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Mitogenic Phospholipase D Activity Is Restricted to Caveolin-Enriched Membrane Microdomains

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Phospholipase D (PLD) activity is elevated in response to the oncogenic stimulus of several signaling oncogenes. PLD activity is also elevated in response to peptide growth factors, indicating that PLD likely plays an important role in mitogenic signaling. Many proteins that mediate mitogenic signaling are localized in caveolin-enriched membrane microdomains (CEMMs). We report here that the elevated PLD activity in NIH 3T3 cells transformed by activated oncogenic forms of Src, Ras, and Raf is largely restricted to the CEMMs. Likewise, the PLD activity stimulated by epidermal growth factor is also restricted to the CEMMs. Although both PLD1 and PLD2 were found in CEMMs, neither was particularly enriched in the CEMMs of the transformed relative to the parental cells, indicating that it is the specific activity of PLD that is increased in the CEMMs. An apparent PLD substrate specificity in transformed cells for phosphatidylcholine lacking arachidonate acyl groups is also explained by the localization of activity in the CEMMs where [^{3}H]arachidonate-labeled PC was excluded. These data indicate that mitogenic signals through PLD are initiated in CEMMs where many signaling molecules colocalize.

Key Words: **caveolae; EGF; oncogene; PLD; transformation.**

Phospholipase D (PLD) activity is elevated in response to many extracellular signals (1) indicating that PLD plays an important role in the transduction of intracellular signals. PLD activity is also elevated in response to the oncoproteins v-Src (2), v-Ras (3–5), v-Fps, (6) and v-Raf (7). PLD is also critical for the transformed phenotype induced by Src and Ras (8, 9) as well as the EGF receptor (10). We demonstrated previously that the PLD activity induced by v-Src could not be detected in cells that were radiolabeled with the polyunsaturated fatty acid arachidonic acid (2, 11). This exclusion occurred in spite of arachidonic acid being incorporated efficiently into phosphatidylcholine (PC) (11), the substrate for PLD. PLD activity in v-Src-transformed cells was only detected when radiolabeled saturated fatty acids like palmitate or myristate were used to label membrane phospholipids. This suggested that either: 1) the PLD activated by v-Src had a substrate preference for PC containing saturated fatty acids or 2) that PC with saturated fatty acids was localized to where the PLD activated by v-Src. v-Src localizes to the plasma membrane by virtue of being doubly acylated with two saturated fatty acids in the amino terminus (12). v-Src has been reported to localize to caveolae or caveolin-enriched membrane microdomains (CEMMs) (13). Caveolae are plasma membrane invaginations that form in the presence of caveolin (14). Caveolae are rich in glycosphingolipids, cholesterol, and phospholipids enriched with saturated fatty acids, and it has been proposed that the stronger van der Waals interactions between the closely packed saturated fatty acids and cholesterol create membrane microdomains that float in the plasma membrane much like a “raft” (15, 16). These lipid rafts contain many of the proteins that facilitate the transduction of intracellular signals and have been proposed to serve as recruitment and assembly sites for intracellular signaling complexes (17). Since v-Src localizes to these CEMMs, the observed preference of the v-Src-induced PLD for PC with saturated fatty acids could be due to localization of these PC species to these membrane microdomains that are rich in phospholipids with higher percentages of saturated fatty acids. In this report, we have investigated whether the PLD activity elevated in response to the oncogenic signals of v-Src, v-Ras, and v-Raf is restricted to the CEMMs, where there is an enrichment for phospholipids with satu-

Abbreviations used: CEMM, caveolin-enriched membrane microdomain; EGF, epidermal growth factor; PC, phosphatidylcholine; PLD, phospholipase D; TLC, thin-layer chromatography.

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rated fatty acids such as the PC utilized as substrate by the v-Src-induced PLD.

MATERIALS AND METHODS

Cells and cell culture conditions. Parental and v-Src-, v-Ras-, and v-Raf-transformed NIH 3T3 cells were described previously (3, 4, 7). 3Y1 rat fibroblasts and 3Y1 rat fibroblasts overexpressing the epidermal growth factor (EGF) receptor were described by Hornia *et al.* (18). All cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% bovine serum (HyClone). Cell cultures were made quiescent to reduce background PLD activity by growing to confluence and then replacing with fresh media containing 0.5% bovine serum for one day.

