisms that lead to the induction of gene expression (17, 41, 43). Protein kinase C (PKC) is a serine/threonine-specific protein kinase that has been implicated in v-Src-induced intracellular signaling (20, 38, 43, 45, 51, 53), although not all intracellular signals activated by v-Src involve PKC (41, 43). PKC is activated by the phospholipid metabolite diglyceride (DG) (37). DG is frequently generated by the phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (see references 16 and 17 for a review); however, v-Src-induced increases in DG are derived from phosphatidylcholine (PC) via the action of a type D phospholipase (PLD) and a phosphatidic acid phosphatase (50). The activation of PLD by v-Src was found to be independent of PKC (48). Thus, it is likely that the DG produced by the PLD-phosphatidic acid phosphatase mechanism is responsible for the activation of PKC by v-Src. How v-Src might induce PLD activity and the subsequent activation of PKC is not known. G proteins have recently been implicated in the transduction of intracellular signals by protein-tyrosine kinases. A pertussis toxin-sensitive G protein has been implicated in the transduction of intracellular signals initiated by the insulin (18, 31, 33, 34), epidermal growth factor (26, 27, 55), and colony-stimulating factor 1 (24, 25) receptors. G proteins have also been implicated in mediating the effects of c-Src on the β-adrenergic response (11, 36). We previously demonstrated that the activation of PKC-dependent gene expression and phosphorylation of the PKC substrate MARCKS by the related protein-tyrosine kinase v-Fps is dependent on a G protein (1). Since PKC is presumably the target of the PLD-generated DG, these data suggest that a G protein could be involved in the activation of PLD activity by v-Src. Additionally, nonhydrolyzable analogs of GTP, which enhance G-protein-mediated signals, have been reported to stimulate PLD activity in vitro (6, 23, 40, 54). In this study, we have employed a permeabilized mammalian cell system to generate data implicating a G protein and phosphorylation in the activation of PLD activity by v-Src.

MATERIALS AND METHODS

Cells and cell culture conditions. BALB/c 3T3 cells and BALB/c 3T3 cells infected with either Rous sarcoma virus (Schmidt-Ruppin D strain) (SRD cells) or the temperature-sensitive LA90 strain of Rous sarcoma virus (35) (LA90 cells) were maintained in Dulbecco's modified Eagle medium supplemented with 10% newborn calf serum (HyClone). Cell cultures were made quiescent by being grown to confluence and then by using fresh media containing 0.5% newborn calf serum for 1 day.

Materials. GTPγS, GDPβS, ATPγS, and ADPβS were purchased from Boehringer Mannheim; saponin was obtained

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from Sigma. [^{3}H]myristate (NET-830) and [^{3}H]arachidonate (NET-2982) were obtained from New England Nuclear. PEt and DG standards were obtained from Avanti Polar Lipids. Precoated silica 60A thin-layer chromatography (TLC) plates were from Scientific Products.

Prelabeling of phospholipids. Unless otherwise indicated, cells in 35-mm-diameter culture dishes were prelabeled for 4 to 6 h in 2 ml of Dulbecco's modified Eagle medium containing 0.5% newborn calf serum. Isotopes were added to the culture media as follows: for [^{3}H]myristate, 3 μCi (40 Ci/mmol); for [^{3}H]arachidonate, 2 μCi (240 Ci/mmol).

Permeabilization. Permeabilization to allow entry of guanine nucleotides was performed by the procedures of Alexandropoulos et al. (1), with modifications. Prelabeled cells were washed once with serum-free medium and incubated for 10 min; the cells were then washed with permeabilization medium [110 mM KCl, 10 mM NaCl, 1 mM KH_2PO_4, 4 mM $MgCl_2$, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.32 mM $CaCl_2$, 1 mM Na_2ATP, 5 mM creatine phosphate, 3 U of creatine kinase per ml, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.0)] without saponin and were then given a 3-min treatment with permeabilization buffer containing saponin (70 μg/ml). Guanine or adenine nucleotides were included in the permeabilization buffer as indicated in the text and figure legends. The permeabilized cells were rinsed twice with permeabilization buffer lacking saponin but containing guanine or adenine nucleotides, and the cells were incubated for 10 min prior to activating the kinase activity of v-Src by temperature shift in LA90 cells. The SRD and BALB/c 3T3 cells were incubated for 15 min at 37°C with guanine or adenine nucleotides. Permeabilization was monitored by trypan blue uptake.

