v-Src activates a unique phospholipase D activity that can be distinguished from the phospholipase D activity activated by phorbol esters

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Phospholipase D (PLD) activity, as measured by the transphosphatidylolation of cellular phospholipids, is elevated in BALB/c 3T3 cells transformed by v-Src. Phorbol esters that activate protein kinase C (PKC) also increase PLC activity in BALB/c 3T3 cells. v-Src-induced PLD activity could be distinguished from phorbol ester-induced PLD activity by differential radiolabelling of phospholipids, which are the substrates of PLD. Both v-Src- and phorbol ester-induced PLD activity could be detected when phospholipids were prelabelled with either radiolabelled myristate or palmitate; however, only phorbol ester-induced PLD activity could be detected when either arachidonate or 1-*O*-alkyl-*sn*-glyceryl-3-phosphorylcholine (alkyl-lysoPC) was used to prelabel the phospholipids. The increased PLD activity in v-Src-transformed cells was not detected when the cells were prelabelled with either arachidonic acid or alkyl-lysoPC, which contains an ether linkage at *sn*-1 of the glycerol backbone. As both arachidonic acid and alkyl-

lysoPC are incorporated into phosphatidylcholine (PC), the substrate for v-Src-induced PLD activity, these data suggest that the PLD activated by v-Src can distinguish PCs lacking arachidonic acid and ether linkages. Consistent with v-Src activating a PLD activity that is distinct from that activated by phorbol esters that activate PKC directly, neither depleting cells of PKC nor treatment with the protein kinase inhibitor, staurosporine, had any effect on v-Src-induced PLD activity, whereas both PKC depletion and staurosporine inhibited phorbol ester-induced PLD activity. Taken together, these data suggest that v-Src activates a PKC-independent PLD activity that is specific for a subpopulation of PC and distinct from the PLD activity induced by PKC activity induced by phorbol esters. The diacylglycerol produced from PC by the action of the v-Src-induced PLD may therefore be responsible for the activation of PKC by v-Src.

INTRODUCTION

Protein kinase C (PKC) activity has been implicated in v-Src-induced intracellular signals (Halsey et al., 1986; Sagara et al., 1986; Wolfman et al., 1987; Spangler et al., 1989; Nori et al., 1990; Qureshi et al., 1991). Diacylglycerol (DG) is a second messenger that leads to the activation of PKC (Nishizuka, 1986). We have previously demonstrated that v-Src-induced increases in DG are derived primarily from phosphatidylcholine (PC) via the action of a phospholipase D (PLD) and phosphatidic acid (PA) phosphatase (Song et al., 1991). Several agonists have been reported to increase intracellular DG levels by activating PC-specific phospholipases (reviewed by Exton, 1990). PC metabolism has been reported to be both dependent on PKC (Cabot et al., 1988; Billah et al., 1989a; Huang and Cabot, 1990; Martinson et al., 1990; Liscovitch, 1989; Price et al., 1989; van Blitterswijk et al., 1991; Hii et al., 1989, 1991; Conricode et al., 1992) and independent of PKC (Billah et al., 1989a; Reinhold et al., 1990; Huang and Cabot, 1990; Yamada et al., 1991; Llahi and Fain, 1992). It has been suggested that PKC is activated in response to a phospholipase C (PLC)-mediated hydrolysis of phosphatidylinositol (PI) 4,5-bisphosphate which is followed by a PKC-dependent activation of a PLD-mediated hydrolysis of PC to produce PA, which is subsequently hydrolysed to DG (Exton, 1990). DG produced from PI phosphates has been reported to be rapidly converted into PA by DG kinase, whereas DGs produced from PC are poor substrates for DG kinase (Ford and Gross, 1990; Lee et al., 1991). Thus there may be significant

functional differences between the DGs produced from PC and PI. These differences can only be in the aliphatic groups attached to the glycerol backbone. DGs produced from PC are apparently more stable and give a more prolonged signal than DGs produced from PI. The greater stability of PC-derived DGs is consistent with the time course for PC-derived DG production induced by v-Src which peaks at 15–30 min after induction (Song et al., 1991).

Although increased levels of inositol of phosphates in v-Src-transformed rat cells have been reported (Johnson et al., 1989), these increased levels were not detected concomitantly with the production of DG (Song et al., 1991; Martins et al., 1989). However, it is still possible that low levels of v-Src-induced DG could be derived from PI. Our previous report (Song et al., 1991) did not establish whether the activation of PKC by v-Src is required for v-Src-induced PC hydrolysis or whether the activation of PKC by v-Src is a consequence of v-Src-induced PC hydrolysis. In this report, we present data suggesting that v-Src activates a PLD activity that is specific for a subpopulation of PC and is independent of PKC activation. The potential significance of the unique DG species generated in v-Src-transformed cells from this subpopulation of PC is discussed.

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

Abbreviations used: alkyl-lysoPC, 1-*O*-alkyl-*sn*-glyceryl-3-phosphorylcholine; DG, diacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PEt, phosphatidylethanol; PI, phosphatidylinositol; PLC, phospholipase C; PLD, phospholipase D; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol 13-acetate ('PMA'); SRD cells, BALB/c 3T3 cells infected with Rous Sarcoma virus.