Materials. Dipalmitoyl-PC, phosphoatidylethanolamine, and phosphatidylinostol-4,5-bisphosphate were purchased from Sigma; [^{3}H]-myristate, [^{3}H]-arachidonate, [^{3}H]-stearate and {choline-methyl-[^{3}H]-dipalmitoyl}-PC were obtained from New England Nuclear. Precoated silica 60A thin layer chromatography plates were from Scientific Products; protease inhibitor cocktail (Set I) was from Calbiochem.

Isolation of membranes. The strategy for separation of light and heavy membrane fractions was based on one developed by Lisanti and colleagues (19) with modifications that excluded the use of sodium carbonate and high pH. Quiescent confluent cells grown in 150 mm dishes were washed twice with ice-cold phosphate-buffered saline, scraped into 2 ml of buffer M (25 mM Mes, pH 6.5; 250 mM sucrose; 1 mM EDTA) with protease inhibitors. Homogenization was carried out by using a Wheaton Dounce homogenizer (20~25 strokes) and then by sonication (three 20-s bursts; VC 300, Sonics & Materials Inc., Danbury, CT). Protein concentration was determined as described previously (18). 5 mg of homogenate protein was diluted to 2 ml in Buffer M, adjusted to 45% sucrose (w/v) by adding 2 ml of 90% sucrose (w/v) prepared in 25 mM Mes, pH 6.5. This solution was then overlaid with 35% and 5% sucrose (w/v) in 25 mM Mes, pH 6.5 to form a discontinuous gradient in an ultracentrifuge tube. The gradient was centrifuged at 39,000 rpm for 16–18 h in an SW41 rotor (Beckman). 1-ml fractions were collected from top to bottom and analyzed for PLD activity and proteins as described in the text. The pellets were sonicated in 1 ml of buffer M and analyzed along with the collected fractions.

Antibodies. Antibodies raised against Caveolin 1, the endoplasmic reticulum binding protein BiP, Na^+,K^+-ATPase βII, the *trans*-Golgi protein TGN38 and Raf were from Transduction Laboratories. The anti-Ras antibody was obtained from Santa Cruz Biotechnology. Antibodies against PLD1 and PLD2 were purchased from Quality Control Biochemicals (QCB). The v-Src antibody was from Calbiochem. A monoclonal antibody to the EGF receptor (LA22) was obtained from Upstate Biotechnology.

Western analysis. Proteins were heated for 3 min at 100°C prior to separation by SDS-polyacrylamide gel electrophoresis using an 8–12% acrylamide separating gel. After transferring to polyvinylidene difluoride (for caveolin) or nitrocellulose membrane (for other proteins), the membrane filters were blocked with 5% non-fat dry milk in phosphate-buffered saline and incubated with the appropriate antibody. Depending upon the origin of the primary antibodies, either anti-mouse or anti-rabbit IgG conjugated with horseradish peroxidase was used, and the bands were visualized using the enhanced chemilluminescent detection system (Pierce).

Assay of PLD activity. A liposome-based *in vitro* PLD assay based largely on strategies used by Brown *et al.* (20) was used to assay PLD activity in cell homogenate or the fractions. PLD activity was determined by examining the ability to convert [^{3}H]-PC in prepared liposomes to phosphatidylethanol in the presence of exogenously provided ethanol. Liposomes were prepared by mixing chloroform solutions of phospholipids containing 1 μCi/reaction of [^{3}H]-PC (42 Ci/mM) and drying under a stream of nitrogen followed by resuspension with sonication for 10 min at room temperature in lysis buffer (25 mM HEPES, pH 7.2; 100 mM KCl; 10 mM NaCl; 0.5 mM EGTA; 0.5 mM EDTA; 1 mM DTT). The liposomes consist of phosphatidylethanolamine/phosphatidylinositol-4,5-bisphosphate/PC in a molar ratio of 16/1.4/1 with PC suspended to a final concentration of 8.6 μM. The reaction buffer consisted of lysis buffer plus 5 mM $MgCl_2$, 0.16 mM $CaCl_2$ and 1% ethanol in a total volume of 0.1 ml. The reaction mixtures were incubated for 45 min at 37°C. The reaction was stopped by adding 400 μl of 10% trichloroacetic acid and 200 μl of 10 mg/ml bovine serum albumin. Precipitated lipids and proteins were removed by centrifugation at 3000 $\times$ g for 10 min at 4°C. An aliquot of the supernatant containing the released choline was removed and analyzed by liquid scintillation spectroscopy.