Extraction of lipids. Extraction of lipids was performed by procedures described by Song et al. (48–50), with minor modifications. Prelabeled LA90, SRD, or BALB/c 3T3 cells were permeabilized and treated as described below. The media were then aspirated, and the cells were immediately chilled on ice. The cells were then taken up into 0.50 ml of methanol (MeOH)–6 N HCl (50:1) in 1.5-ml Eppendorf tubes. Lipids were extracted by the addition of 0.50 ml of chloroform. Phase separation was obtained by adding 0.15 ml of 1 M NaCl. The organic phase was reextracted with 0.10 ml of 0.35 M NaCl, 0.10 ml of MeOH–6 N HCl (50:1), and 0.35 ml of H_2O; recovered; dried under N_2; and redissolved in $CHCl_3$-MeOH (9:1).

Characterization of phospholipid metabolites by TLC. Extracts of phospholipid metabolites were characterized by TLC (silica gel 60A plates) as described previously (4, 5, 50). Lipid standards were visualized by treating TLC plates with iodine vapor. TLC plates were sprayed with En^{3}Hance (Dupont) and exposed to Kodak XAR-5 film at −70°C for 2 to 3 days. To quantitate metabolically labeled PEt and DG, appropriate regions of TLC plates corresponding to PEt or DG were scraped, counted in a scintillation counter, and normalized to total counts per minute incorporated into cellular lipid. Total counts per minute were determined by taking an aliquot of the initial chloroform extract. The following solvent systems were used: for DG, hexane-diethyl ether-MeOH-glacial acetic acid (90:20:3:2); for PEt, the organic phase of ethylacetate-trimethylpentane-acetic acid-H_2O (100:50:20:100).

Bacterial toxin and aluminum fluoride treatment. The effect of cholera toxin (obtained from Sigma) was examined over a concentration range of 20 to 150 ng/ml as described previously (42). Pertussis toxin (also obtained from Sigma) was used over a concentration range of 10 to 250 ng/ml. Pertussis and cholera toxins were administered 10 h prior to harvesting cells. Aluminum fluoride (10 mM NaF, 20 μM $AlCl_3$) was added with the permeabilization medium with and without saponin.

RESULTS

v-Src-induced increases in PLD activity and DG production are blocked by GDPβS. PLDs catalyze the transphosphatidylation of substrate phospholipids to PEt in the presence of exogenous ethanol (30, 44). This assay has been used extensively to demonstrate PLD activity (4, 5, 9, 30, 32, 44, 48–50, 54). We previously demonstrated that activating the protein-tyrosine kinase activity of v-Src induces the transphosphatidylation of PC to PEt in the presence of exogenously provided ethanol (48, 50). These data implicated a PC-specific PLD in the transduction of intracellular signals initiated by v-Src. Since G proteins have been implicated in the activation of PLD (6, 23, 40, 54), we wished to determine whether v-Src-induced PLD activity requires a G protein. To accomplish this, we employed GDPβS, a nonhydrolyzable analog of GDP that inhibits signals mediated by G proteins in permeabilized cells (19). In Fig. 1a, it is shown that activating the protein-tyrosine kinase activity of v-Src by temperature shift induced PLD activity in LA90 cells that had been treated with saponin to make the cells permeable to guanine nucleotides. The magnitude of v-Src-induced PLD activity in the saponin-permeabilized cells was roughly the same as that observed in the intact cells (data not shown). GDPβS inhibited the v-Src-induced increase in PLD activity in a dose-dependent manner (Fig. 1a). Substitution of ADPβS for GDPβS failed to block v-Src-induced PLD activity (Fig. 1a), demonstrating a specific effect of the guanine nucleotide.

