Phospholipase Resistance of the Glycosyl-Phosphatidylinositol Membrane Anchor on Human Alkaline Phosphatase

Yee Wah Wong and Martin G. Low

Alkaline phosphatase (ALP) is attached to the cell surface in mammalian tissues via a glycosyl-phosphatidylinositol (GPI) anchor and can be released from the membrane by GPI-specific phospholipases. In a range of cultured human cell lines, however, the sensitivity of ALP to phospholipases was observed to be variable in magnitude (~20–90%). The mechanism of phospholipase resistance was explored with phospholipases of different bond specificities. The results suggest that phospholipase resistance is the result of acylation of the inositol ring in the GPI anchor. The occurrence of phospholipase-resistant forms of ALP may have important implications for the in vivo release and disposition of plasma ALP.

Additional Keyphrases: GPI anchor · isoenzymes · phospholipase C · phospholipase D

Most of the alkaline phosphatase (ALP) in mammalian tissues is located on the cell surface.2 The enzyme is anchored in the plasma membrane by means of a covalent linkage to a portion of membrane phospholipid moieties called glycosyl-phosphatidylinositosols (GPIs). Although the existence of this type of membrane anchorage was formally demonstrated only in 1985, it has now been identified on >100 proteins from a wide variety of eukaryotic organisms (1, 2). Collectively, the proteins linked to this rather complex membrane anchor perform diverse functions in the cell, and it has therefore been difficult to assign a unique functional role to the GPI anchor. However, one characteristic of GPI anchors is the ability to be cleaved by specific phospholipases. These enzymes remove the hydrophobic portion of the GPI lipid, with the result that the protein is released from the membrane. In fact, it was this effect of a bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) on ALP that eventually led to the discovery of the GPI anchor (1).

The ability of exogenous bacterial phospholipases to release ALP from cell membranes in an active form led us to speculate whether a similar function could be performed by an endogenous anchor-degrading phospholipase. An activity that could degrade the anchor on ALP was identified first in pig kidney (3) over a decade ago and subsequently in rat liver, kidney, and heart (4–6). However, the identity of this enzyme was uncertain until studies with human placental ALP (7) indicated that the anchor-degrading activity was attributable to a GPI-specific phospholipase D (GPI-PLD). The enzyme was subsequently shown to be abundant in plasma, and it is probable that much of the ALP anchor-degrading activity in tissues is due to blood contamination (8, 9). The GPI-PLD has been purified from human and bovine plasma (10, 11), and recently a cDNA encoding the enzyme has been cloned and sequenced (12).

Although the functional significance of protein release at the cell surface mediated by the anchor-degrading phospholipases is still uncertain, these enzymes could be responsible for the release of ALP into the plasma. Thus, GPI-PLD circulating in the plasma might degrade the GPI anchor of ALP on cell membranes and thereby release the enzyme into the plasma. The amount of ALP in serum samples would thus reflect the amount and activity of GPI-PLD in the plasma as well as the amount of ALP expressed by particular tissues. Any one of these factors might be affected as a result of pathological processes.

The amount of ALP released would also be dependent on the sensitivity of the GPI anchor to degradation by phospholipases. Most early studies on the release of ALP by PI-PLC were performed with tissues from a range of mammalian species (but not human), and ALP was shown to be highly sensitive to release by PI-PLC (1). However, in certain cells, notably the human erythrocyte, a large majority of the GPI-anchored protein molecules is resistant to release by PI-PLC (13–19). Although human erythrocytes are not a significant source of ALP, studies with human placental microsomes and human tumor cells that express the placental ALP isoenzyme indicate that a large proportion of the ALP on these cells may also occur in a PI-PLC-resistant form (7, 20, 21). Relatively little information is available concerning the GPI-PLD sensitivity of human ALP, and therefore it is not certain what effect phospholipase resistance is likely to have on ALP release in vivo. We have therefore performed a systematic investigation of the presence of phospholipase-resistant ALP in cultured human cell lines and attempted to determine the molecular mechanism of such resistance.