In vivo PLD activity was determined by the transphosphatidylation reaction in the presence of 0.8% butanol as described previously (11). Cells in 100-mm culture dishes were prelabeled with [^{3}H]-myristate for 4–6 h in Dulbecco's modified Eagle medium containing 0.5% bovine serum. Lipids were extracted and characterized by thin layer chromatography as described below. Relative levels of PLD activity were then determined by measuring the intensity of the corresponding phosphatidylbutanol band in the autoradiograph using a Molecular Dynamics scanning densitometer and Image-Quant software.

Characterization of phospholipid metabolites by thin layer chromatography (TLC). Phospholipid metabolites were characterized by TLC (silica gel 60A plates) using procedures described previously (2, 11). Lipid standards were visualized by treating TLC plates with iodine vapor. The following solvent systems were used: For PC, $CHCl_3$:methanol:glacial acetic acid:H_2O (50:25:8:4;v/v); for phosphatidylbutanol, the upper phase of ethylacetate:trimethylpentane: acetic acid:H_2O (90:50:20:100;v/v).

RESULTS

Elevated levels of PLD activity in transformed cells is restricted to the CEMMs. PLD activity is elevated in cells transformed by several signal transducing oncogenes including v-Src (2), v-Ras (3–5), and v-Raf (7). The level of induction of PLD activity in these cells was generally rather small (2- to 3-fold) relative to the level of other responses induced by mitogenic signals. Since PLD activity has been implicated in other cellular functions such as protein trafficking (21), it is possible that the low level of PLD activity induced in response to mitogenic signals is due to a high background PLD activity involved in other cellular activities. Many proteins implicated in mitogenic signaling including Src, Ras and Raf have been reported to localize to CEMMs (14, 17). We therefore wanted to examine PLD activity in different membrane fractions from both normal and transformed cells. NIH 3T3 cells and NIH 3T3 cells transformed by v-Src, v-Ras and v-Raf were harvested and membranes isolated, broken up by sonication and centrifuged over a discontinuous sucrose gradient to separate the light membrane CEMM fraction from other membranes. The distribution of proteins known to localize to caveolae (caveolin 1), the plasma membrane (Na^+,K^+-ATPase βII), the *trans*-Golgi network (TGN38) and the endoplasmic reticulum (BiP) was examined in NIH 3T3 cells. As shown in Fig. 1A, caveolin 1 was present predominantly in fractions 4 and 5,

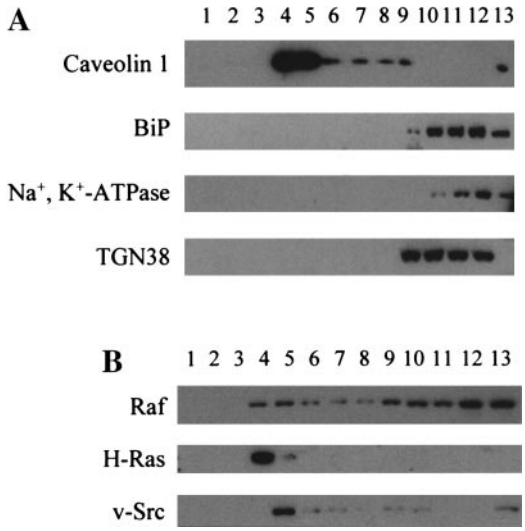

FIG. 1. Distribution of proteins in light and heavy membrane fractions. A. NIH 3T3 cells were disrupted by Dounce and Polytron homogenization and then sonicated as described under Materials and Methods. The membrane fragments were then run over a discontinuous gradient of 5%, 35% and 45% sucrose. 12 fractions and a pellet were recovered and subjected Western blot analysis using antibodies to caveolin 1, the endoplasmic reticulum protein BiP, the plasma membrane protein Na^+, K^+-ATPase $\beta 2$, and the *trans*-Golgi network protein TGN38. The amount of material loaded onto the gels was normalized by volume from each of the fractions. The figure shown is a representative of three independent experiments. B. NIH 3T3 cells transformed by v-Src, v-Ras, or v-Raf were subjected to the same fractionation procedure as in A, and then subjected to Western blot analysis using antibodies raised against Src, Ras, and Raf as shown.