The DG produced in response to v-Src is derived from phosphatidic acid, the primary metabolite of PLD activity, which is hydrolyzed to DG by phosphatidate phosphatase (50). Since v-Src-induced PLD activity is blocked by GDPβS, the phosphatidic acid production and the subsequent production of DG should also be blocked by GDPβS. As shown in Fig. 1b, GDPβS blocked v-Src-induced DG production to approximately the same extent as that observed for v-Src-induced PLD activity. These data further implicate a G protein in the activation of PLD activity by v-Src.

It has been widely reported that phorbol esters that activate PKC also induce PLD activity (see reference 16 for a review). We previously demonstrated that the PLD activity induced by v-Src could be distinguished from that induced by the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (48). This suggested that TPA might activate PLD activity via a mechanism that is different from the one used by v-Src. We therefore investigated the sensitivity of TPA-induced PLD activity to GDPβS in BALB/c 3T3 cells. As shown in Fig. 1c, TPA-induced PLD activity was insensitive to GDPβS. Thus, GDPβS does not block all PLD and suggests some specificity for the PLD activity activated by v-Src. The data also suggest that the PLD induced by the PKC isoforms activated by phorbol esters is activated by a mechanism that is different from that used by v-Src. It was recently reported that TPA-induced PLD activity does not require ATP (14). Thus, TPA-induced PLD activity is apparently independent of both kinase and GTPase activity.

A nonhydrolyzable GTP analog enhances v-Src-induced PLD activity. Nonhydrolyzable analogs of GTP sustain G-protein-mediated signals because they cannot be hydrolyzed by the GTPase activity of the G protein to the inactive GDP-bound state (19). If a G protein is required for v-Src-induced PLD activity, then a nonhydrolyzable analog of GTP should enhance v-Src-induced PLD activity. In BALB/c 3T3 cells

