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Partial Isolation From Intact Cells of a Cell Surface-Exposed Lysophosphatidylinositol-Phospholipase C

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Abstract A novel cell surface phosphoinositide-cleaving phospholipase C (ecto-PLC) activity was isolated from cultured cells by exploiting its presumed external exposure. Biotinylation of intact cells followed by solubilization of the biotinylated proteins from a membrane fraction and recovery onto immobilized-avidin beads, allowed assay of this cell surface enzyme activity apart from the background of the substantial family of intracellular PLCs. Several cell lines of differing ecto-PLC expression were examined as well as cells stably transfected to overexpress the glycosylphosphatidylinositol (GPI)-anchored protein human placental alkaline phosphatase (PLAP) as a cell surface enzyme marker. The resulting bead preparations from ecto-PLC positive cells possessed calcium-dependent PLC activity with preference for lysophosphatidylinositol (lysoPI) rather than phosphatidylinositol (PI). The function of ecto-PLC of intact cells evidently is not to release GPI-anchored proteins at the cell surface, as no detectable Ca^{2+}-dependent release of overexpressed PLAP from ecto-PLC-positive cells was observed. To investigate the cell surface linkage of the ecto-PLC itself, intact cells were treated with bacterial PI-PLC to cleave simple GPI anchors, but no decrease in ecto-PLC activity was observed. High ionic strength washes of biotinylated membranes prior to the generation of bead preparations did not substantially reduce the lysoPI-PLC activity. The results verify that the ecto-PLC is truly cell surface-exposed, and unlike other members of the PLC family that are thought to be peripheral membrane proteins, this novel lysoPI-PLC is most likely a true membrane protein. J. Cell. Biochem. 65:550–564.

Key words: ecto-PLC; ecto-enzyme; phosphoinositide-specific phospholipase C; cell surface enzyme; lyso-PI-cleaving PLC

Since the discovery of the phosphoinositide-dependent signal transduction pathway in eukaryotic cells, the phosphoinositide-specific phospholipases C (PLCs) have been the subject of intense interest [reviewed in Crooke and Bennett, 1989; Cockroft and Thomas, 1992; Rhee, 1993]. A working model for this pathway involves soluble cytoplasmic PLCs, which in response to the activation of specific cell surface receptors, bind to the internal face of the plasma membrane and cleave phosphorylated derivatives of phosphatidylinositol (PI; see Fig. 1) to generate potent second messengers [Cockroft and Thomas, 1992]. It is now clear that this model, while appropriate for the most widely studied PLC isozymes, does not necessarily apply to all members of the PLC family. In particular, a number of studies have provided evidence for the presence of PLCs in other locations both within the cell [McBride et al., 1991; Payrastre et al., 1992; Mazzoni et al., 1992] and extracellularly. Reports of the latter include both a cell surface–bound activity [Ting and Pagano, 1990, 1991; Volwerk et al., 1992; Birrell et al., 1993] and a soluble activity released from intact cells [Birrell et al., 1995].

The extracellular members of the PLC family are particularly intriguing, as their function is unknown, and their relationship to each other and to the intracellular enzymes remains to be determined. Work from two laboratories on the mouse and rat fibroblast cell surface PLC does

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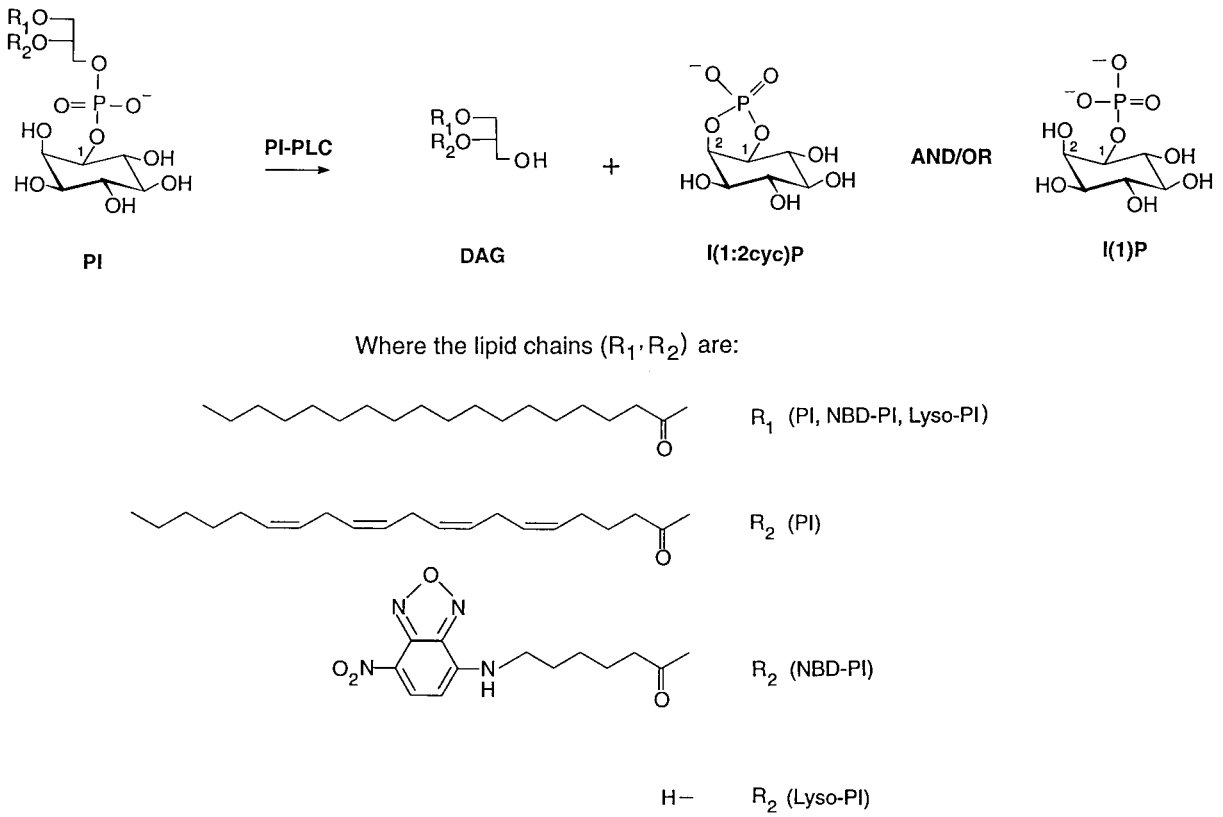

Fig. 1. Diagram illustrating the basic scheme for the cleavage of PI by a PI-PLC. The products formed, which in mammalian cells are potent second messengers, are diacylglycerol (DAG) and inositol phosphates [inositol 1,2 cyclic monophosphate [I(1:2cyc)P] and inositol 1-monophosphate [I(1)P]]. *B. cereus* PI-PLC is known to generate the cyclic IP first and to subsequently catalyze conversion to the noncyclic form [Volwerk et al., 1990]; the details of this reaction remain to be determined for mammalian PI-PLCs. Also indicated are the structures of the lipid chains, R_1 and R_2, for beef liver PI, lysoPI, and the fluorescent PI derivative NBD-PI.

appear to describe the same cell surface enzyme. In experiments based on two different approaches [a fluorescence assay using NBD-PI: Ting and Pagano, 1990, 1991; and a biochemical assay using radiolabeled lysoPI: Volwerk et al., 1992], the expression of cell surface PLC activity correlated with the onset of quiescence in normal fibroblasts, while virally or chemically transformed fibroblasts did not express the activity. A subsequent study of normal rat brain astrocytes and the C6 rat glioma line using the lysoPI-based assay revealed a cell surface PLC activity (ecto-PLC) in glial cells as well, with a constitutive rather than growth-dependent pattern of expression but otherwise similar to the mouse fibroblast ecto-PLC [Birrell et al., 1993]. To date, isolation of this novel extracellular PLC from intact cells, and determination of the nature of its linkage to the cell surface, has not been accomplished for any cell type.