which contains about 5–6% of the total proteins (not shown) and represents the light membrane fraction containing the CEMMs. BiP, Na^+,K^+-ATPase, and TGN38, as expected, were found in heavier membrane fractions (Fig. 1A), indicating that the fractionation procedure was able to separate the CEMMs from the heavier membrane fractions. Similar data were obtained with the transformed cells except that, as described previously (13), caveolin 1 levels were substantially reduced (data not shown). We also investigated the distribution of v-Src, v-Ras and v-Raf in the gradient and as shown in Fig. 1B, all three oncoproteins could be found in the light membrane fraction. v-Src and H-Ras were found almost exclusively in the light membrane fraction, whereas Raf was detected all fractions throughout the gradient.

To examine the PLD activity in isolated membranes, we needed to use an *in vitro* PLD assay. We showed previously (22), that the elevated PLD activity observed in cells transformed by v-Src and v-Ras was still observed in broken cell lysates. To confirm this observation and to establish that this was also the case in v-Raf-transformed cells, both *in vivo* and *in vitro* PLD assays were compared in the transformed and parental NIH 3T3 cells. As shown in Fig. 2A, similar increases in PLD activity were observed using either the *in vivo* transphosphatidylation reaction or the *in vitro* choline release assay. These data indicate that the elevated PLD activity seen in the transformed cells is maintained in this broken cell system.

We next examined the PLD activity in the parental and transformed NIH 3T3 cells in fractions obtained from the sucrose gradient. Although elevated PLD activity relative to the NIH 3T3 cells could be detected in the intermediate density fractions (fractions 6–8), the most significant increase in PLD activity was seen in the light membrane fractions 4 and 5 (Fig. 2B). As much as an 8-fold increase in PLD activity in the cells

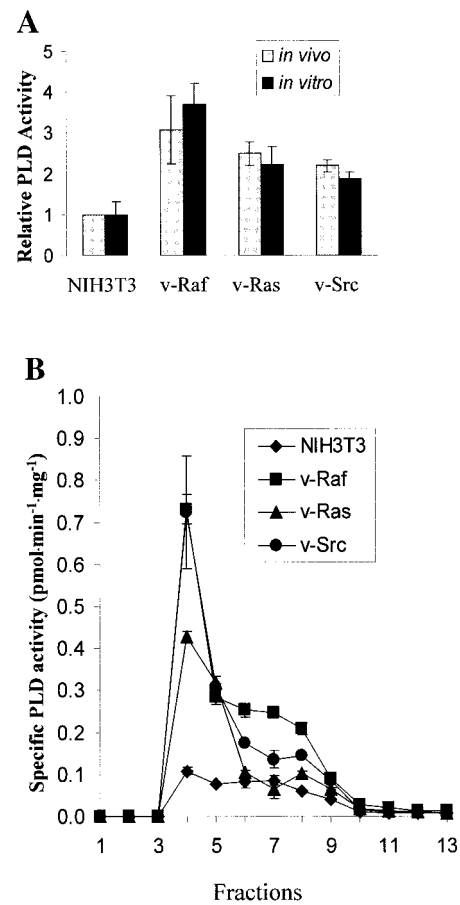

FIG. 2. Elevated levels of PLD activity in transformed cells are restricted to the CEMMs. A. PLD activity in NIH 3T3 cells and NIH 3T3 cells transformed by v-Src, v-Ras, or v-Raf was determined by transphosphatidylation (*in vivo*) or choline release (*in vitro*) as described under Materials and Methods. Error bars represent standard deviations for duplicate experiments repeated three times (*in vivo*) or four times (*in vitro*). B. NIH 3T3 cells and NIH 3T3 cells transformed by v-Src, v-Ras or v-Raf were subjected to the fractionation procedure described in Fig. 1A. Fractions were recovered and the PLD activity in the different fractions was determined by adding liposomes containing [^{3}H]-choline-labeled PC and measuring choline release as described under Materials and Methods. PLD activity in each fraction was normalized for total protein in each fraction. Error bars represent the range for duplicate results from a representative of two independent experiments.