Materials and Methods

Materials. L-Homoarginine, L-phenylalanine, L-leucine, diethanolamine, p-nitrophenyl phosphate, sodium α-naphthyl phosphate, sodium butyrate, hydrocorti-
sone, prednisolone, and Fast Blue BB salt were obtained from Sigma (St. Louis, MO); electrophoresis-grade boric acid, Tris, and polyacrylamide premix from Bio-Rad (Richmond, CA); Dulbecco's minimum essential medium (DMEM), Ham's F-12 medium, l-glutamine, and penicillin-streptomycin from Meditech (Washington, DC); fetal calf serum (FCS) from Gibco (Grand Island, NY); and Centriprep 30 and Centricon 30 from Amicon (Beverly, MA).

PI-PLC was purified from the culture supernates of Bacillus subtilis BG2320 that had been transformed with a high-copy number plasmid encoding the gene for B. thuringiensis PI-PLC as described previously (21, 22). GPI-PLD was purified by immunoaffinity chromatography from bovine serum as described previously (10, 21).

Cell lines. HeLa S3 (human cervical epithelial carcinoma) and WISH (human amnion with possible HeLa contamination) cell lines were obtained from American Type Culture Collection (Rockville, MD) (Table 1). HeLa S3L and JEG3L (human choriocarcinoma) were gifts from J. Chou (National Institutes of Health, Bethesda, MD) and were maintained in DMEM supplemented with FCS, 40 mL/L. D98Au2.19 (a HeLa subline), SKG3a (human cervical epithelial carcinoma), and HeLa TCRC-1 cell lines were provided by J. L. Millán (La Jolla Cancer Research Foundation, La Jolla, CA). ROS 17/2.8 (rat osteogenic sarcoma) was obtained from R. Majeska (Mount Sinai Medical Center, New York, NY). HeLa and WISH lines maintained in our laboratory for a long period showed a spontaneous increase in their resistance to PI-PLC (21) and for the purpose of this study are arbitrarily designated as HeLa R and WISH R. D98Au2.19, TCRC-1, WISH, HeLa R, and WISH R were maintained in DMEM with 100 mL/L FCS. ROS, SKG3a, and HeLa S3 cell lines were maintained in Ham's F-12 medium with 100 mL/L FCS. All culture media were supplemented with 2 mmol/L L-glutamine, streptomycin (5 μg/mL), and penicillin (50 IU/mL). In induction experiments, media were supplemented with inducing agents at the concentrations indicated in Table 2.

Release of ALP activity from intact cells. Cells (5 × 10^9/well) were cultured in 96-well flat-bottomed plates and 0.2 mL of medium at 37 °C and under 5% CO₂ for 24 h. Cells were then washed once with serum-free medium. For determination of PI-PLC sensitivity, the cells were treated (in quadruplicate) with either PI-PLC (1.5 U/mL) in 40 μL of buffer A [0.15 mmol/L NaCl, 0.1 mmol/L MgCl₂, 0.01 mmol/L zinc acetate, 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid·NaOH (pH 7.0)], or with buffer A alone, for 60 min at 37 °C. After the treatment, the plate was centrifuged at 12 000 × g for 5 min at 4 °C. Thirty microliters of the supernate was harvested, and the cells were washed once with buffer A and recentrifuged as above. Aliquots of buffer A (40 μL and 10 μL, respectively) were added to the cell and supernatant fractions, so as to adjust the volume of each to 40 μL. For the amino acid inhibition assay, cells were preincubated in 40 μL of the amino acids specified in Table 1 (dissolved in buffer A) for 10 min, with shaking.