One of the challenges in isolation and characterization of a cell surface–exposed PLC is to separate the enzyme from the potentially contaminating (and substantial) background provided by the intracellular PLC family. While the majority of the PLCs studied have been purified from the cytosolic fraction, tightly membrane-bound (presumably through ionic interactions) PLCs are also found. Thus, purification procedures that are optimal for isolation of membrane proteins rather than soluble cytosolic proteins are not sufficiently selective for preferential isolation of a membrane-bound ecto-enzyme. In this report, we have employed a procedure in which biotinylation of intact cells is used as a first step in the recovery of cell surface–exposed proteins onto avidin-methacrylate or -agarose beads. To provide a readily detectible control cell surface enzyme, ecto-PLC-positive rat glial cells (cell line C6), which were stably transfected to overexpress human placental alkaline phosphatase (PLAP), were also used. We show that this procedure can be used to recover PLC activity from biotinylated ecto-

PLC-positive, but not ecto-PLC-negative or non-biotinylated, cells. The results verify this approach and indicate that the activity is in fact due to a cell surface–exposed PLC enzyme, which can be studied in an in vitro assay system apart from intact cells.

MATERIALS AND METHODS

Cells and Cell Culture

The C6 rat glioma, Swiss 3T3 mouse fibroblast, MCF-7 human breast carcinoma, 3T3L1 mouse preadipocyte, V79 hamster fibroblast, CCL 105 human adrenal cortex carcinoma, and K. Balb transformed mouse fibroblast cell lines were all obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose (Sigma, St. Louis, MO) and 10% iron-supplemented calf serum (Summit Biotech, Greeley, CO).

The C6PLAP and SwissPLAP cell lines expressing human placental alkaline phosphatase (PLAP) were derived from the C6 and Swiss 3T3 cell lines, respectively, by infection with a replication-defective retroviral vector, PinaPLAP513 [Mace et al., 1992]. The PinaPLAP513 vector is based on Moloney murine leukemia virus (M-MuLV) and produces the human PLAP protein along with the bacterial neomycin phosphotransferase (neo) gene, which confers resistance to the drug geneticin (G418) when expressed in mammalian cells. Infections of C6 rat glioma cells and Swiss 3T3 mouse fibroblasts were performed with virus particles assembled in the replication-defective Psi2 retrovirus vector packaging cell line [Mann et al., 1983] and infected cells were selected in growth media with 0.8–1.0 mg/ml of 50% pure G418 (Gibco, Grand Island, NY). Infected, G418-resistant colonies of C6 and 3T3 were separately pooled to generate the C6PLAP and SwissPLAP cell lines, and assayed for PLAP activity as described previously [Mace et al., 1992]. The C6PLAP and Swiss PLAP cell lines were subsequently subcloned in the presence of G418 and subclones selected for use on the basis of homogeneous labeling for cell surface PLAP by immunofluorescence (monoclonal antibody MAB 102, Chemicon, Temacula, CA).

Chemicals and Materials

The lysophosphatidylinositol substrate ([^{3}H]-lysoPI) was prepared from a mixture of liver phosphatidylinositol (PI; Avanti Polar Lipids, Birmingham, AL) and [^{3}H]-PI radiolabeled in the head group (DuPont NEN, Boston, MA) through the action of phospholipase A_2 as described [Volwerk et al., 1992]. When PI was examined as a potential substrate, care was taken to prepare it from the same lots of cold PI and radiolabeled PI as was the lysoPI substrate, in order to give the same specific radioactivity (typically 6–7Ci/mol). As a control for head group specificity, lysophosphatidylcholine ([^{3}H]-lysoPC) was also used in these assays. Phospholipase A_2 was used to generate the lysoPC substrate from a mixture of liver PC (Sigma) and head group radiolabeled [^{3}H]-PC (DuPont NEN) by the same procedure as used to prepare the lysoPI substrate. Recombinant PI-PLC from *Bacillus cereus* was obtained as previously reported [Koke et al., 1991]. Biotinylation of intact cells was accomplished with one of the water-soluble, nonpermeant biotinylating agents, NHS-ss-biotin or Sulfo-NHS-biotin (Pierce, Rockford, IL), with similar results. Immobilization of biotinylated membrane proteins for the experiments shown here was performed with Soft-Link Soft Release Avidin Resin (Promega Corporation, Madison, WI). Immunopure Immobilized Streptavidin (Pierce) was also used, with similar results. Purified Triton X-100 was from Boehringer Mannheim (Mannheim, Germany), sodium deoxycholate from Calbiochem (La Jolla, CA), sodium cholate from US Biochemical (Cleveland, OH), and sodium dodecylsulfate (SDS) from BDH Chemicals (Poole, UK).

Ecto-PLC Assay on Intact Cells

Confluent, healthy cultures of cells in 60 mm tissue culture dishes ("Falcon," Becton Dickinson, Lincoln Park, NJ) were washed twice with 3 ml of ice-cold buffer A (10 mM HEPES, pH 7.4, 4 mM KCl, 126 mM NaCl) and then twice with the same buffer containing either $CaCl_2$ (typically 1 mM, unless specified otherwise) or 1 mM EGTA (for negative controls). After the last wash, the dishes received 0.6 ml of their respective final wash buffer and were placed on a rocker at 7°C. To start the assay, substrate ([^{3}H]-lysoPI) was added at a final concentration of 25 µM with swirling, the dishes were rocked for 4 min at 7°C, and then the assay buffer was removed to microfuge tubes. Any floating cells were removed by 3 min of centrifugation at 11,600*g*, and a 0.5 ml aliquot of the supernatant taken for chloroform-methanol-HCl extraction

followed by extraction with chloroform alone, to separate the water-soluble [^{3}H]-inositol phosphate from the unreacted substrate for quantitation [Volwerk et al., 1992].

Cells which were exposed to bacterial PI-PLC prior to assay were treated as follows. Four 60-mm diameter dishes of identically seeded C6 cell cultures were washed with cold sterile buffer A. Two of the dishes received 0.5 ml of buffer A containing 1 U/ml PI-PLC from *B. cereus;* the remaining two dishes received 0.5 ml of buffer A only (mock-treated control). The cultures were rocked at 7°C for 15 min, aspirated free of buffer, washed 3× in cold buffer A, and returned to DMEM containing 10% serum at 37°C. After 2 h of incubation in culture medium, the PI-PLC-treated and mock-treated dishes were assayed for ecto-PLC activity (one of each pair in 1 mM Ca^{2+} and the other in 1 mM EGTA), as described above.

To test whether biotinylation altered the ecto-PLC (lysoPI-PLC) activity of intact cells, parallel 60-mm cultures of C6PLAP cells were treated (or mock-treated) with biotinylating agent as per the procedure in the following section. Immediately after this treatment, these cell cultures were assayed for ecto-PLC activity as described above. There was no evidence that biotinylation affected the ecto-PLC activity of intact cells.