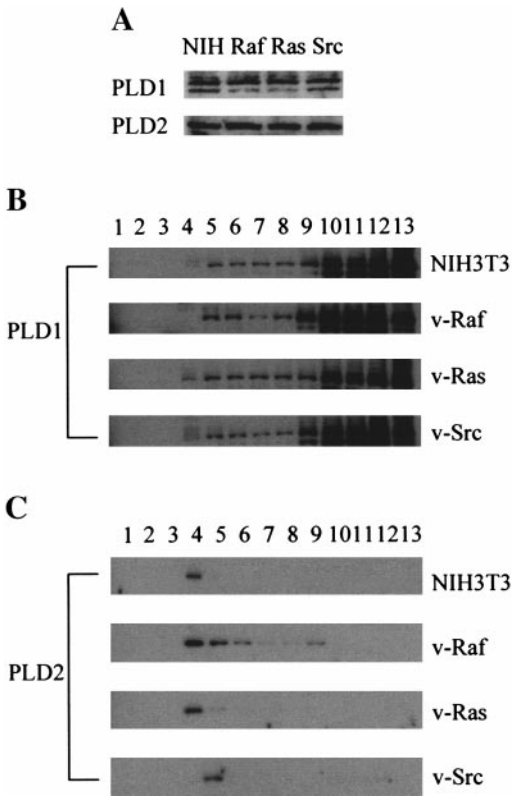

FIG. 3. Increased PLD activity is not due to increased levels of either PLD1 or PLD2. Lysates from NIH 3T3 cells and NIH 3T3 cells transformed by v-Src (Src), v-Ras (Ras), or v-Raf (Raf) were subjected to Western blot analysis using antibodies raised against either PLD1 or PLD2 (A). The parental and transformed NIH 3T3 cells were then subjected to fractionation procedures described in the legend to Fig. 1A and the recovered fractions were subjected to Western blot analysis using antibodies raised against either PLD1 (B) or PLD2 (C). The data shown are representative experiments of three (for PLD1) or two (for PLD2) independent experiments.

transformed by v-Raf was seen in fraction 4 whereas only 2- to 3-fold increase was seen in the intermediate fractions. Similar differences were observed for the v-Src- and v-Ras-transformed cells relative to the parental NIH 3T3 cells.

Increased PLD activity is not due to increased levels of either PLD1 or PLD2. We next investigated the levels of PLD1 and PLD2 in the transformed and parental NIH 3T3 cells. In Fig. 3A, it is shown that there was no significant difference in the levels of either PLD1 or PLD2 in the parental or transformed cells that could account for elevated PLD activity in the transformed cells. Both PLD1 and PLD2 have been reported to be present in CEMMs (23–25). We therefore examined the levels of PLD1 and PLD2 in the CEMM fractions of the NIH 3T3 and transformed NIH 3T3 cells. Both PLD1 (Fig. 3B) and PLD2 (Fig. 3C) could be detected in the CEMM fraction. PLD1 was present in both the heavy and light membrane fractions whereas PLD2 was largely restricted to the light membrane fraction. There did not appear to be any dramatic changes in the distribution of either PLD1 or PLD2 in the transformed cells relative to the parental NIH 3T3 cells that could account for the increased PLD activity observed in the light membrane fractions. These data suggest that the increased PLD activity in the transformed cells is due to a change in the activity of PLD rather than to an increase in either PLD1 or PLD2 protein.