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the temperature-sensitive LA90 strain of Rous sarcoma virus were maintained in Dulbecco's modified Eagle medium supplemented with 10 % newborn calf serum (Gibco).

Materials

[³H]Myristate (NET-830), [³H]palmitate (NET-043), [³H]arachidonate (NET-2982) and [³H]alkyl-lysoPC (NET-878) were obtained from New England Nuclear. PI, PC, phosphatidylserine, phosphatidylethanolamine and DG standards were obtained from Sigma. Phosphatidylethanol (PEt) was obtained from Avanti Polar Lipids. T.l.c. plates (Silica gel 60A) were from Scientific Products.

Prelabelling of phospholipids

Unless otherwise indicated, confluent 60 mm culture dishes were prelabelled for 3–5 h in 5 ml of medium containing 0.5 % newborn calf serum. Isotopes were added to the culture media as follows: [³H]myristate, 5 μ Ci (40 Ci/mmol); [³H]palmitate, 5 μ Ci (60 Ci/mmol); [³H]arachidonate, 2 μ Ci (240 Ci/mmol); [³H]alkyl-lysoPC, 2 μ Ci (45 Ci/mmol). Prelabelling was in the presence of 0.1 % ethanol.

Extraction of phospholipase products

Extraction of lipids was performed as described by Billah et al. (1989b) with minor modifications previously described (Song et al., 1991). Cells were washed with isotonic Tris/saline buffer and rapidly treated with 0.6 ml of methanol/6 M HCl (25:1, v/v). Lipids were extracted by the addition of 0.6 ml of chloroform. Phase separation was obtained by adding 200 μ l of 1 M NaCl. The organic phase was re-extracted with 0.6 ml of 0.35 M NaCl and 0.2 ml of methanol/6 M HCl (50:1, v/v) and recovered, dried under N₂ and redissolved in chloroform/methanol (9:1, v/v).

Characterization of phospholipid metabolites by t.l.c.

Phospholipid metabolites were characterized by t.l.c. (silica gel 60A plates) using procedures described previously (Song et al., 1991). Lipid standards were visualized by treating t.l.c. plates with iodine vapour. To quantify metabolically labelled compounds, the appropriate regions of t.l.c. plates were scraped off and counted in a liquid-scintillation counter. The following solvent systems were used: for DG, hexane/diethyl ether/methanol/acetic acid (90:20:3:2, by vol.); for PC, PI, phosphatidylserine and phosphatidylethanolamine, chloroform/methanol/acetic acid/water (50:25:8:4, by vol.); for PEt, the upper phase of ethyl acetate/trimethylpentane/acetic acid/water (9:5:2:1, by vol.).

PKC depletion

Cells were depleted of PKC by treatment with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (100 ng/ml, 26 h). The cells were maintained in 100 ng/ml TPA until the time of harvest as described above. The effectiveness of depleting cells of PKC after prolonged treatment with phorbol ester was determined by Western-blot analysis using a polyclonal antibody raised against a peptide from a conserved region of the kinase domain of PKC (Ballester and Rosen, 1985) as described previously (Joseph et al., 1992). We have found that the α isoform is the predominant species in the cell lines used here (C. Joseph and D. A. Foster, unpublished work). The prolonged phorbol ester treatment eliminated the α isoform within the limits of our ability to detect

by Western-blot analysis. We have used this treatment several times to inhibit the induction of v-Src-responsive genes and promoter elements (Spangler et al., 1989; Qureshi et al., 1991, 1992). Thus this treatment is able to eliminate PKC species used for v-Src-induced intracellular signals.

RESULTS

PLD activity is elevated in v-Src-transformed cells

In the presence of a critical concentration of ethanol, PLDs catalyse the transphosphatidylation of substrate phospholipids to PEt (Kobayashi and Kanfer, 1987). This procedure has been used extensively to demonstrate PLD activity (Billah et al., 1989a,b; Hii et al., 1989, 1991; Liscovitch, 1989; Martinson et al., 1990; Randall et al., 1990; Huang and Cabot, 1990; Welsh et al., 1990; Reinhold et al., 1990; Balsinde and Mollinedo, 1991; Yamada et al., 1991). Phorbol esters that activate PKC have been shown to induce transphosphatidylation in the presence of ethanol (Billah et al., 1989a; Hii et al., 1989, 1991; Liscovitch, 1989; Huang and Cabot, 1990; Reinhold et al., 1990; Balsinde and Mollinedo, 1991; Yamada et al., 1991). In BALB/c 3T3 cells, the addition of the phorbol ester, TPA, led to a large increase in PEt that was dependent on ethanol (Figure 1a). We have previously demonstrated that activating the protein-tyrosine kinase activity of v-Src in BALB/c 3T3 cells transformed by a temperature-sensitive derivative of v-Src led to an increase in PLD activity in BALB/c 3T3 cells (Song et al., 1991). To characterize PLD activity in v-Src-transformed cells, we compared PLD activity in BALB/c 3T3 cells and BALB/c 3T3 cells transformed with the Schmidt-Rupin-D strain of Rous sarcoma virus (SRD cells). As shown in Figure 1(b), the increased ethanol concentration that produced the TPA-induced increase in PEt led to a large increase in PEt levels in the SRD cells compared with the slight increase observed in the parental BALB/c 3T3 cells. To determine whether the protein-tyrosine kinase activity of v-Src was responsible for the increased PLD activity in SRD cells, we treated them with increasing concentrations of herbimycin A, which is an antibiotic that has been reported to reverse oncogenic transformation induced by v-Src (Uehara et al., 1989a,b). It inhibits tyrosine phosphorylation mediated by v-Src and increases v-Src turnover (Uehara et al., 1989c). We have previously found that treating SRD cells with herbimycin A led to a reduction in v-Src protein and v-Src kinase activity *in vitro* which was detected within 4–6 h (Joseph et al., 1992). Therefore in this study, v-Src-transformed BALB/c 3T3 cells were treated with herbimycin A for 6 h. It inhibited transphosphatidylation in SRD cells by 60 % but had no effect on transphosphatidylation induced by TPA (Figure 1c). These data suggest that the constitutive PLD activity in v-Src-transformed BALB/c 3T3 cells is a direct consequence of the protein-tyrosine kinase activity of v-Src. These data also distinguish v-Src-induced PLD activity from TPA-induced PLD activity in BALB/c 3T3 cells.