Isolation of Cell Surface-Exposed Membrane Proteins From Intact Cells

The standard procedure used was adapted from Hare and Taylor [1992]. Depending on the size of the planned experiment, cell cultures were grown in either 100- or 150-mm tissue culture dishes (Corning, Corning, NY). A single 100-mm confluent culture can be used to prepare a bead preparation with readily measurable lysoPI-PLC activity, but for assays with multiple variables (which require splitting of the sample into many fractions), it is best to start with one or two confluent 150-mm cultures. The cultures were washed 3 times in ice-cold buffer A (3 or 5 ml depending on dish size), then once in ice-cold buffer A adjusted to pH 8.3. To all dishes other than mock-biotinylated dishes, 0.5 mg/ml biotinylating agent (either sulfo-NHS-biotin or NHS-ss-biotin) was added to the cells in ice-cold pH 8.3 buffer A and the cultures rocked at 7°C for 15 min. The cells remained attached and spread during this process. Following aspiration of the biotinylating agent, the cultures were washed 3 times in buffer A and then scraped up in 1 ml of ice cold homogenization buffer (0.27 M sucrose, 0.02 M HEPES, pH 7.4, 1 mM EDTA, and 20 µg/ml each leupeptin and aprotinin). Cells were broken open by 40 strokes in glass Dounce homogenizers on ice, then transferred to 15 ml tubes and bath sonicated (model G112SP1G, Laboratory Supplies, Hicksville, NY) on ice for 3 min. Each sample was diluted to 5 ml with additional homogenization buffer and then spun at 700g for 3 min to pellet intact cells and nuclei. If large pellets were present, the pellets were rehomogenized and recentrifuged, and the respective supernatants combined. The supernatants (containing membrane fragments) were transferred to ultracentrifuge tubes and centrifuged at 100,000*g* for 45 min at 4°C. The high-speed supernatants were discarded and the pellets were stirred up with the aid of a hand-held vortex/stirrer (Bio-Vortexer, RPI, Mt. Prospect, IL) in 1 ml of solubilization buffer (0.02 M Tris-HCl, pH 7.5, containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM EDTA, 1 mM phenylmethylsulfonylfluoride). After transfer to microfuge tubes, the samples were bath sonicated on ice for 5 min followed by centrifugation at 11,600*g* for 5 min to remove any insoluble material. The supernatants were then mixed with immobilized avidin "Soft-Link" beads (previously washed in solubilization buffer), at a ratio of 0.2–0.4 ml packed beads per ml of solubilized sample, and agitated overnight at 7°C. The beads were subsequently washed as follows: twice in 1.5 ml solubilization buffer, twice in solubilization buffer containing 0.5 M NaCl, twice in solubilization buffer diluted 1:10 with ddw, and twice in buffer A, prior to aliquoting for assay.

A variation on the standard procedure was to omit the SDS from the solubilization buffer, which did not significantly change the final results. In a few experiments, after the initial spin at 100,000*g* for 45 min, parallel membrane pellets from biotinylated cell cultures were vortexed (with a hand-held vortex/stirrer) on ice in standard homogenization buffer or homogenization buffer with 1.5 M NaCl. This was followed by a second spin at 100,000*g* for 45 min, prior to a final solubilization in solubilization buffer and incubation with avidin beads as previously described.

Assay of PI-PLC Activity on Beads

After aliquoting evenly into the desired number of assay tubes (silanized microfuge tubes, Coast Scientific, San Diego, CA), the beads were washed twice in assay buffer of the desired pH and then twice in final assay buffer containing either 1 mM $CaCl_2$ or EGTA. For single pH experiments at pH 7.0 or 7.5, buffer A was used for assays; for wider pH ranges, TMS buffer was used (50 mM Tris-maleate, 50 mM NaCl). The activity obtained with the lysoPI substrate was relatively unaffected by the addition of sodium deoxycholate up to 1 mg/ml, while assays with the PI substrate showed optimal activity in the 0.5–1 mg/ml range. Although these deoxycholate concentrations are at the low end of the expected CMC values for deoxycholate (0.5–3 mg/ml, depending on the buffer system) [Thomas and Christian, 1980] our observations are consistent with previous observations of a PI-PLC optimally active at 1 mg/ml [Manning and Sun, 1983]. Both activities declined significantly in the presence of 2 mg/ml deoxycholate (data not shown); thus 0.5 mg/ml deoxycholate was routinely included in the assay buffer. After the final wash, enough buffer was added back to make a total volume of 0.25 ml (including the typical packed bead pellet volume of 50 µl), substrate (either [^{3}H]-lysoPI or [^{3}H]-PI) was added from a stock to make a final concentration of 50 µM, and the tubes were rotated at 37°C using a Heto Rotamix (Gydevang Allerød, Denmark) for the desired time. At the end of this time, the reaction mixture was centrifuged for 3 min in the cold to pellet the beads, 0.2 ml of supernatant was transferred to silanized glass culture tubes, brought up to 0.5 ml volume with buffer A, and extracted first with 2.5 ml chloroform-methanol-HCl (66:33:1; vol/vol). The upper phase (0.9 ml) was then re-extracted, with 0.9 ml chloroform alone. Radioactivity in 0.55 ml of the aqueous phase, which contained the inositol phosphate products, was measured by liquid scintillation counting.

Assay of Alkaline Phosphatase Activity

Bead preparations to be assayed for PLAP activity (usually the same beads that were assayed for PLC activity) were washed twice in buffer A and twice in dw, before addition of 1 ml per sample of 1 M diethanolamine, pH 9.8, containing 1 mM $MgCl_2$ and 0.5 mg/ml *p*-nitrophenylphosphate. The samples were rotated at 37°C for the desired time (typically 10 and 20 min), followed by 3-min centrifugation in the cold to pellet the beads and transfer of the supernatants to cuvettes for measurement of the optical absorption at 405 nm using a Beckman DU-40 spectrophotometer. Samples demonstrating PLAP activity typically required a 1:10 or 1:20 dilution for OD measurements, and the assay used was linear to at least 30 min.

To assay alkaline phosphatase activity released from cells, 60- or 100-mm diameter confluent cultures of C6PLAP cells were washed 4× with buffer A, then incubated at 7°C in 1.25 (or 1.5 ml for the larger dishes) of buffer A containing 10 µg/ml leupeptin and 1 U/ml of PI-PLC from *B. cereus*. At various time points, aliquots were removed for assay with the chromogenic substrate *p*-nitrophenylphosphate in diethanolamine substrate buffer, by continuous monitoring of the increase in OD at 405 nm with temperature control (28° or 37°C, depending on the experiment). Assays of alkaline phosphatase in the absence of bacterial PI-PLC were carried out similarly except that the incubation with the cell cultures was for 1 h prior to assay in buffer A containing either 1 mM Ca^{2+} or 1 mM EGTA and protease inhibitors. Concentration of the buffer (in Centricon-30 spin concentration devices; Amicon, Beverly, MA) was not a useful approach for detecting low levels of PLAP release, as even in the presence of protease inhibitors, PLAP activity in concentrates of conditioned buffer from cells degraded rapidly. Therefore, in order to increase the sensitivity of the assay, in some experiments the fluorescent alkaline phosphatase substrate fluorescein diphosphate [Huang et al., 1992] (Molecular Probes, Eugene, OR) was used (28 µM) in place of *p*-nitrophenylphosphate. Continuous fluorescence measurements were made using a Hitachi F-4500 (Hitachi, Danbury, CT) fluorescence spectrophotometer, with an excitation wavelength of 476 nm and an emission wavelength of 518 nm.

Calculation of the number of PI-PLC-releasable PLAP enzymes per cell in C6PLAP cultures was based on the *p*-nitrophenylphosphate/diethanolamine assay at 37°C, with the assumptions of a specific activity of 1,600 µmoles of substrate hydrolyzed per minute per mg of purified enzyme at 37°C and a molecular weight of 70,000 for PLAP [Costa et al., 1993].

Identification of Water-Soluble Reaction Products

Assay buffer (from either intact cells or enzyme preparations isolated on beads) was extracted with chloroform and methanol (2:1; vol/vol) followed by chloroform only, to separate the water-soluble [^{3}H]-inositol-containing products from unreacted substrate and any soluble protein, followed by chromatography on Dowex AG 1-X8 ion exchange resin as described previously [Birrell et al., 1995].