EGF-induced PLD activity is predominantly in CEMMs. EGF causes an increase in PLD activity (18, 26, 27). The EGF receptor has also been reported to be present in CEMMs and upon EGF treatment, the receptor leaves the CEMMs and internalized (28). We examined the distribution of the EGF receptor in 3Y1 rat fibroblasts that overexpress the EGF receptor (18). As shown in Fig. 4A, the EGF receptor was present in the light membrane fraction 5. However, there was also a substantial amount of the receptor in the intermediate density fractions 8 and 9 (Fig. 4A). We next examined the PLD activity in the different fractions of the 3Y1 cells overexpressing the EGF receptor, and as shown in Fig. 4B, the most significant increase in PLD activity was observed in the light membrane fraction 5.

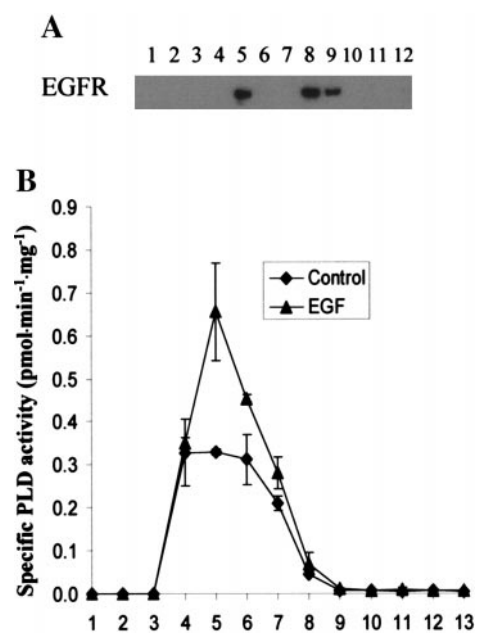

FIG. 4. EGF-induced PLD activity is predominantly in CEMMs. A. 3Y1 cells overexpressing the EGF receptor were subjected to the fractionation procedures used in Fig. 1A and the presence of the EGF receptor in the different fractions was determined by Western blot analysis. B. The 3Y1 cells overexpressing the EGF receptor were treated with EGF (100 ng/ml, 4 min), subjected to fractionation and the PLD activity in the different fractions was determined as in Fig. 2B. The PLD activity in each fraction was normalized for total protein in each fraction. Error bars represent the range for duplicate results from a representative of two independent experiments.