v-Src-induced PLD activity can be distinguished from phorbol-ester-induced PLD activity by differential substrate utilization

We have previously demonstrated that PC-derived DG produced in response to v-Src could not be detected when cells were prelabelled with [³H]arachidonic acid (Song et al., 1991). Although [³H]arachidonic acid is incorporated preferentially into PIs (Swendsen et al., 1987; Takayama et al., 1987; Song et al., 1991), it is also incorporated into the PC of murine fibroblasts (Song et al., 1991). We therefore examined whether the transphosphatidylation products, observed in Figure 1(b) using

Figure 1 PLD activity is elevated in v-Src-transformed cells

(a) BALB/c 3T3 cells (▨) and BALB/c 3T3 cells treated with TPA (100 ng/ml, 30 min) (▩) were prelabelled with [3 H]myristate in 0.1% ethanol as described in the Materials and methods section. Ethanol to 1.5% (v/v) was added as shown. Cellular lipids were extracted 30 min after the addition of either ethanol or TPA. Transphosphatidylated products were separated by t.l.c. and quantified by measuring the radioactivity incorporated into PET. The data are presented as fold increase over the baseline c.p.m. values for PET in the presence of 0.1% ethanol. Increases were normalized to this baseline value. (b) BALB/c 3T3 cells (BALB) and SRD cells were prelabelled with [3 H]myristate. Ethanol to 1.5% (v/v) was added as shown and PET levels were determined as in (a). (c) BALB/c 3T3 cells treated with TPA (100 ng/ml, 30 min) (□) and SRD cells (■) were prelabelled with [3 H]myristate. Ethanol (1.5%) was added 30 min before harvesting of cellular lipids as in (a). Increasing concentrations of herbimycin A were added 6 h before the harvesting of cellular lipids, and PET levels were determined as in (a). For this experiment, the label was added 5 h before the herbimycin to minimize any effects of herbimycin on the uptake of [3 H]myristate. Qualitatively similar results were obtained with 24 h labelling.

[3 H]myristate to prelabel phospholipids, could be detected when cells were prelabelled with [3 H]arachidonate. PET levels were examined in the presence of both low (0.1%) and high (1.5%) concentrations of ethanol in SRD cells and BALB/c 3T3 cells in the presence and absence of TPA. As shown in Figure 2(a), transphosphatidylation was not detected in v-Src-transformed cells prelabelled with [3 H]arachidonate. In contrast, TPA-induced transphosphatidylation was readily detected in both BALB/c 3T3 and SRD cells prelabelled with [3 H]arachidonate (Figure 2a). The inability to detect transphosphatidylation in SRD cells prelabelled with [3 H]arachidonate was not due to a reduced incorporation of arachidonate into the phospholipids of these cells. As shown in Table 1, [3 H]arachidonate was incorporated efficiently into the PC of both BALB/c 3T3 and SRD cells. In addition, the induction of PET production by TPA in SRD cells was easily detected when [3 H]arachidonate was used to prelabel phospholipids (Figure 2a).