RESULTS

Isolation of a Cell Surface LysoPI-Cleaving Activity From Intact Cells Onto Avidin-Linked Beads

Biotinylation of intact cells with a membrane-impermeant biotinylating agent is an increasingly used method for identifying and isolating plasma membrane proteins with an extracellular domain [e.g., Meier et al., 1992; Fujimoto et al., 1992]. Since previous studies have provided evidence of a PLC on the external cell surface of normal quiescent mouse fibroblasts [Ting and Pagano, 1991; Volwerk et al., 1992] and on rat glial cells [Birrell et al., 1993], a major goal of this study was to use cell surface biotinylation as a means of isolating this PLC activity from the complex environment of the whole cell. The approach used here is to biotinylate washed, intact cells, homogenize the cells, prepare a plasma membrane fraction, then solubilize the membrane proteins and incubate the solubilized proteins with avidin-methacrylate (Soft-Link) beads to immobilize the biotinylated membrane proteins. Following washes to remove loosely associated non-specifically bound proteins from the beads, the bead preparations are aliquoted into assay buffer, suspended by continuous agitation, and incubated with substrate under the desired conditions. At various time points following the addition of substrate, the reactions are terminated by centrifuging the beads out of the buffer followed by separation of water-soluble radioactive products from unreacted substrate and quantitation by scintillation counting, as described in Materials and Methods.

Figure 2 is an example of the results obtained when biotinylated membrane proteins from C6 rat glioma cells (an ecto-PLC positive cell type) and from a comparable number of V79 hamster fibroblasts (an ecto-PLC negative cell type) are immobilized on beads and assayed for Ca^{2+}-dependent phosphoinositide-cleaving activity. Figure 2A shows that among the biotinylatable cell surface membrane proteins from C6 cells is an enzyme that cleaves lysophosphatidylinositol (lysoPI) in a time- and calcium-dependent manner (Fig. 2A; open circles: +1 mM $CaCl_2$; closed circles: +1 mM EGTA). The biotinylatable cell surface membrane proteins from V79 hamster fibroblasts, in contrast, have essentially background levels of lysoPI-cleaving activity that do not increase with time (Fig. 2A; open squares: +1 mM $CaCl_2$; closed squares: +1 mM EGTA). LysoPI-cleaving activity was also present on bead preparations made from confluent C6PLAP rat glioma cells (C6 cells expressing human placental alkaline phosphatase) as well as from confluent/quiescent Swiss3T3, 3T3L1, and SwissPLAP (Swiss 3T3 cells expressing human placental alkaline phosphatase) cell cultures (all ecto-PLC-positive). Negligible lysoPI-cleaving activity was observed with bead preparations made from confluent MCF-7, CCL 105, and K. Balb cell cultures, as well as non-quiescent Swiss 3T3 cell cultures (all ecto-PLC-negative) (data not shown). Thus, there is a good correlation between the ecto-PLC activity of the intact cell type and the lysoPI-cleaving activity of bead preparations generated from the cell surface proteins of that cell type.

Surprisingly, when PI was used as the substrate in place of lysoPI, bead preparations from all cell types examined exhibited a low but definite calcium-dependent PI-cleaving activity (shown in Fig. 2B for C6 rat glial cells and V79 hamster fibroblasts). Since there was no correlation between the presence or absence of lysoPI-cleaving activity on beads and the presence of PI-cleaving activity on beads, it is possible that the latter is due to the presence of a separate enzyme. To provide a readily detectible independent cell surface enzyme control for the bead preparation procedure, C6PLAP cells (which express the cell surface enzyme human PLAP) were employed. Bead preparations were generated from one biotinylated and one mock-treated culture of C6PLAP cells, and assayed for calcium-dependent cleaving of lysoPI and PI (solid bars, Fig. 3, and for PLAP activity (open bars, Fig. 3). Both lysoPI-cleaving activity and PLAP activity were strongly biotinylation-dependent, indicating that these activities were due to the isolation of cell surface proteins through the biotinylation process. The presence

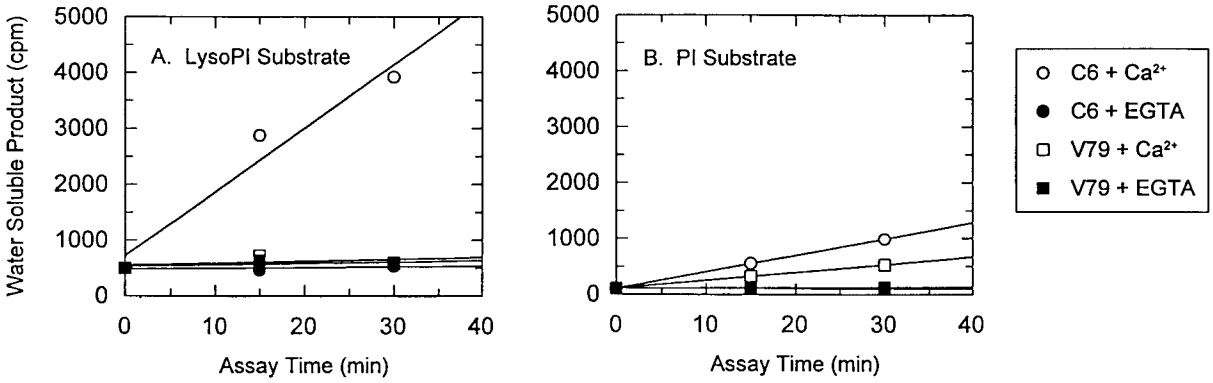

Fig. 2. Formation of water soluble radiolabeled product as a function of assay time at pH 7.5 for biotinylated membrane preparations isolated on Soft-Link (immobilized avidin) beads. Two cell lines were used: ecto-PLC-positive C6 rat glioma cells and ecto-PLC-negative V79 hamster lung fibroblasts. **A:** Substrate = [^{3}H]-lysoPI. **B:** Substrate = [^{3}H]-PI. +Ca^{2+} and +EGTA indicate that the assays were carried out in buffer containing 1 mM Ca^{2+} and 1 mM EGTA, respectively. The results with lysoPI-cleaving activity of C6-derived bead preparations were representative of 8 separate experiments; the comparison to lysoPI-cleaving activity of bead preparations derived from an ecto-PLC-negative cell-derived bead preparation was representative of 5 separate experiments.

of PI-cleaving activity on beads, on the other hand, is only partially dependent on biotinylation (Fig. 3). This suggests that a substantial part of the PI-cleaving activity may be due to nonspecific but tight binding to the beads of an enzyme, which is independent of the lysoPI-cleaving enzyme, possibly originating from the cytoplasmic face of the plasma membrane rather than the biotinylatable extracellular face. For this reason the PI-cleaving activity present on bead preparations was not pursued further. In addition to PI itself, [^{3}H]-lysoPC was examined as a potential substrate for the lysoPI-PLC on bead preparations of C6PLAP cells. However no time- or calcium-dependent water-soluble product was observed to be generated from lysoPC (data not shown).