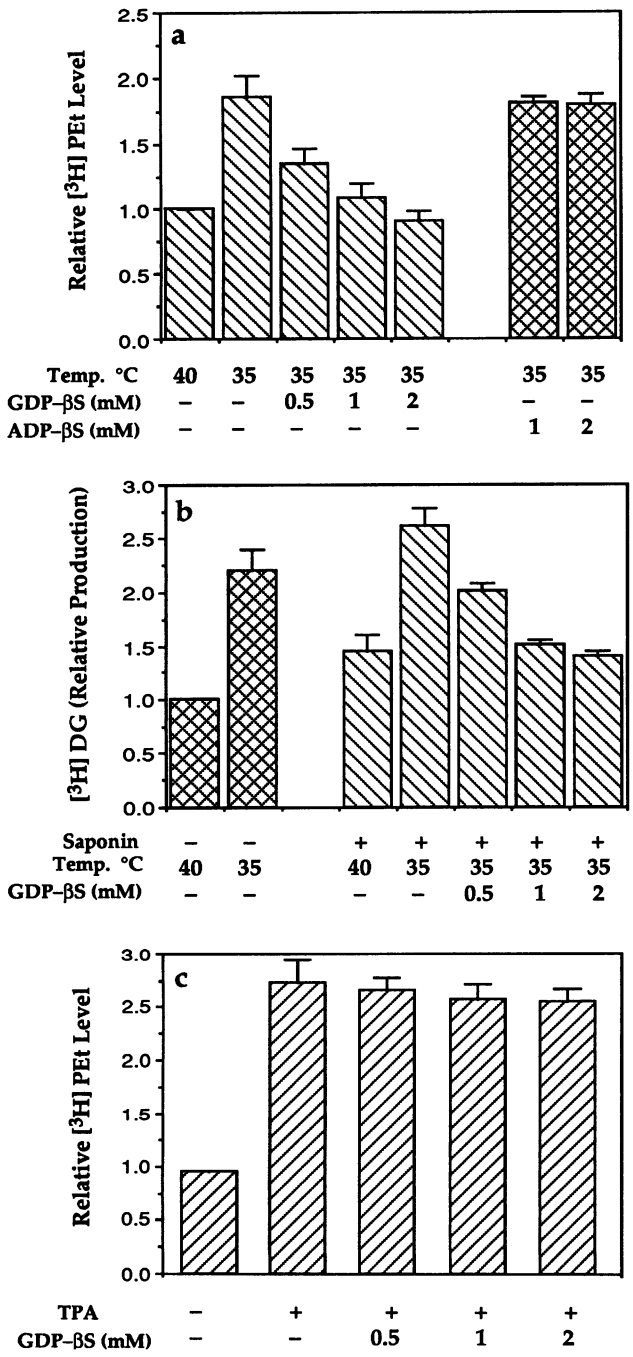

FIG. 1. The inhibition of v-Src-induced PLD activity by GDPβS but not ADPβS. The induction of PLD activity, as measured by the transphosphatidylation of PC to PEt, was performed in LA90 cells that had been prelabeled with [^{3}H]myristate. The cells were permeabilized in the presence or absence of guanine or adenine nucleotides as described in Materials and Methods. LA90 cells were then incubated for 10 min at the nonpermissive temperature for v-Src and then shifted to the permissive temperature for 10 min, at which time PLD activity is approaching maximal levels (50). The production of PEt (in the presence of 1% ethanol) (a) and DG (b) was then examined in the presence or absence of GDPβS or ADPβS, as indicated. (c) BALB/c 3T3 cells were permeabilized in the presence of GDPβS as described in Materials and Methods and then stimulated with TPA (100 ng/ml, 15 min). [^{3}H]PEt or [^{3}H]DG levels were determined as described in Materials and Methods. For each experiment, duplicate samples were prepared from different dishes. The mean values for [^{3}H]PEt and [^{3}H]DG in LA90 cells maintained at the nonpermissive temperature for v-Src (a and b) and BALB/c 3T3 cells without TPA stimulation (c) were assigned a value of 1. The means of duplicate samples obtained at the permissive temperature for v-Src in the presence of either GDPβS or ADPβS were then normalized to the control value. The data represent the means of [^{3}H]PEt or [^{3}H]DG from duplicate dishes relative to the control values with standard error for at least three independent experiments. Control counts per minute for individual experiments varied by less than 30%. The mean baseline values were 2,254 ± 170, 9,528 ± 720, and 2,140 ± 198 cpm for a, b, and c, respectively.

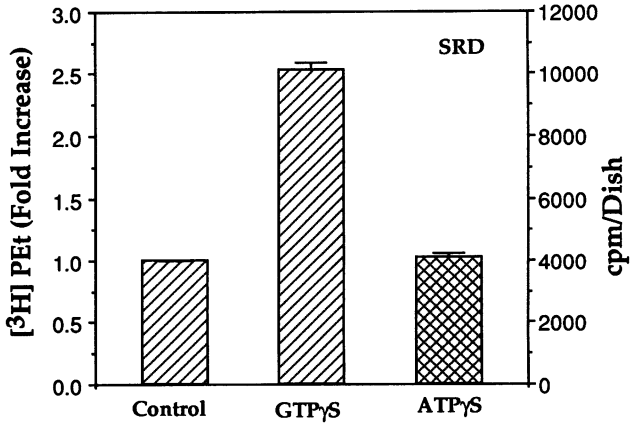

FIG. 2. The nonhydrolyzable GTP analog, GTPγS, stimulates PLD activity in v-Src-transformed cells. v-Src-transformed SRD cells were prelabeled with [^{3}H]myristate and permeabilized in the presence or absence of either GTPγS or ATPγS (100 μM). [^{3}H]PEt levels were determined as described in Materials and Methods. The [^{3}H]PEt values from cells permeabilized in the absence of either GTPγS or ATPγS were used as controls. The counts per minute ± standard errors are presented along with relative values normalized to the control value. The data are the means of duplicates determined from a representative experiment that was performed at least three times.