Transphosphatidylation of phospholipids to PET was readily detected in v-Src-transformed cells when either [3 H]myristate (Figure 2b) or [3 H]palmitate (Figure 2c) was used to prelabel the cells. TPA also induced transphosphatidylation of phospholipids that had incorporated [3 H]myristate and [3 H]palmitate to the same extent as that observed with [3 H]arachidonate-labelled phospholipids (Figures 2b and 2c). We next examined the ability of v-Src and TPA to induce transphosphatidylation of phospholipids with either linkages in the *sn*-1 position of PC. Ether linkages are common in the *sn*-1 position of PC and can account for as much as 70% of PC in some cells (Vance and Vance, 1985; Chilton and Connell, 1988). BALB/c 3T3 and SRD cells were prelabelled with [3 H]alkyl-lysoPC. As shown in Figure 2(d), no PET was detected in the v-Src-transformed SRD cells, whereas PET was detected in both BALB/c 3T3 and SRD cells treated with TPA. These data cannot be explained by differential incorporation of the labelled phospholipid precursors into SRD cells and BALB/c 3T3 cells as both cell lines incorporate the labelled precursors into phospholipids with approximately the same efficiency (Table 1). The distribution of label was similarly unaffected by the addition of TPA (results not shown). The induction of transphosphatidylation by TPA in cells prelabelled with [3 H]arachidonate, in which v-Src-induced transphosphatidylation could not be detected, was of the same magnitude as that observed in cells prelabelled with [3 H]myristate or [3 H]palmitate, in which v-Src-induced transphosphatidylation could be detected. Therefore it is unlikely that the apparent differential substrate utilization by the PLD(s) activated by v-Src is the result of differential degrees of isotope incorporation. The data presented in Figure 2 suggest that the PLD activated by v-Src has a more selective substrate range than the PLD(s) activated by TPA. The data also suggest that the substrates utilized by the PLD activated by v-Src do not contain arachidonic acid or ether linkages at the *sn*-1 position.

v-Src- and TPA-activated PLD activity are differentially sensitive to depleting cells of PKC and the protein kinase inhibitor staurosporine

As the data presented above suggest that the PLD activated by v-Src may be different from that activated by TPA, we examined whether v-Src could activate PLD activity in cells that had been depleted of PKC. Prolonged treatment (20–48 h) with TPA

The c.p.m. values for PET in SRD cells and TPA-treated BALB/c 3T3 cells in the presence of 1.5% ethanol and the absence of herbimycin A were assigned a value of 100% for the fully induced level and all other values were normalized to this value. The data are from representative experiments performed in duplicate and repeated at least three times. Error bars indicate S.E.M.

Figure 2 v-Src and phorbol esters activate distinguishable PLD activities

PET levels in SRD and BALB/c 3T3 cells in the presence or absence of TPA were examined after prelabelling with different fatty acid precursors. Ethanol was present at either 0.1 or 1.5% as shown. SRD-transformed and untransformed BALB/c 3T3 cells were prelabelled with (a) [³H]arachidonate, (b) [³H]myristate, (c) [³H]palmitate or (d) [³H]alkyl-lysoPC as described in the Materials and methods section. Ethanol (1.5%) or TPA (100 ng/ml) were added as shown 30 min before harvesting of cellular lipids. The c.p.m. values for PET in 0.1% ethanol in the absence of TPA was assigned a value of 1 and all other values were normalized to this value. Data are the means of at least two independent experiments. Error bars indicate S.E.M.

Table 1 Relative incorporation of phospholipid precursors into cellular phospholipids of BALB/c 3T3 and SRD cells

BALB/c 3T3 cells and SRD cells were prelabelled with the indicated phospholipid precursor. The percentage of label incorporated into PC, PI, phosphatidylethanolamine (PE) and phosphatidylserine (PS) was calculated by determining the radioactivity incorporated into either PC, PI, PE, and PS and dividing this value by the total c.p.m. present in the chloroform/methanol lipid extract as described previously (Song et al., 1991). Total c.p.m. were determined before t.l.c. and the c.p.m. for PC, PI, PE and PS were determined after t.l.c. Alkyl-lysoPC has the choline headgroup already and is incorporated exclusively into PC (results not shown). Therefore total radioactivity contains some background radioactivity and thus the absolute percentages are likely to be higher than the values shown. Data are the means of two separate experiments where values did not vary by more than 20%.

Percentage of [³ H]phospholipid precursor incorporated into:						
SRD cells			BALB/c 3T3 cells			
	[³ H]Arachidonate	[³ H]Myristate	[³ H]Palmitate	[³ H]Arachidonate	[³ H]Myristate	[³ H]Palmitate
PC	33	32	35	32	49	45
PI	11	0.9	2.2	32	0.3	8.4
PE	8.7	3.5	5.0	14	4.9	22
PS	2.3	0.6	1.8	13	0.5	1.3

(100 ng/ml) leads to the down regulation of PKC and this treatment has been used extensively to demonstrate PKC requirements in a variety of systems (Rodriguez-Pena and

Rozengurt, 1984; Nishizuka, 1986; Spangler et al., 1989; Qureshi et al., 1991). BALB/C 3T3 and SRD cells were depleted of PKC as described in the Materials and methods section. We have

Figure 3 v-Src- and phorbol ester-induced PLD activity are differentially sensitive to PKC depletion and staurosporine

PET induced by v-Src and TPA (100 ng/ml) was examined after prelabelling with [3 H]palmitate. PET production in the presence of ethanol (1.5%) was compared in cells that were either treated or not with prolonged exposure to TPA (100 ng/ml, 26 h) to deplete cells of PKC (PKC depl) (a) or with staurosporine (500 ng/ml) (b). Ethanol (0.1% or 1.5%) and TPA (100 ng/ml) were added 30 min before harvesting of cellular lipids. The c.p.m. value for PET in 0.1% ethanol was assigned a value of 1 and all other values were normalized to this value. Data are the means of at least three independent experiments; error bars indicate S.E.M.

demonstrated previously (Joseph et al., 1992) by Western-blot analysis using an antibody raised against a conserved region of the catalytic domain of PKC (Ballester and Rosen, 1985) that this treatment reduces PKC to undetectable levels. Ethanol-dependent transphosphatidylase activity in v-Src transformed cells was not inhibited by depleting cells of PKC activity, whereas TPA-induced transphosphatidylase in BALB/C 3T3 cells was sensitive to PKC depletion (Figure 3a). In fact, there was a small, but reproducible, increase in transphosphatidylase in v-Src-transformed cells that had been depleted of PKC (Figure 3a).