Table 1. Characterization of ALP Expressed in Cultured Cell Lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Heat treatment*</th>
<th>L-Homo-arginine*</th>
<th>L-Phenylalanine*</th>
<th>L-Leucine*</th>
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<tr>
<td></td>
<td>(10 mmol/L)</td>
<td>(10 mmol/L)</td>
<td>(10 mmol/L)</td>
<td>(10 mmol/L)</td>
</tr>
<tr>
<td>WISH R</td>
<td>88.5</td>
<td>113 ± 5.0</td>
<td>51.0 ± 5.7</td>
<td>65.0 ± 5.7</td>
</tr>
<tr>
<td>HeLa R</td>
<td>82.0</td>
<td>112 ± 9.1</td>
<td>53.7 ± 7.9</td>
<td>77.3 ± 8.3</td>
</tr>
<tr>
<td>HeLa S3L</td>
<td>79.5</td>
<td>107 ± 5.9</td>
<td>31.0 ± 5.7</td>
<td>54.0 ± 8.6</td>
</tr>
<tr>
<td>D98Au2.19</td>
<td>52.5</td>
<td>128 ± 15.8</td>
<td>68.3 ± 16.5</td>
<td>72.3 ± 2.4</td>
</tr>
<tr>
<td>WISH</td>
<td>98.0</td>
<td>98.0 ± 5.4</td>
<td>61.3 ± 8.0</td>
<td>65.0 ± 5.7</td>
</tr>
<tr>
<td>HeLa S3</td>
<td>86.0</td>
<td>119 ± 11.3</td>
<td>46.7 ± 3.9</td>
<td>54.3 ± 6.9</td>
</tr>
<tr>
<td>HeLa TCRC-1</td>
<td>69.0</td>
<td>108 ± 10.6</td>
<td>50.3 ± 2.9</td>
<td>69.0 ± 5.1</td>
</tr>
<tr>
<td>SKG3a</td>
<td>86.0</td>
<td>102 ± 3.3</td>
<td>58.7 ± 8.8</td>
<td>82.3 ± 2.6</td>
</tr>
<tr>
<td>ROS</td>
<td>&lt;1.0</td>
<td>82.0 ± 5.0</td>
<td>112 ± 2.1</td>
<td>137 ± 10.7</td>
</tr>
</tbody>
</table>

* Mean of two experiments.

In the heat-sensitivity assay, cells were incubated in 40 μL of buffer A at 56 °C for 15 min and then cooled to room temperature prior to assay for ALP activity. ALP activity was determined by adding 60 μL of substrate solution [8.35 mmol/L p-nitrophenyl phosphate in 1.5 mL Triton X-100, 1.67 mmol/L MgCl₂, 1.67 mol/L diethanolamine·HCl (pH 10.0)] to each well at room temperature; the reaction was stopped by adding 0.1 mL of a solution containing 1.5 mmol/L NaOH, 7.5 mmol/L EDTA, and 0.5 g/L sodium deoxycholate. The absorbance of the microtiter plate was monitored with a Microplate Reader MR600 (Dynatech Lab, Chantilly, VA) at 410 nm. Two features of the experimental design made it impracticable to use a standard incubation period for the ALP assay: the cell lines varied markedly in their expression of ALP, and it was not possible to control precisely the amount of ALP activity (i.e., number of cells) in each well. Consequently, the incubation time (3–60 min) was adjusted for each cell line so that readings were taken in the linear range of the ALP assay. However, values for controls and treated samples of the same cell line were incubated for the same length of time.

Butanol extraction of alkaline phosphatase. A confluent culture in a 75-cm² flask was rinsed twice with serum-free medium and then incubated at 37 °C for 30 min. The supernate was discarded and the cells were scraped from the flask with three 2-mL aliquots of 0.15 mol/L NaCl, 0.1 mmol/L MgCl₂, 0.01 mmol/L zinc acetate, 50 mmol/L Tris-HCl (pH 8.2). The pooled cell scrapings were extracted with 3.6 mL of ice-cold butanol on ice for 30 min with occasional vortex-mixing. The mixture was then centrifuged at 13 000 × g for 30 min. The aqueous layer was harvested and dialyzed against 0.15 mol/L NaCl, 0.1 mmol/L MgCl₂, 0.01 mmol/L zinc acetate, 0.2 mL/L Nonidet P-40, and 10 mmol/L Tris-HCl (pH 7.0). The resulting extract was concentrated to the desired volume (~1.5 mL/flask of cells) with a Centriprep 30 and then a Centricon 30 as suggested by the manufacturer. The extensive washing prior to extraction and the alkaline pH during the extraction are
include to minimize GPI anchor degradation by the GPI-PLD present in culture media.