transformed by the Schmidt-Ruppin D strain of Rous sarcoma virus (SRD cells), the nonhydrolyzable GTP analog, GTPγS, stimulated PLD activity, whereas ATPγS had no effect upon PLD activity in SRD cells (Fig. 2). To establish whether the effect of GTPγS was on v-Src-induced PLD activity, the effect of GTPγS on PLD activity in SRD cells was compared with the effect of GTPγS on PLD activity in the parental BALB/c 3T3 cells. To distinguish v-Src-induced PLD activity from other PLD activities, the cellular phospholipids were differentially prelabeled with either [^{3}H]myristate or [^{3}H]arachidonate. [^{3}H]myristate is incorporated almost exclusively into PC (48–50), the primary substrate phospholipid for v-Src-induced PLD activity (50), whereas [^{3}H]arachidonate is incorporated into phospholipids not recognized by the PLD activated by v-Src (48, 50). Figure 3a shows that in cells prelabeled with [^{3}H]myristate, there was a dose-dependent increase in PLD activity in response to GTPγS that was much greater in the v-Src-transformed SRD cells than in the parental BALB/c 3T3 cells. In contrast, if the cells were prelabeled with [^{3}H]arachidonate, no difference between GTPγS-induced PLD activities in SRD cells and BALB/c 3T3 cells was observed (Fig. 3b). Thus, the ability to detect a significant GTPγS effect upon PLD activity was dependent on prelabeling with a phospholipid precursor ([^{3}H]myristate) that is incorporated into PC species that are substrates for the v-Src-induced PLD. The effect of GTPγS on

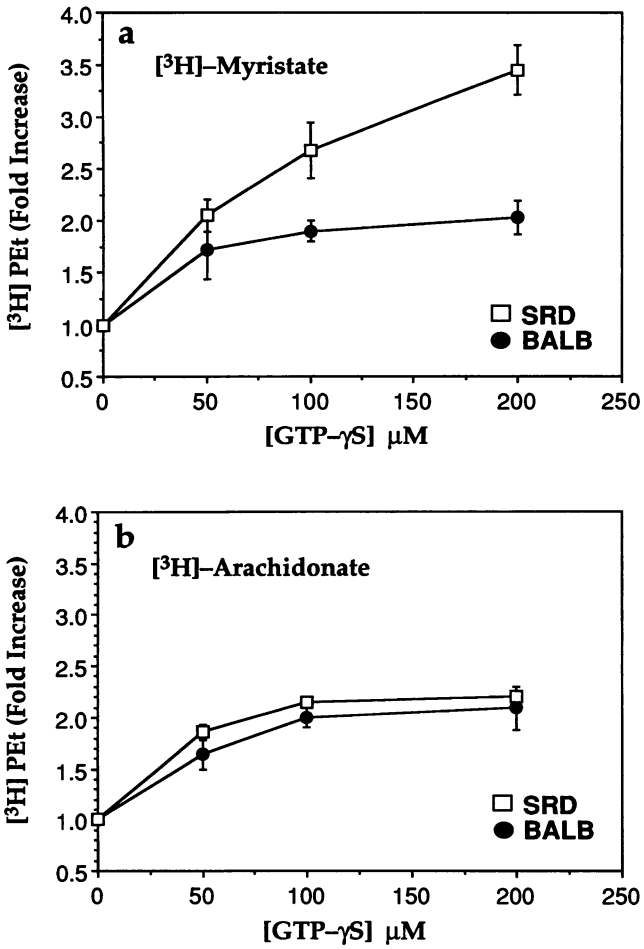

FIG. 3. GTPγS preferentially enhances PLD activity in v-Src-transformed cells relative to that in the parental BALB/c 3T3 cells. v-Src-transformed SRD cells and the parental BALB/c 3T3 cells were permeabilized in the presence of increasing concentrations of GTPγS cells that were prelabeled with either [^{3}H]myristate (a) or [^{3}H]arachidonate (b). After permeabilization with saponin (3 min), the cells were washed as described in Materials and Methods and incubated for 15 min in the presence of the indicated concentration of GTPγS and 1% ethanol (EtOH). [^{3}H]PEt values were then determined as for Fig. 1. Mean [^{3}H]PEt values from cells permeabilized in the absence of GTPγS were used as controls and were assigned a value of 1. All other means were normalized to the control values. The data represent the means of [^{3}H]PEt values relative to control values from at least three independent experiments. Baseline values for [^{3}H]myristate were 4,882 ± 715 and 1,860 ± 216 cpm for SRD and BALB/c 3T3 cells, respectively. Baseline values for [^{3}H]arachidonate were 2,160 ± 382 and 1,684 ± 235 cpm for SRD and BALB/c 3T3 cells, respectively.