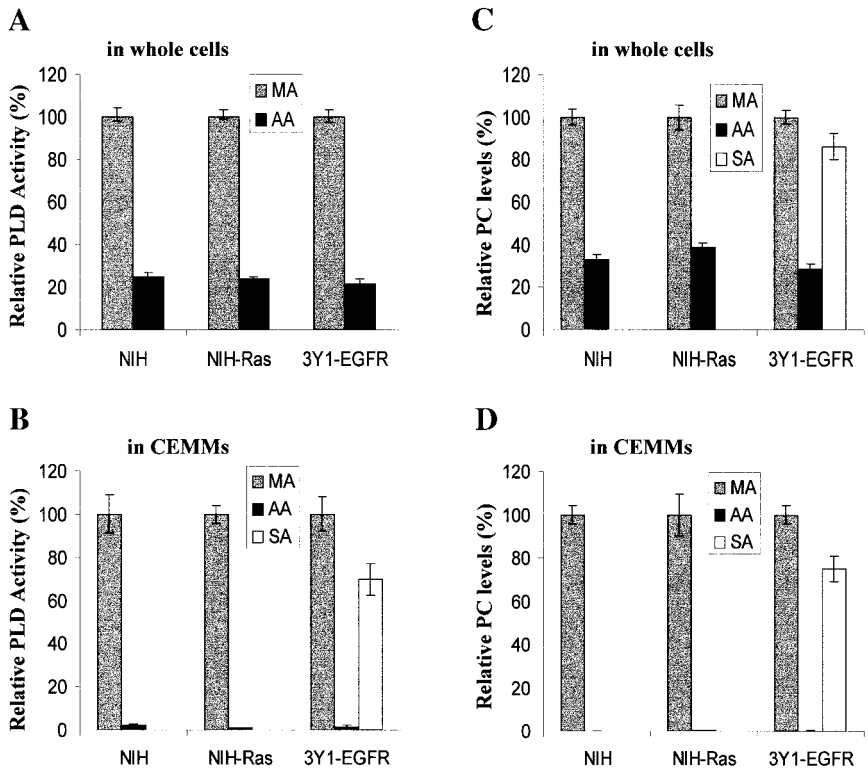

FIG. 5. PC prelabeled with [^{3}H]-arachidonic acid is excluded from CEMMs. NIH 3T3 (NIH), Ras-transformed NIH 3T3 (NIH-Ras), and 3Y1-EGFR cells were prelabeled with [^{3}H]-myristic acid (MA), [^{3}H]-arachidonic acid (AA), or [^{3}H]-stearic acid (SA). PLD activity was then determined in whole cells (A) or in the CEMM fraction (fractions 4 and 5) of isolated membranes (B) by measuring the production of phosphatidylbutanol by TLC as described under Materials and Methods. The PLD activity in [^{3}H]-arachidonic acid- and [^{3}H]-stearic acid-labeled cells was normalized to the PLD activity in the [^{3}H]-myristic acid-labeled cells, which was assigned a value of 100% for each cell line. Relative PC levels in whole cells (C) and in the CEMM fraction (D) was determined scanning of autoradiographs from TLC plates where PC had been resolved. The PC from the [^{3}H]-arachidonic acid- and [^{3}H]-stearic acid-labeled cells was normalized to the PC in the [^{3}H]-myristic acid-labeled cells, which was assigned a value of 100% for each cell line. The data represent the average of three independent experiments ± standard deviation.

Interestingly, there was very little increase seen in the intermediate fractions 8 and 9 where there was also substantial EGF receptor. This population of EGF receptor likely represents internalized EGF receptor. These data indicate that the PLD activity elevated in response to EGF occurs in the CEMMs where EGF receptor is present prior to internalization.

Substrate specificity for PC lacking arachidonate is explained by exclusion from CEMMs. We previously reported that the PLD activity elevated in transformed cells, as measured by transphosphatidylation, could not be detected when PC was prelabeled with the polyunsaturated fatty acid arachidonic acid (2, 11). In contrast, PLD activity elevated in response to TPA could readily be detected when substrate PC was prelabeled [^{3}H]-arachidonic acid. This result could have been due to exclusion of PC containing the polyunsaturated arachidonic acid from the light membrane fraction, which is enriched with membrane lipids containing saturated fatty acids (15, 16). We therefore investigated the distribution of PC prelabeled with either the polyunsaturated arachidonate, or the saturated fatty acids myristate and stearate. We first examined the PLD activity in either whole cells or in isolated CEMMs (fractions 4 and 5). As shown in Fig. 5A, the PLD activity in whole cells, prelabeled with [^{3}H]-arachidonic acid, ranged from 20% to 30% of that observed in cells labeled with [^{3}H]-myristic acid. In contrast, there was virtually no PLD activity detected in the CEMM fraction when cells were labeled with [^{3}H]-arachidonic acid (Fig. 5B). We next examined the level of [^{3}H]-arachidonate-, [^{3}H]-myristate-, and [^{3}H]-stearate-labeled PC in whole cells and the CEMM fraction. As shown in Fig. 5C, the PC detected in whole cells prelabeled with [^{3}H]-arachidonic acid was approximately 30% of that observed in cells labeled with either [^{3}H]-myristic or [^{3}H]-stearic acid. In contrast, virtually no PC could be detected in the CEMM fraction when cells were labeled with [^{3}H]-arachidonic acid (Fig. 5D). These data suggest that the apparent substrate specificity of mitogenic PLD activity for PC lacking arachidonic acid (11) was due to exclusion of arachidonate-containing PC from the membrane microdomain where the mitogenic PLD is activated.