Characterization of the LysoPI-Cleaving Activity on Bead Preparations as a Phospholipase C

Phospholipases are classified according to the type of product generated from cleavage of lipid substrates. The ecto-PLC activity on intact cells has been previously shown to have the specificity of a phospholipase C, as the water-soluble product generated from the cleavage of [^{3}H]-inositol-containing-lysoPI is almost exclusively [^{3}H]-inositol phosphate (IP) [Volwerk et al., 1992; Birrell et al., 1993]. To determine the nature of the phospholipase activity observed on bead preparations, Dowex ion-exchange column analysis of the water-soluble products formed from [^{3}H]-lysoPI was performed. Optimal enzyme activity in these assays is observed over a range between pH 6 and 8 (with a slight preference for pH 7; data not shown), and two pHs within this range were chosen for analysis. As a representative example, Figure 4 shows the elution profile for the products of the lysoPI-cleaving activity of C6 bead preparations. The elution positions of inositol (which would be produced by a phospholipase D) and of cyclic and noncyclic inositol phosphates (both produced by the action of phospholipase C) are indicated. Under the experimental conditions used, glycerophosphoinositol (produced by nonspecific esterases) would be found in fractions 2 and 3 in nearly equal amounts. Calcium-dependent production of inositol phosphates was observed at both assay pHs, with slightly higher activity observed at pH 7.5 than at 6.1. A shift from predominately noncyclic IP production at pH 7.5 towards noncyclic IP at the more acidic pH was observed, as has been reported for certain of the intracellular mammalian phospholipases C [Kim et al., 1989] as well as for an extracellular soluble PI-cleaving PLC released by mouse fibroblasts [Birrell et al., 1995]. These results are consistent with the presence on bead preparations of one type of phospholipase activity acting on the lysoPI substrate, and that this activity is a phospholipase C.

Determining Whether GPI Anchors Are Substrates for Ecto-PLC

Among possible substrates for ecto-PLC are the relatively large numbers of GPI-anchored proteins known to be present on the cell sur-

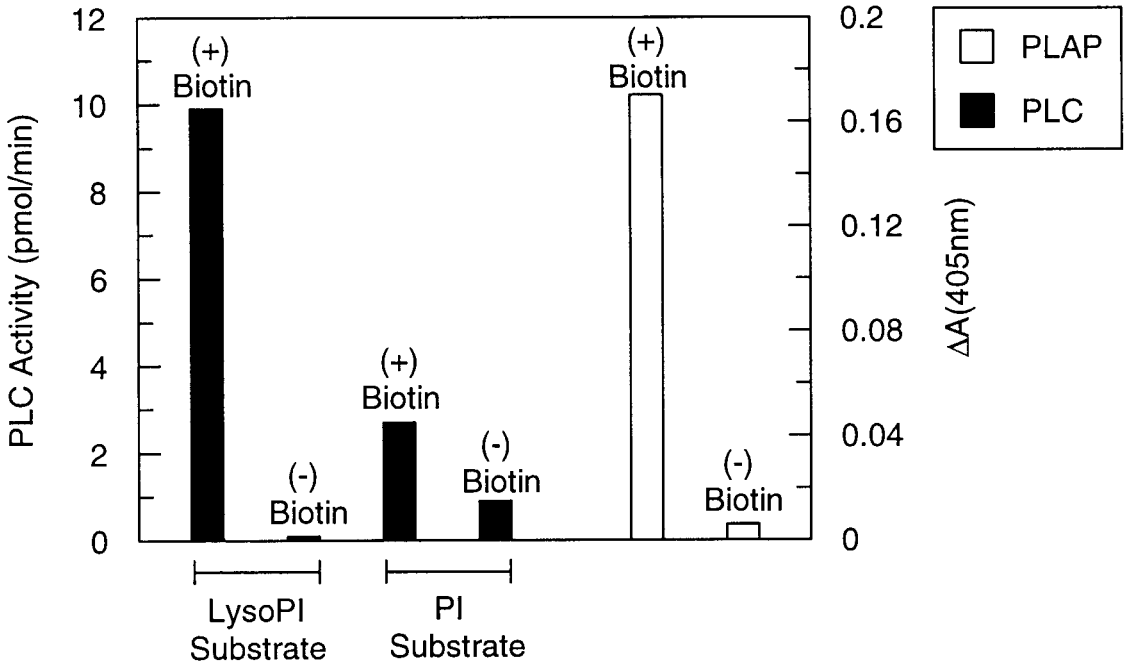

Fig. 3. Phosphoinositide-specific phospholipase C (solid bars) and alkaline phosphatase (open bars) activities isolated from C6PLAP cells onto avidin-linked beads. Cell preparations that were biotinylated before membrane isolation are labeled (+) biotin; mock-biotinylated negative controls are designated (−) biotin. Phospholipase C assays were carried out at pH 7.5; alkaline phosphatase assays were performed in diethanolamine buffer at pH 9.8. Similar results were obtained with an experiment carried out on bead preparations of SwissPLAP cells.

face, of which those with a "simple" GPI anchor are susceptible to cleavage by bacterial PI-PLC. To test whether bacterial PI-PLC-susceptible GPI anchors are readily cleaved by ecto-PLC on the intact cell surface, we examined whether there is a Ca^{2+}-dependent release of GPI-anchored PLAP from C6PLAP cells. The number of releaseable PLAPs present on each cell was first estimated by measuring the maximal bacterial PI-PLC-releasable PLAP activity from a known number of cells, as described in Materials and Methods. From this we estimate that there are roughly 1 million PI-PLC-removable PLAP molecules per C6PLAP cell. Using a fluorescence assay of PLAP activity (fluorescein diphosphate as substrate) [Huang et al., 1992], which in our hands is about 10-fold more sensitive than the chromogenic assay, we calculate that we should be able to readily detect the release of approximately 50–60 PLAP enzymes per cell from a population of 1×10^7 C6PLAP target cells. However, when parallel dishes of cells were then incubated in buffer containing either 1 mM Ca^{2+} or 1 mM EGTA for 1 h in the presence of protease inhibitors, and the buffers subsequently assayed for PLAP activity, no difference in PLAP activity was detectable by either the chromogenic assay (shown in Fig. 5 relative to bacterial PI-PLC-releasable PLAP) or the fluorescence assay (not shown). Therefore, it appears that little if any GPI-linked PLAP is released from C6PLAP cell surfaces by the action of ecto-PLC.

Mode of Attachment of the Ecto-PLC to the Cell Surface

A biotinylatable cell surface membrane-associated protein such as the lysoPI-PLC we isolate on bead preparations could be either a peripheral (bound by ionic interactions at the hydrophilic membrane face) or true membrane protein (bound by an anchor interacting with the hydrophobic membrane domain). High salt washes are an effective way to reduce the association of peripherally-bound proteins to membranes. A comparison of biotinylated C6 membrane pellets treated with or without 1.5M NaCl in an additional homogenization/wash step indicated there was little difference in the lysoPI-cleaving activity on the final bead preparations. This was in contrast to the independent (and presumably nonspecifically bound) PI-cleaving activity, which dropped by one-third for the NaCl-washed preparations (data not shown).