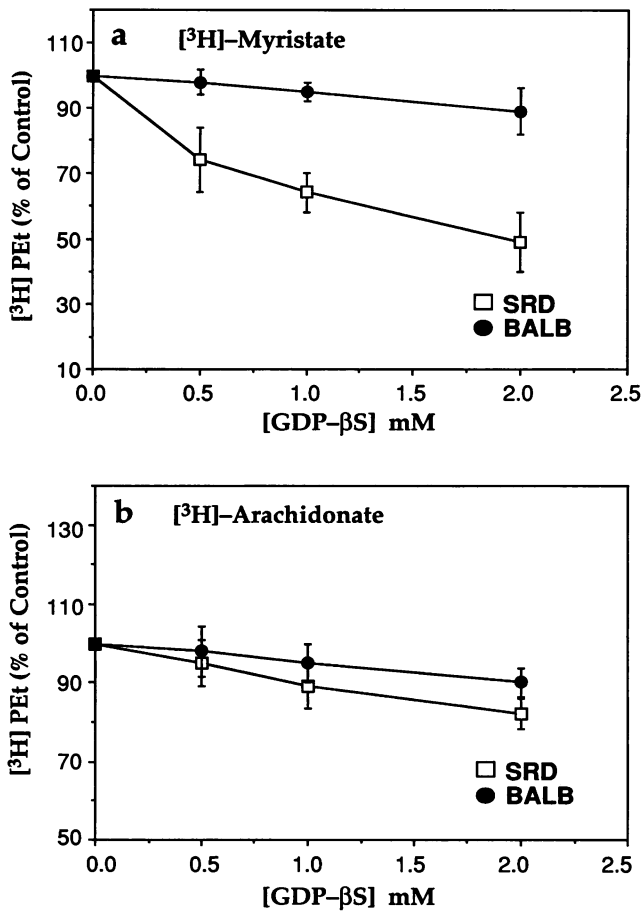

FIG. 4. GDPβS preferentially inhibits v-Src-induced PLD activity in v-Src-transformed cells. v-Src-transformed SRD and BALB/c 3T3 cells were prelabeled with either [^{3}H]myristate (a) or [^{3}H]arachidonate (b). The effect of increasing concentrations of GDPβS on [^{3}H]PEt levels was determined in permeabilized cells as for Fig. 1. Mean control values in the absence of GDPβS were given a value of 100%. All other mean values were normalized to the control values. The data represent the means of [^{3}H]PEt values relative to control values from at least two independent experiments. Baseline counts per minute were the same as those for Fig. 3.

TPA-induced PLD activity was barely additive (data not shown) and taken together with the lack of effect of GDPβS on TPA-induced PLD activity suggests that GTPγS does not stimulate TPA-induced PLD activity. These data further implicate a G protein as a mediator of v-Src- but not TPA-induced PLD activity.

GDPβS preferentially inhibits v-Src-induced PLD activity in v-Src-transformed cells. The role of G proteins in mediating v-Src-induced PLD activity was further examined by comparing the effect of GDPβS on PLD activity in SRD and BALB/c 3T3 cells prelabeled with either [^{3}H]myristate or [^{3}H]arachidonate. In [^{3}H]myristate-prelabeled cells, the observed inhibition of PEt formation by GDPβS was approximately 60% in SRD cells, whereas in BALB/c 3T3 cells, the observed inhibition of PEt formation by GDPβS was less than 20%. In contrast, if the cells were prelabeled with [^{3}H]arachidonate, the observed inhibition of PEt formation by GDPβS was less than 20% in both SRD cells and the parental BALB/c 3T3 cells (Fig. 4b). The greater effect of GDPβS on PLD activity in SRD cells relative to that in BALB/c 3T3 cells observed only in [^{3}H]myristate-prelabeled cells is consistent with the hypothesis that the elevated PLD activity in v-Src-transformed cells is dependent on a G protein.