We next examined the effect of the protein kinase inhibitor staurosporine on v-Src- and TPA-induced transphosphatidylase. Staurosporine has been reported to inhibit PKC *in vivo* (Tamaoki et al., 1986) and we have found that at 500 nM, staurosporine blocks TPA-induced phosphorylation of the PKC substrate MARCKS (C. Joseph and D. A. Foster, unpublished work). Staurosporine, like PKC depletion, had a small, but reproducible, stimulatory effect on transphosphatidylase in v-Src-transformed cells, while inhibiting TPA-induced transphosphatidylase (Figure 3b). To establish that the lack of sensitivity of v-Src-induced PLD activity to PKC depletion was not simply a reflection of a difference in the different cell lines used, we examined the effect of PKC depletion and staurosporine on v-Src-induced PLD activity in BALB/c 3T3 cells transformed by the temperature-sensitive LA90 strain of Rous sarcoma virus (Maroney et al., 1992). As shown in Table 2, activating the kinase activity of v-Src induced increased PET levels within 30 min as described previously (Song et al., 1991). The v-Src-induced increase in PET levels was insensitive to both PKC depletion and staurosporine. When the LA90 cells were labelled with [3 H]arachidonate, v-Src-induced increases in PET were not observed. However, TPA-induced increases in PET in LA90 cells can be detected and these increases were sensitive to PKC depletion (Table 2). The data in Figures 3(a) and 3(b) and Table 2 suggest that the increased PLD activity in v-Src-transformed cells is independent of PKC. These data further distinguish the PLD activity induced by v-Src from that induced by phorbol esters that activate PKC and demonstrate that the activation of PLD activity by v-Src is independent of PKC.

We have previously demonstrated that DG produced in response to v-Src is derived from PC via a PLD/PA phosphatase-mediated mechanism (Song et al., 1991). The primary metabolite of PLD activity is PA, which can be converted into compounds other than DG, such as lyso-PA (van Corven et al., 1990). Therefore it was possible that the v-Src-induced PA produced in response to the PLD activity observed in Figure 3 was not

Table 2 v-Src-induced increases in PLC activity are independent of PKC

LA90 cells, which express a temperature-sensitive derivative of v-Src (Maroney et al., 1992), maintained at the non-permissive temperature for v-Src (40 °C) were either shifted to the permissive temperature for v-Src (34 °C) or treated with TPA (100 ng/ml) for 30 min, and PET levels were determined in cells labelled with either [3 H]myristate or [3 H]arachidonate. The TPA induction of PET levels in [3 H]arachidonate-labelled cells was performed on cells maintained at 34 °C. Cells were depleted of PKC by prolonged exposure to TPA (100 ng/ml, 26 h), or treated with staurosporine (500 nM, added 15 min before stimulation) as in Figure 3. The fold increase in PET in response to either v-Src or TPA is presented. Data are the means of two separate experiments where values did not vary by more than 20%. N.D., not determined.

	Fold increase in PET					
	[3 H]Myristate			[3 H]Arachidonate		
	Control	PKC depleted	Staurosporine	Control	PKC depleted	Staurosporine
v-Src	1.73	1.63	2.25	0.95	1.05	1.30
TPA	12	1.42	4.80	7.6	1.30	N.D.

Table 3 v-Src-induced increases in DG are independent of PKC

LA90 cells maintained at the non-permissive temperature for v-Src (40 °C) were either shifted to the permissive temperature for v-Src (34 °C), or treated with TPA (100 ng/ml) for 30 min as in Table 2. The fold increase in DG in response to either v-Src or TPA in untreated LA90 cells, LA90 cells that had been depleted of PKC or LA90 cells that had been treated with staurosporine was calculated. LA90 cells were prelabelled with [³H]myristate, which is incorporated almost exclusively into PC, as described previously (Song et al., 1991). The mean $\pm$ S.E.M. of at least three experiments is reported. Similar data were obtained when TPA was added to BALB/c 3T3 cells.