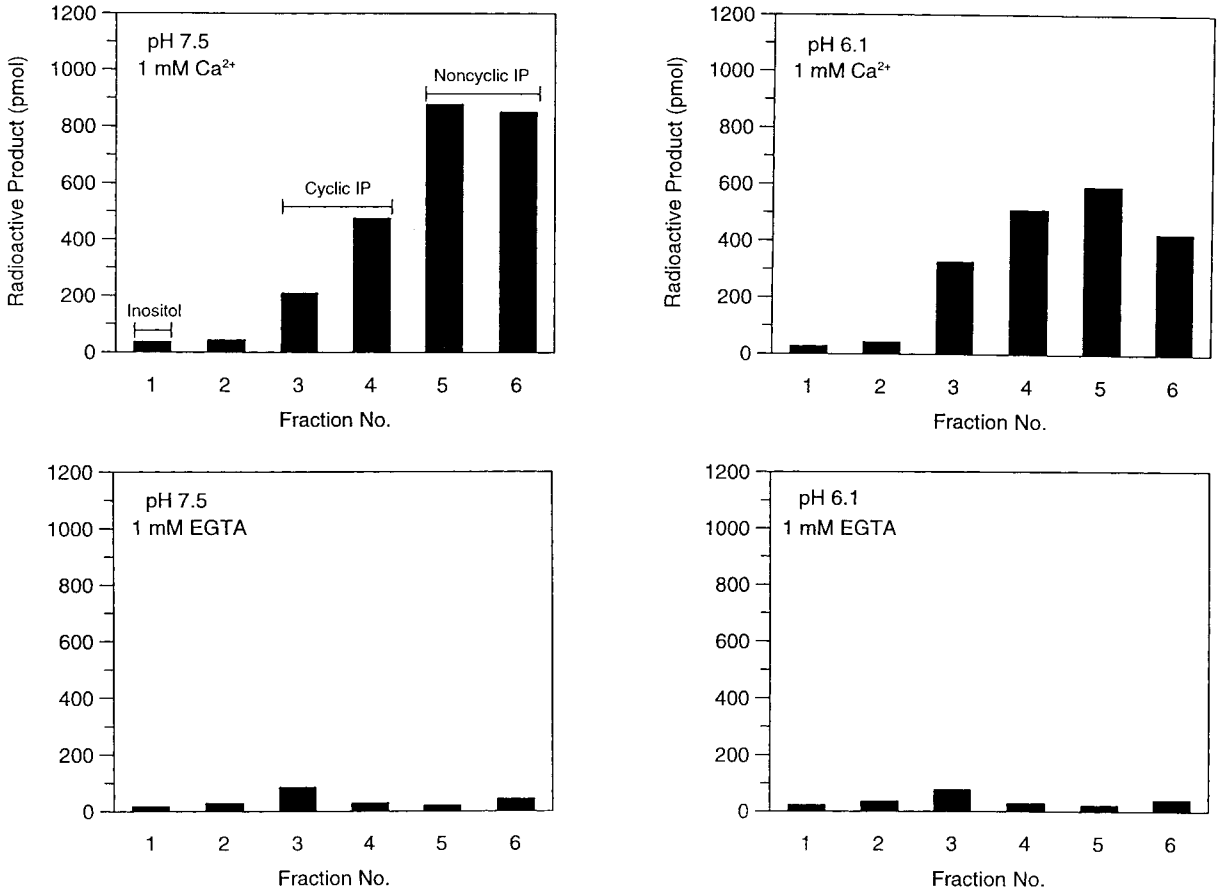

Fig. 4. Analysis of water-soluble radiolabeled products (containing [^{3}H]-inositol) generated by biotinylated cell membranes isolated from C6 cells onto avidin-linked beads. Samples were applied to a Dowex AG1-X8 anion exchange column and eluted with increasing concentrations of ammonium formate. Assays were run at pH 6.1 and 7.5 in Tris-maleate-saline buffer containing either 1 mM Ca^{2+} or 1 mM EGTA. Under the conditions used, almost all of the cyclic inositol monophosphate formed is eluted in fractions 3 and 4 and noncyclic inositol monophosphate is in fractions 5 and 6.

Thus, lysoPI-PLC on the cell surface (ecto-PLC) is probably a true rather than a peripheral membrane protein.

Two well-known classes of hydrophobically-anchored membrane proteins are those that are linked to the membrane by hydrophobic polypeptide residues (including transmembrane proteins), and those that are attached to the membrane via a lipid anchor (glycosylphosphatidylinositol (GPI)-linked proteins). To better understand the manner in which ecto-PLC is attached to the cell membrane, we measured ecto-PLC activity after treatment of the cells with bacterial PI-PLC, which cleaves unmodified GPI anchors [Guther et al., 1994]. Four parallel cultures of C6 cells were either mock-treated or treated with PI-PLC from *B. cereus* at 7°C. The conditions used were previously determined to be sufficient to cleave all PLC-removable PLAP activity from C6PLAP or SwissPLAP cell cultures (data not shown). After thorough washing with cold buffer, the cultures were returned to serum-containing culture medium in the incubator for 2 h before assaying for ecto-PLC activity. One dish each of the untreated and PI-PLC-treated cells were assayed in the presence of 1 mM Ca^{2+} and the remaining two dishes assayed in buffer containing 1 mM EGTA. The hydrophilic radiolabeled products of lysoPI cleavage were separated out and chromatographed on Dowex anion exchange resin under conditions that permit the separation of cyclic and noncyclic inositol phosphates.

As shown in Figure 6A, control cells which had not been exposed to bacterial PI-PLC exhibited high levels of Ca^{2+}-dependent ecto-PLC activity, which was almost completely abol-

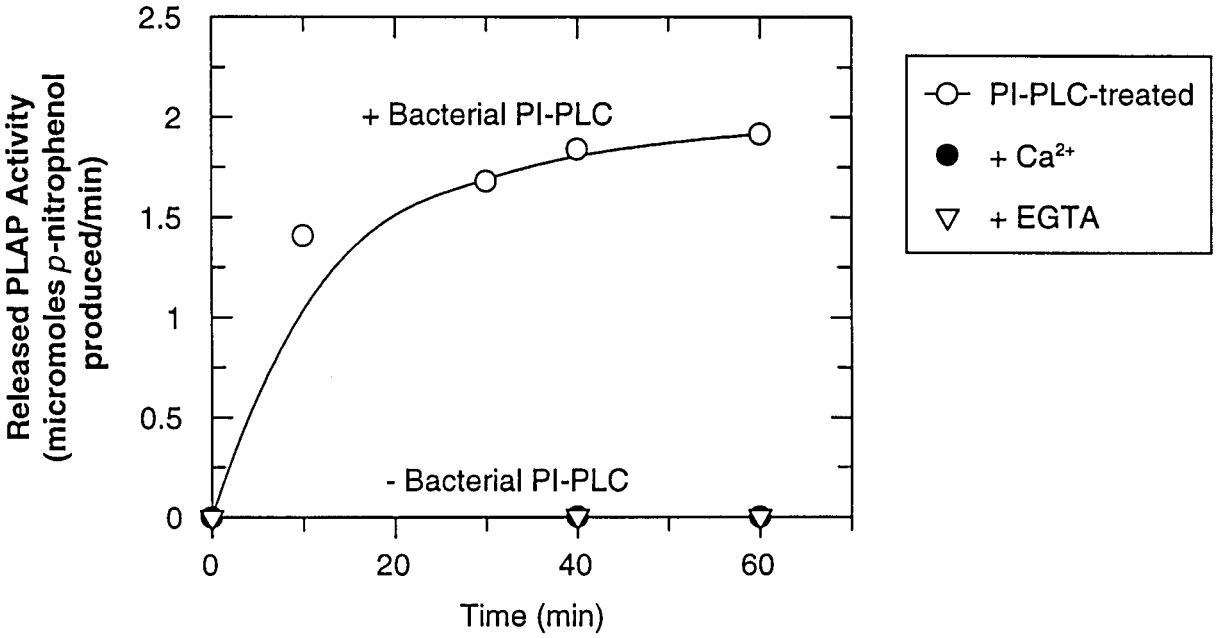

Fig. 5. Amount of placental alkaline phosphatase (PLAP) activity released as a function of time in cold buffer A, from cultures of C6PLAP cells. Closed circles/open triangles indicate spontaneous release of PLAP in the presence or absence of Ca^{2+}, while open circles indicate PLAP release catalyzed by bacterial PI-PLC. The experiment was performed three times with similar results.