ATP is required for the GTP-dependent increase in PLD activity induced by v-Src. The activation of PLD activity by PKC was reported to be independent of phosphorylation (14). It has also been reported that ATP potentiates GTPγS-induced PLD activity (32). This potentiation was sensitive to the protein-tyrosine kinase inhibitor herbimycin A. Thus, PLD activity can apparently be activated by protein kinases via mechanisms that are both dependent on and independent of

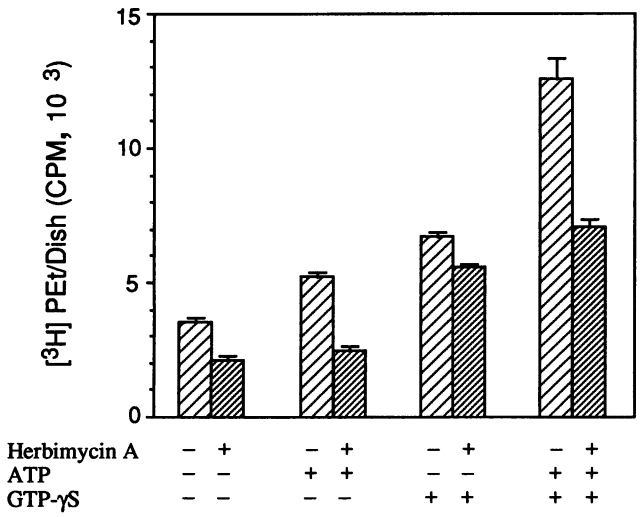

FIG. 5. ATP dependence and herbimycin A sensitivity of PLD activity in SRD cells. PLD activity in [^{3}H]myristate-prelabeled SRD cells was determined as for Fig. 2 in the presence and absence of herbimycin A (1 μg/ml, 16 h), ATP (1 mM), and GTPγS (100 μM) as shown. The conditions for herbimycin A inhibition and downregulation of v-Src have been worked out previously (28, 48). The data are mean counts per minute ± standard errors for duplicate samples from a representative experiment that was performed twice.

phosphorylation. The experiments whose results are presented in Fig. 1 to 4 involved ATP and an ATP-regenerating system. We therefore determined whether the presence of ATP was essential for the effects observed in Fig. 1 to 4 and whether herbimycin A could block the effects. As shown in Fig. 5, PLD activity is maximal in the presence of both GTP and ATP. More significantly, the inhibitory effect of herbimycin A is drastically reduced in the absence of ATP; herbimycin A had very little effect when GTPγS was used in the absence of ATP (Fig. 5). These data suggest that the protein-tyrosine kinase activity of v-Src is required for the GTP-dependent increase in PLD activity in the v-Src-transformed SRD cells.

PLD activity in SRD cells is insensitive to both cholera and pertussis toxins and to aluminum fluoride. Several heterotrimeric class G proteins are substrates for ADP ribosylation by bacterial toxins (19). ADP ribosylation by cholera toxin blocks GTP hydrolysis and therefore stimulates signals mediated by G proteins that are substrates for cholera toxin. We previously demonstrated that cholera toxin does not induce phosphorylation of the PKC substrate MARCKS in either avian or murine fibroblasts (42, 51). Since the activation of PLD activity presumably results in the activation of PKC, these data suggest that the G protein implicated here is not a cholera toxin substrate. Consistent with our previous observations, cholera toxin had no effect on PLD activity in either SRD or BALB/c 3T3 cells (data not shown). Pertussis toxin, which blocks some G-protein-mediated signals (19), also had no effect on PLD activity in SRD cells (data not shown). Therefore, the G protein implicated here is not likely a substrate for either cholera or pertussis toxin. Aluminum fluoride (10 mM NaF, 20 μM $AlCl_3$), which has been reported to specifically enhance heterotrimeric G-protein-mediated intracellular signals (9, 29), did not enhance PLD activity in SRD cells (data not shown). Although these data do not identify the putative G protein implicated here, they eliminate a large number of candidate heterotrimeric G proteins.