	Fold increase in DG		
	Control	PKC depleted	Staurosporine
v-Src	2.08 $\pm$ 0.27	1.86 $\pm$ 0.10	1.89 $\pm$ 0.09
TPA	2.12 $\pm$ 0.13	1.25 $\pm$ 0.16	1.38 $\pm$ 0.06

metabolized to the DG. We therefore examined whether v-Src-induced increases in DG were also insensitive to PKC depletion and staurosporine. To examine v-Src-induced DG levels, we used the LA90 cells expressing the temperature-sensitive derivative of v-Src. As shown in Table 3, v-Src-induced increases in DG were not significantly affected by either PKC depletion or staurosporine. In contrast, TPA-induced increases in DG were inhibited by these treatments (Table 3). Thus it is likely that the PLD activity detected in v-Src-transformed cells generates PA which is efficiently metabolized to generate the increases in DG seen in response to v-Src. It is of interest that, although TPA induces PLD activity to a far greater extent than does v-Src, the induction of DG by v-Src and TPA is comparable. This suggests that the PA generated by v-Src is converted more efficiently into DG than the PA generated by TPA. These data further suggest differences in the PLDs, which may include specificity at the level of the aliphatic components of the phospholipid substrates.

DISCUSSION

It has recently become apparent that PLD activity plays a significant role in the transduction of intracellular signals [see Exton (1990) or Foster (1993) for review]. PLD activity leads to the production of PA, which can be converted into DG or other biologically active molecules including lyso-PA (van Corven et al., 1990). PA has been reported to be biologically active by itself (Moritz et al., 1992). We have previously demonstrated that v-Src-induced increases in DG are derived from PC via a PLD/PA phosphatase mechanism. As v-Src activates intracellular signals that are dependent on PKC (Spangler et al., 1989; Qureshi et al., 1991; Nori et al., 1990), we suggested that the DG produced by this mechanism might be responsible for the activation of PKC (Song et al., 1991). However, several reports have demonstrated that the activation of PLDs by a variety of agonists is blocked by depleting cells of PKC (Billah et al., 1989a; Hii et al., 1989, 1991; Liscovitch, 1989; Huang and Cabot, 1990; Reinhold et al., 1990; Balsinde and Mollinedo, 1991; Yamada et al., 1991) or that PLD is activated by the addition of PKC to isolated membranes (Conricode et al., 1992). In this report, we have shown that PLD activated by v-Src and that activated by phorbol esters can be distinguished by using different phospholipid precursors to prelabel cellular phospholipids. The PLD activated by v-Src was unable to catalyse the transphosphatidylation of cellular phospholipids prelabelled with either arachidonate or alkyl-lysoPC, whereas TPA was able to induce the transphosphatidylation of phospholipids prelabelled with both of these compounds. Thus the PLD activated by v-Src

probably has a more restricted substrate range than the PLD(s) activated by phorbol esters with regard to the aliphatic composition of the phospholipid substrates. We also demonstrated that v-Src-induced PLD activity was insensitive to, or perhaps even enhanced by, depleting cells of PKC. These data are consistent with the hypothesis that v-Src activates a PLD that is specific for a subpopulation of PC lacking both arachidonic acid and ether linkages.

As the production of DG from PC in response to v-Src is independent of PKC, it is possible that this PC-derived DG activates PKC. The DG produced in response to v-Src increases gradually to a maximum level approx. 30 min after activation of the kinase activity of v-Src (Song et al., 1991). This gradual increase is slower and longer lived than that reported for the DGs produced by PLC-mediated hydrolysis of PIs (Exton, 1990; Fukami and Takenawa, 1989; Augert et al., 1989; Lee et al., 1991). DGs produced in response to a variety of stimuli can vary considerably in their aliphatic components (Pessin and Rabin, 1989; Pessin et al., 1990; Leach et al., 1991). It has also been shown that different classes of DGs, based on aliphatic composition, are differentially metabolized by DG kinase to PA (Ford and Gross, 1990). Additionally, it has been shown that DGs produced from PCs are poor substrates for DG kinase relative to the DGs produced from PIs (Lee et al., 1991). Thus it may be important that the DG produced in response to v-Src is derived from PC (Song et al., 1991). It may also be important in this regard that v-Src-induced increases in both DG and PEt were not observed when cells were prelabelled with arachidonate (Figure 2a). Although arachidonate is incorporated preferentially into PI (Swendsen et al., 1987; Takayama et al., 1987), it is also incorporated into the PC of these cells (Table 1). In human neutrophils, PC containing arachidonate is predominantly of the *sn*-1 alkyl ether class of phospholipid (Chilton and Connell, 1988). Thus there may be a relationship between the lack of observable PLD activity when either alkyl-lysoPC or arachidonate is used to prelabel phospholipids. The activation by v-Src of a PLD that is specific for PCs lacking arachidonate and ether linkages may be important for producing DGs that are poor substrates for DG kinase, and thus lead to a more prolonged DG signal. In this regard, it may be important that, although TPA induced greater increases in transphosphatidylation than v-Src, the induction of DG production by v-Src and TPA were comparable (see Figures 2b and 2c and Table 3). Thus the PA produced in response to TPA is probably metabolized differently from that generated in response to v-Src, which is rapidly hydrolysed to DG (Song et al., 1991). This further implicates the aliphatic composition of phospholipids as being biologically important, because, without the head group, the only structural differences are in the aliphatic components.