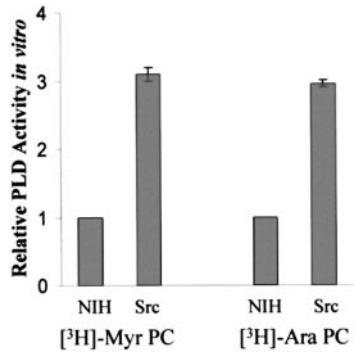

FIG. 6. V-Src-induced increase in PLD activity has no substrate specificity *in vitro*. [^{3}H]-PC was isolated from cells labeled with either [^{3}H]-arachidonic acid or [^{3}H]-myristic acid as described under Materials and Methods. The [^{3}H]-arachidonic acid- and [^{3}H]-myristic acid-labeled PC was then used to prepare [^{3}H]-PC-labeled liposomes ([^{3}H]-Myr PC or [^{3}H]-Ara PC as shown). Membranes from either parental or v-Src-transformed NIH 3T3 cells were added and PLD activity, as determined by transphosphatidylation in the presence of 1% butanol, was determined as described previously (4, 30). The data represent the range of two independent experiments.

To demonstrate that there was no inherent substrate specificity of the PLD activated by mitogenically activated PLD, we examined PLD activity in membranes from parental and v-Src-transformed NIH 3T3 cells using substrate PC in generated liposomes. Substrate PC was generated by labeling v-Src-transformed NIH 3T3 cells with either [^{3}H]-myristate or [^{3}H]-arachidonate and then recovering TLC-resolved PC. Membranes from either parental or v-Src-transformed NIH 3T3 cells were then mixed with liposomes containing the recovered [^{3}H]-myristate- or [^{3}H]-arachidonate-labeled PC. As shown in Fig. 6, the increased PLD activity in the membranes from the v-Src-transformed cells was observed using either [^{3}H]-myristate- or [^{3}H]-arachidonate-labeled PC. These data indicate the PLD activated in response to v-Src does not have any intrinsic substrate specificity that would exclude PC containing the polyunsaturated arachidonic acid. The data further support the hypothesis that the PLD elevated in response to mitogenic signals takes place in CEMMs, which are enriched with phospholipids containing saturated fatty acids.

DISCUSSION

In this report we have shown that the elevated PLD activity in transformed cells is largely restricted to CEMMs where many signaling molecules aggregate to form signaling complexes (17). As reported previously (23, 24), both PLD1 and PLD2 could be detected in the CEMM fraction, however there was no dramatic increase in either PLD1 or PLD2 in these fractions that would account for the increased activity. The apparent substrate specificity of mitogenically-stimulated PLD for PC lacking arachidonic acid (11) could also be explained by exclusion of arachidonate-containing PC from the CEMMs. These data provide evidence that the PLD activity elevated in response to mitogenic signals takes place in CEMMs, where many of the proteins involved in the initiation of mitogenic signals are localized.

The restriction of elevated PLD activity in the transformed cells to the light membrane CEMM fraction explains our previous observation that elevated PLD activity in transformed cells was not observed when cells were radiolabeled with the polyunsaturated fatty acid arachidonic acid (2, 11). Data presented here demonstrates that arachidonate-labeled PC was absent in the light membrane fraction. Moreover, the elevated PLD activity in v-Src-transformed cells had no substrate preference in an *in vitro* PLD assay. Thus, the apparent substrate specificity for PC lacking arachidonic acid observed previously for mitogenically stimulate PLD activity is likely due to the localization of this PLD activity to the CEMMs that are enriched with phospholipids containing saturated fatty acids.

The PLD activity elevated in response to Src, Ras, Raf, and EGF is dependent upon the small GTPase RalA (4, 7, 10, 18, 29). We previously demonstrated that PLD1, but not PLD2, could be precipitated by immobilized GST-RalA fusion protein (4, 30). We also demonstrated that the PLD associated with RalA was responsive to Arf (30), which is also a characteristic of PLD1 (31, 32). These data indicated that the PLD activity mediated by RalA was the Arf-responsive PLD1. The data presented here show that while PLD1 is present in the CEMMs, only a small percentage of the total PLD1 is present in this fraction. In contrast, almost all of the PLD2 is in light membrane fractions. Thus, while we believe that the Arf-responsive, RalA-associated PLD1 is likely involved in mitogenically stimulated PLD activity, an additional role for PLD2, which is apparently localized almost exclusively to the CEMM fraction can not be ruled out. It is possible that PLD1 and PLD2 work together in response to the mitogenic signals initiated in the CEMMs.

ACKNOWLEDGMENTS

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