DISCUSSION

In recent years, it has become apparent that a substantial number of biological functions are regulated by GTPase activity (7, 8). Data presented here suggest that the induction of PLD activity induced by the protein-tyrosine kinase activity of v-Src is regulated by a G protein. We recently demonstrated that HaRas is required for the transduction of at least two distinguishable intracellular signals activated by v-Src (41). These v-Src-induced intracellular signals could be distinguished on the basis of a differential sensitivity to PKC and a dominant negative mutant of Raf-1 in murine fibroblasts (41, 43). A PKC-dependent intracellular signal activated by v-Src was sensitive to a dominant negative HaRas mutant, with HaRas functioning downstream from PKC (3). A Raf-1-dependent intracellular signal activated by v-Src was also sensitive to the dominant negative HaRas mutant, with HaRas functioning upstream from Raf-1 (2, 41). Since the G-protein-dependent DG produced in response to v-Src is likely responsible for the activation of PKC (48), the G protein implicated here likely functions upstream from PKC. Thus, the data presented here implicate a third GTPase-requiring step in the transduction of intracellular signals activated by v-Src. G proteins have also been implicated in the ability of c-Src to modify the β-adrenergic response (11, 36). Thus, GTPase activity apparently regulates at least four distinguishable effects of Src.

There are two major classes of G proteins: heterotrimeric and monomeric (7, 19). The involvement of G proteins of the heterotrimeric class in the transduction and amplification of intracellular signals has been well established for intracellular signals initiated by membrane receptors of the seven transmembrane domain classes (8, 19); however, there have been several reports suggesting the involvement of heterotrimeric class G proteins in some of the signals activated by protein-tyrosine kinases, including the receptors for insulin (17, 31, 33, 34), epidermal growth factor (26, 27, 55), colony stimulating factor 1 (24, 25) and the *fps* and *src* gene products (1, 11, 36). In addition, it has been reported that α subunits of heterotrimeric class G proteins can be phosphorylated on tyrosine residues by the insulin receptor (31) and c-Src (21). Thus, it is becoming apparent that the role of heterotrimeric G proteins may extend beyond intracellular signals initiated by the seven transmembrane domain receptors. While no heterotrimeric class G protein that mediates intracellular signals initiated by protein-tyrosine kinases has been unambiguously identified, there are a large number of heterotrimeric class G proteins that have been identified on the basis of sequence homology to known heterotrimeric class G proteins without a defined function (46). Thus, there are many candidate G proteins for protein-tyrosine kinase-initiated signaling systems for which heterotrimeric G proteins have been implicated. The recent report of tyrosine phosphorylation of G protein α subunits by c-Src (21) suggests the possibility that the G protein required for the v-Src-induced activation of PLD activity could be a direct substrate of v-Src. The lack of stimulatory effect by either cholera toxin or aluminum fluoride toxin or an inhibitory effect by pertussis toxin would tend to rule out a substantial number of heterotrimeric class G proteins, including G_S, the G protein phosphorylated by c-Src in vitro (21); however, there is substantial homology between the heterotrimeric class G proteins (46), and if c-Src can phosphorylate G_S, v-Src might phosphorylate another heterotrimeric G protein.

The monomeric G protein HaRas has been implicated in the transduction of intracellular signals initiated by v-Src (15, 39, 41, 47, 52). We previously demonstrated that HaRas functions

downstream from PKC in an intracellular signaling pathway that led to the transcriptional activation of TPA response elements (3, 41). Thus, it is unlikely that HaRas is the G protein implicated here, which functions upstream from PKC. In addition, it has been reported that an inhibitory antibody to HaRas did not block the GTP-dependent activation of PLD activity in rat liver membranes (23). It was recently reported that PLD activity could be activated in neutrophils by the Ras family monomeric G protein Rho (9). The monomeric G protein family of ADP ribosylation factors has also been shown to activate PLD in vitro (10, 13). Thus, a growing body of evidence implicates monomeric G proteins in the activation of PLD. The lack of a stimulatory effect by aluminum fluoride on v-Src-induced PLD activity supports the involvement of a monomeric G protein, since aluminum fluoride has been reported to have no effect upon monomeric G proteins (29). However, the lack of an effect of aluminum fluoride is a negative result, and it is possible that a nonspecific effect of aluminum fluoride is masking a stimulatory effect by this compound which has not been thoroughly studied. Thus, it is still possible that a heterotrimeric G protein could be involved. Although the putative G protein that mediates v-Src-induced PLD activity remains to be identified, the data presented here define an additional GTP-dependent event in v-Src-induced intracellular signals and further demonstrate the importance of GTPase activity as a master switching mechanism in the transduction of intracellular signals initiated by protein-tyrosine kinases.

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