TPA was able to induce PLD activity when alkyl-lysoPC was used to prelabel cells. Therefore it is capable of activating a PLD that recognizes PC. As v-Src activates a PLD activity that is specific for PC (Song et al., 1991) but does not recognize the alkyl-PCs, the data presented here suggest that there are PLDs with substrate specificities for PCs with different aliphatic composition. The TPA-activated PLD(s) recognize PCs containing ether linkages in the *sn*-1 position and possibly arachidonate-containing PC, whereas v-Src-activated PLD(s) do not recognize PCs containing arachidonate or ether linkages, but do recognize PCs containing myristate and palmitate. The extent to which the specificity of PLDs for the aliphatic components of PC exists remains to be determined; however, the data presented here suggest that there are PLDs that can distinguish between PCs on the basis of their aliphatic makeup.

PKC has been implicated in the transduction of intracellular

signals activated by v-Src (Halsey et al., 1986; Sagara et al., 1986; Wolfman et al., 1987; Spangler et al., 1989; Nori et al., 1990; Qureshi et al., 1991). If v-Src activates PKC, it might be predicted that v-Src would activate the PLD(s) that are activated by PKC in response to phorbol esters and that the transphosphatidylolation products produced in response to TPA should also be produced in response to v-Src. This was clearly not the case, as TPA produced a greater variety of transphosphatidylolation products than v-Src (see Figure 2). Thus TPA probably activates additional PLD activity not activated by v-Src. Phorbol esters have been demonstrated to activate a PLD that hydrolyses PIs (Balsinde et al., 1988; Balsinde and Mollinedo, 1990), which preferentially incorporate labelled arachidonate (Swendsen et al., 1987; Takayama et al., 1987; Song et al., 1991). Thus TPA might be activating a PLD that is specific for PIs that are efficiently labelled with arachidonic acid. The lack of induction of this TPA-induced PLD by v-Src might be the result of additional signals, activated by v-Src, that down-regulate the PLD(s) activated by direct activation of PKC with phorbol esters. In this regard, it may be important that v-Src also activates PKC-independent as well as PKC-dependent intracellular signals (Qureshi et al., 1991). The PKC-independent signals activated by v-Src could modify or restrict the PLD activity that is activated in response to PKC. It is also possible that, as the DG produced in response to a v-Src differs from that produced in response to phorbol esters that activate PKC, the subspecies of DG produced in response to v-Src do not induce the same effects that phorbol esters induce. Kolesnick and Paley (1987) have previously demonstrated that phorbol esters and DGs can induce differential effects. Molecular details of this apparently complex intracellular signalling phenomenon remain to be determined, but preliminary data from our laboratory suggest that v-Src activates only a subset of PKC isoforms present in murine fibroblasts. Thus it is possible that the apparent aliphatic specificity demonstrated here may contribute to a selective activation of PKC isoforms.