ished when the assay was carried out in 1 mM EGTA (Fig. 6C). The Dowex column profile of Figure 6A shows that >80% of the product formed by untreated cells is noncyclic IP. Interestingly, cells pretreated with bacterial PI-PLC show no decrease in the production of IP, but rather appear to produce additional product in the form of cyclic IP (Fig. 6B). However, the source of the additional cyclic IP becomes evident by examination of the column profile from the assay performed in the presence of 1 mM EGTA (Fig. 6D). Bacterial PI-PLC does not require Ca^{2+} for activity, and is known to produce almost exclusively cyclic IP from a PI substrate [Volwerk et al., 1990]. Thus, the activity occurring when the assay is carried out in 1 mM EGTA almost certainly arises from bacterial PI-PLC bound tightly to the cell surface and not removed by the extensive washing employed prior to the assay. Subtracting the EGTA column profile (Fig. 6D) from that obtained in the presence of calcium ion (Fig. 6B) makes it possible to determine the amount of IP that is formed independently of the bacterial PI-PLC. The difference profile obtained (Fig. 6F) is very similar to that of the mock-treated control (Fig. 6E), indicating that bacterial PI-PLC treatment of the cells has little or no effect on ecto-PLC activity. We conclude from this result that ecto-PLC is not cleaved from the cell surface by bacterial PI-PLC. Although from this data we cannot rule out the possibility of a complex GPI linkage, the attachment of the enzyme to the cell surface is clearly not through a simple GPI anchor susceptible to cleavage by bacterial PI-PLC.

DISCUSSION

Ecto-enzymes are enzymes anchored to the external surfaces of cells with their active sites directed outside the cell [Kenny and Turner, 1987]. Independent work from two laboratories has shown that certain cultured cell types have a cell surface activity, which meets the criteria for an external plasma membrane PLC, or ecto-PLC [Ting and Pagano, 1990, 1991; Volwerk et al., 1992; Birrell et al., 1993]. The purpose of this study was to show that ecto-PLC activity could be isolated from intact cells on the basis of the presumed physical location of this enzyme on the external cell surface. The activity could then be examined apart from the substantial background of lipid-directed enzyme activities present in whole cells, with the eventual goal of isolation and cloning of this novel protein. The use of transfected mouse and rat cell lines expressing PLAP activity provided an easily measured independent assay, for purposes

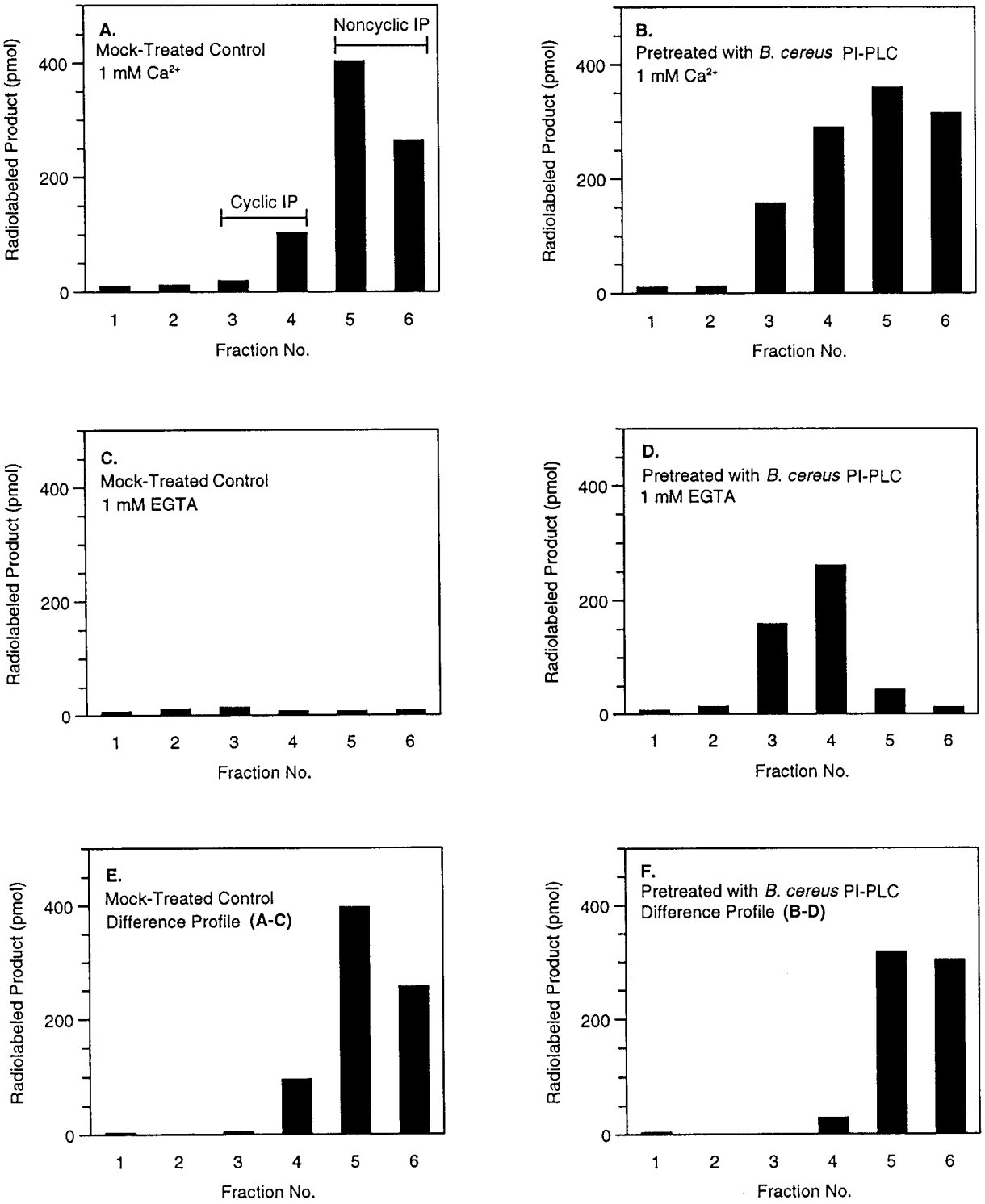

Fig. 6. Effects of pretreatment of C6 cells with bacterial PI-PLC on ecto-PLC activity. **A,C,E:** Mock-treated cells; **B,D,F:** bacterial PI-PLC-treated cells. Following treatment, the cell cultures were washed 3× with buffer and returned to culture medium for 2 h prior to assaying for ecto-PLC as described in Materials and Methods. The water-soluble products were separated on a Dowex anion exchange column as described in Materials and Methods; at this pH (7.4) the inositol phosphate product of bacterial PI-PLC cleavage of lysoPI is predominately cyclic IP eluting in fractions 3 and 4; that of the ecto-PLC is predominately noncyclic, eluting in fractions 5 and 6. The experiment was performed twice with similar results.

of comparison, of an additional cell surface enzyme with a defined membrane linkage.

Figure 2 demonstrates that it is possible to use the biotinylation procedure to isolate a lysoPI-cleaving activity from the surfaces of intact ecto-PLC positive cells, and that cells which are not ecto-PLC positive do not yield this activity. Similarly, Figure 3 shows that biotinylation is a required step in order to recover either lysoPI-cleaving activity or, as a cell surface enzyme control, PLAP activity on avidin beads. Figure 4 confirms that the lysoPI-cleaving activity isolated onto beads, like the ecto-PLC of intact cells, is consistent with its identification as a calcium-dependent phospholipase C activity yielding IP as a product. These results indicate that lysoPI-PLC on bead preparations does in fact originate from a bona fide externally exposed cell surface enzyme.