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REFERENCES

- Augert, G., Bocckino, S. B., Blackmore, P. F. and Exton, J. H. (1989) *J. Biol. Chem.* **264**, 21689–21698
- Ballester, R. and Rosen, O. M. (1985) *J. Biol. Chem.* **260**, 15194–15199
- Balsinde, J. and Mollinedo, F. (1990) *FEBS Lett.* **259**, 237–240
- Balsinde, J. and Mollinedo, F. (1991) *J. Biol. Chem.* **266**, 18726–18730
- Balsinde, J., Diez, E. and Mollinedo, F. (1988) *Biochem. Biophys. Res. Commun.* **154**, 502–508
- Billah, M. M., Pai, J.-K., Mullmann, T. J., Egan, R. W. and Siegel, M. I. (1989a) *J. Biol. Chem.* **264**, 9069–9076
- Billah, M. M., Eckel, S., Mullmann, T. J., Egan, R. W. and Siegel, M. I. (1989b) *J. Biol. Chem.* **264**, 17069–17077
- Cabot, M. C., Welsh, C. J., Cao, H.-T. and Chabbot, H. (1988) *FEBS Lett.* **233**, 153–157
- Chilton, F. H. and Connell, T. R. (1988) *J. Biol. Chem.* **263**, 5260–5265
- Conricode, K. M., Brewer, K. A. and Exton, J. H. (1992) *J. Biol. Chem.* **267**, 7199–7202
- Exton, J. H. (1990) *J. Biol. Chem.* **265**, 1–4
- Foster, D. A. (1993) *Cell Signalling* **5**, 389–399
- Ford, D. A. and Gross, R. W. (1990) *J. Biol. Chem.* **265**, 12280–12286
- Fukami, K. and Takenawa, T. (1989) *J. Biol. Chem.* **264**, 14985–14989
- Halsey, D. L., Girard, P. R., Kuo, J. F. and Blackshear, P. J. (1986) *J. Biol. Chem.* **261**, 2234–2241
- Hii, C. S. T., Kokke, Y. S., Pruimboom, W. and Murray, A. W. (1989) *FEBS Lett.* **257**, 35–37
- Hii, C. S. T., Edwards, Y. S. and Murray, A. W. (1991) *J. Biol. Chem.* **266**, 20238–20243
- Huang, C. and Cabot, M. C. (1990) *J. Biol. Chem.* **265**, 14858–14863
- Johnson, R. M., Waislenko, W. J., Mattingly, R. M., Weber, M. J. and Garrison, J. C. (1989) *Science* **246**, 121–124
- Joseph, C. K., Qureshi, S. A., Wallace, D. W. and Foster, D. A. (1992) *J. Biol. Chem.* **267**, 1327–1330
- Kobayashi, M. and Kanfer, J. N. (1987) *J. Neurochem.* **48**, 1597–1603
- Kolesnick, R. N. and Paley, A. E. (1987) *J. Biol. Chem.* **262**, 9204–9210
- Leach, K. L., Ruff, V. A., Wright, T. M., Pessin, M. S. and Rabin, D. M. (1991) *J. Biol. Chem.* **266**, 3215–3221
- Lee, C., Fisher, S. K., Agranoff, B. W. and Hajra, A. K. (1991) *J. Biol. Chem.* **266**, 22837–22846
- Liscovitch, M. (1989) *J. Biol. Chem.* **264**, 1450–1456
- Llahi, S. and Fain, J. N. (1992) *J. Biol. Chem.* **267**, 3679–3685
- Maroney, A. C., Qureshi, S. A., Foster, D. A. and Brugge, J. S. (1992) *Oncogene* **7**, 1207–1214
- Martins, T. J., Sugimoto, Y. and Erickson, R. L. (1989) *J. Cell Biol.* **108**, 683–691
- Martinson, E. A., Trilivas, I. and Brown, J. H. (1990) *J. Biol. Chem.* **265**, 22282–22287
- Moritz, A., De Graan, P. N. E., Gispén, W. H. and Wirtz, K. W. A. (1992) *J. Biol. Chem.* **267**, 7207–7210
- Nishizuka, Y. (1986) *Science* **233**, 305–312
- Nori, M., Shawver, L. K. and Weber, M. J. (1990) *Mol. Cell. Biol.* **10**, 4155–4162
- Pessin, M. S. and Rabin, D. M. (1989) *J. Biol. Chem.* **264**, 8729–8738
- Pessin, M. S., Baldassare, J. J. and Rabin, D. M. (1990) *J. Biol. Chem.* **265**, 7959–7966
- Price, B. D., Morris, J. D. H., Marshall, C. J. and Hall, A. (1989) *J. Biol. Chem.* **264**, 16638–16643
- Qureshi, S. A., Joseph, C. K., Rim, M.-H., Maroney, A. and Foster, D. A. (1991) *Oncogene* **6**, 995–999
- Qureshi, S. A., Alexandropoulos, K., Rim, M., Joseph, C. K., Bruder, J., Rapp, U. and Foster, D. A. (1992) *J. Biol. Chem.* **267**, 17635–17639
- Randall, R. W., Bonser, R. W., Thompson, N. T. and Garland, L. G. (1990) *FEBS Lett.* **264**, 87–90
- Reinhold, S. L., Prescott, S. M., Zimmerman, G. A. and McIntyre, T. M. (1990) *FASEB J.* **4**, 208–214
- Rodríguez-Pena, A. and Rozengurt, E. (1984) *Biochem. Biophys. Res. Commun.* **120**, 1053–1059
- Sagara, J., Yamada, K. M. and Kakanuga, T. (1986) *Cancer Res.* **46**, 5292–5296
- Song, J., Pfeffer, L. M. and Foster, D. A. (1991) *Mol. Cell. Biol.* **11**, 4903–4908
- Spangler, R., Joseph, C. K., Qureshi, S. A., Berg, K. B. and Foster, D. A. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 7017–7021
- Swendsen, C. L., Chilton, F. H., O'Flaherty, J. T., Surles, J. R., Piatadosi, C., Waite, M. and Wykle, R. L. (1987) *Biochim. Biophys. Acta* **919**, 79–89
- Takayama, H., Gimbrone, M. A. and Schafer, A. I. (1987) *Biochim. Biophys. Acta* **922**, 314–322
- Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. and Tomita, F. (1986) *Biochem. Biophys. Res. Commun.* **135**, 397–402
- Uehara, Y., Fukazawa, H., Murakami, Y. and Mizuno, S. (1989a) *Biochem. Biophys. Res. Commun.* **163**, 803–809
- Uehara, Y., Murakami, Y. U., Mizuno, S. and Kawai, S. (1989b) *Virology* **164**, 294–298
- Uehara, Y., Murakami, Y. U., Sugimoto, Y. and Mizuno, S. (1989c) *Cancer Res.* **49**, 780–785
- van Blitterswijk, W. J., Hilkmann, H., de Widt, J. and van der Bend, R. L. (1991) *J. Biol. Chem.* **266**, 10337–10343
- van Corven, E. J., Groenink, A., Jalink, K., Eichholtz, T. and Moolenaar, W. H. (1990) *Cell* **59**, 45–54
- Vance, D. E. and Vance, J. (eds.) (1985) *Biochemistry of Lipids and Membranes*, Benjamin/Cummings, Menlo Park, CA
- Welsh, C. J., Schmeichel, K., Cao, H.-T. and Chabbot, H. (1990) *Lipids* **25**, 675–684
- Wolfman, A., Wingrove, T. G., Blackshear, P. J. and Macara, I. G. (1987) *J. Biol. Chem.* **262**, 16546–16552
- Yamada, K., Kanaho, Y., Miura, K. and Nozawa, Y. (1991) *Biochem. Biophys. Res. Commun.* **175**, 159–164