The presence of ecto-PLC activity on normal mouse 3T3 fibroblasts was originally reported by Pagano's laboratory, in a study with an assay based on the cleavage of a fluorescent derivative of phosphatidylinositol (C_6-NBD-PI, Fig. 1) [Ting and Pagano, 1990]. Unlike native PI, which undergoes very little partitioning into intact plasma membranes in the cold in the absence of detergent, NBD-PI has sufficient aqueous-solubility for uptake by the plasma membranes of intact cells. In our laboratory, the ecto-PLC is detected in intact cells by an assay based on radiolabeled lysoPI, which like NBD-PI, has the aqueous solubility needed to partition into the membrane [Stafford et al., 1989]. The specificity of the ecto-PLC for the inositol headgroup has been previously demonstrated by showing that neither NBD-PC [Ting and Pagano, 1990] nor radiolabeled lysoPC [Volwerk et al., 1992] will serve as a substrate. Consistent with those findings, we also find that the ecto-PLC isolated onto bead preparations will not cleave lysoPC (data not shown).

Neither of these assays in intact cells address the question of whether ecto-PLC on the intact cell surface will in fact cleave native PI, or whether a lysoPI-like lipid is the preferred substrate. A comparison of Figure 2A and B shows that while PI-cleaving activity at low levels can be detected on bead preparations generated from biotinylated membrane proteins, its presence does not correlate with that of the lysoPI-PLC. In fact, some PI-cleaving activity, unlike the lysoPI-PLC, can be recovered onto avidin-methacrylate beads without previous biotinylation of cell surface proteins. Similarly, cell types that have no ecto-PLC activity as intact cells (e.g., V79 hamster fibroblasts) can serve as a source of bead preparations that do possess PI-cleaving activity. This suggests that the observed PI-cleaving activity originates from an enzyme that is independent of the lysoPI-PLC, and that it largely derives from tight nonspecific binding of an enzyme with an intracellular source. While we cannot rule out the possibility that the lysoPI-PLC will act on PI to a limited extent, the results indicate that lysoPI rather than PI is a preferred substrate for this ecto-enzyme.

LysoPI is a naturally occurring lipid in mammalian cells, and there is evidence suggesting that lysoPI and its phosphorylated derivatives may have an important role in cell physiology [Wheeler and Boss, 1990]. Little is known regarding the amount of endogenous lysoPI in the outer leaflet of the plasma membrane, although approximately 9% of the total cellular PI of bovine aortic endothelial cells is known to be present in the outer leaflet of the plasma membrane [Sillence and Low, 1994]. The action of extracellular phospholipase A_2, for example, that present in plasma and synovial fluids [Wong and Dennis, 1990] on this PI would be one potential means for generating outer leaflet lysoPI. The true role of an ecto-PLC acting on lysoPI is subject to speculation, but it is understandable why an enzyme would exist to regulate the levels of this potent molecule in the plasma membrane. The detergent-like properties of lysolipids, including lysoPI, can lead to cell lysis at sufficiently high concentrations, so at the least, the ecto-PLC may serve a protective role. LysoPI is also known to alter ion channel function in model membrane systems [Lundbaek and Andersen, 1994], inhibit the phosphatases that hydrolyze the phosphorylated derivatives of lysoPI [Palmer, 1987], enhance the ability of the Ras oncogene to activate adenyl cyclase (in a yeast system) [Resnick and Tomaska, 1994], and stimulate the growth of K-ras-transformed rat cells [Falasca and Corda, 1994]. Thus a lysoPI-PLC may have as yet unidentified important regulatory roles in both the pathways of inositol lipid formation, and in regulation of the levels of lysoPI as a second messenger.

Phospholipases C specific for lyso-PI are known to exist. LysoPI-PLCs have been reported to be present in porcine platelet mem-

branes [Murase and Okuyama, 1985] and rat brain [Tsutsumi et al., 1994, 1995], but these phospholipases appear to be less common than the well-characterized intracellular enzymes that hydrolyze PI, phosphatidylinositol-4-phosphate, and phosphatidylinositol-4,5-bisphosphate. The latter phospholipases have relatively sharp regions of maximal activity at acidic pH (pH 5–6) [Ryu et al., 1987], whereas the previously reported lysoPI-PLCs and that described here have maximal activity over a broad range of pH with peak activity centered around pH 7. The previously reported lysoPI-PLCs, however, were isolated from membrane fractions of homogenized cells or tissues without a means of identifying whether any were associated with the extracellular face of the plasma membrane.

Another logical substrate for a cell surface PI-PLC would be the GPI anchor of many cell surface proteins. Examples of spontaneous release of GPI-anchored proteins from intact cell surfaces are known [e.g., Th'eveniau et al., 1992; Nemoto et al., 1996; Muller et al., 1994], and implicate the presence of an external, yet-unidentified phospholipase (C or D) with GPI-cleaving potential. However, if the ecto-PLC is involved in release of GPI-anchored proteins, the experiments presented here indicate that it occurs at very low levels, below the detection of our activity assay. This result may be due either to an inability of ecto-PLC to cleave GPI anchors or to low accessibility of the enzyme to the target proteins.

The association of proteins with membranes include three types: peripheral binding through ionic interactions, insertion into and/or through membranes via hydrophobic sections of the polypeptide chain, and covalent binding to a lipid anchor (e.g., GPI anchor). To date, the PLCs that have been isolated and sequenced all appear to be of the first category [Noh et al., 1995]. In contrast to these intracellular enzymes, the cell surface lysoPI-PLC is apparently not a peripheral membrane protein, as we find that washing biotinylated cell membranes in 1.5 M salt does not substantially decrease the amount of activity present in the final bead preparations.

Of the true membrane proteins, those with unmodified GPI anchors can be readily cleaved by bacterial PI-PLC, but no reduction of ecto-PLC activity was observed on intact cells treated with bacterial PI-PLC (Fig. 6). Interestingly, the bacterial enzyme bound tightly enough to the treated cells to be readily detectable in an ecto-PLC assay of the cell culture even after several washes with buffer and subsequent incubation in culture medium at 37°C for an additional 2 h. However, at pH 7.5 the products of *B. cereus* PI-PLC action on lysoPI are almost exclusively cyclic IP compared to the predominately noncyclic IP generated by the ecto-PLC. This observation combined with the difference in calcium-dependence makes it relatively simple to distinguish the two activities, with the result that there was no apparent reduction in the amount of ecto-PLC present on the cell surface due to the action of the bacterial PI-PLC. Although this indicates that the ecto-PLC is not linked to the cell surface via a "simple" GPI anchor, it does not rule out the possibility that a modified anchor resistant to bacterial PI-PLC cleavage might be involved. One such modification has been reported to result from the coupling of a fatty acid (e.g., palmitic acid) to the inositol ring at or near the 2-position via a hydroxyester linkage [Roberts et al., 1988]. During the cleavage reaction, bacterial PI-PLC is thought to use the hydroxyl group at the 2-position for nucleophilic attack on the phosphodiester phosphorus, generating a 1,2-cyclic inositol phosphate product. Palmitoylation of the inositol in the vicinity of the 2-position apparently interferes with this process, since GPI anchors containing this modification are resistant to cleavage [Guther et al., 1994].

In conclusion, the results indicate that the ecto-PLC activity expressed by certain cell types is an externally exposed cell surface protein, which can be isolated from cells by virtue of its physical location. The activity can be assayed directly on immobilized avidin beads, and, like the ecto-PLC of intact cells, is a calcium-dependent lysophosphatidylinositol-cleaving PLC. Control experiments show that the presence of low levels of phosphatidylinositol-cleaving activity observed on some bead preparations is due primarily to the nonspecific binding of an independent, most likely intracellular, phospholipase rather than the ecto-PLC. Thus the ecto-PLC appears to be a lysoPI-PLC rather than a phosphatidylinositol-PLC. In addition to its extracellular location, this enzyme is also distinguished from the intracellular PLCs by its apparent true membrane linkage, rather than being an ionically bound peripheral, membrane protein.

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