Phospholipase treatment of butanol-extracted alkaline phosphatase. The butanol extract was digested (in triplicate) with PI-PLC (1.5 U/mL), GPI-PLD (25 U/mL), or PI-PLC and GPI-PLD together, in buffer A (final volume 50 μL) containing 0.1 mL/L NP-40, at 37 °C, for 60 min. For sequential digestions of both phospholipases the extract was first digested with one phospholipase for 60 min at 37 °C and then for an additional 60 min at 37 °C with the other phospholipase. The samples were then analyzed by Triton X-114 phase separation or polyacrylamide gel electrophoresis as described below.

Pretreatment of butanol extracts with hydroxylamine was performed in 1 mL/L hydroxylamine (pH adjusted to 10.8–11.2 with NaOH) at 4 °C for 18–24 h. The samples were dialyzed against 1 mL/L Triton X-100 and 0.1 mL/L TRIS·HCL (pH 7.4) and concentrated prior to digestion with phospholipases. In some experiments, the butanol extracts were digested with bromelain (final concentration, 2 mg/mL) in buffer A containing 0.1 mL/L Nonidet-P40 at 37 °C for 60 min.

Triton X-114 phase separation of butanol-extracted ALP. The digest was adjusted to 250 μL with ice-cold buffer A, and an equal volume of ice-cold 20 mL/L Triton X-114 in buffer A was added. The samples were mixed, and 100-μL portions were saved for determination of total ALP activity (T). The tubes were incubated at 37 °C for 10 min and then centrifuged for 5 min at 13 000 × g; 100 μL of the upper, detergent-poor phase was harvested (S). ALP activity was determined by adding 0.4 mL of a solution containing 6.25 mmol/L p-nitrophenyl phosphate, 1 mL/L Triton X-100, 1.25 mmol/L MgCl₂, and 1.25 mL/L diethanolamine·HCl (pH 10.0) to samples and incubating at 37 °C for 60 min. The reaction was stopped by adding 1.5 mL of 1 mL/L NaOH and 5 mL/L EDTA, and 50 μL of 10 mL/L sodium deoxycholate. The ALP activity was determined from the absorbance at 410 nm. The amount of anchor degradation was calculated from the ratio of the absorbances for the S and T samples, after correction for control incubations that did not contain phospholipase.

Gel electrophoresis. Samples were preincubated with an equal volume of sample buffer (0.5 mL/L TRIS·HCL (pH 6.8), 10 mL/L Triton X-100, 200 g/L sucrose, 0.05 g/L bromophenol blue) for 10 min before application to the gel. The separating gel (7.5% acrylamide) contained 0.375 mL/L TRIS·HCL (pH 8.8) and 5 mL/L Triton X-100, and the stacking gel (3.8% acrylamide) contained 0.125 mL/L TRIS·HCL (pH 6.8) and 5 mL/L Triton X-100. Samples were subjected to electrophoresis at 150 V for 2 h with a tank buffer consisting of 89 mmol/L TRIS·borate (pH 8.6) and 5 mL/L Triton X-100. ALP activity was detected by incubating the gel in 30 mL of 3 mmol/L sodium α-naphthyl phosphate, 2.4 mmol/L Fast Blue BB salt, 0.5 mmol/L MgCl₂, and 0.38 mL/L TRIS·borate (pH 9.5), with gentle shaking, until the desired color developed (~5 h).

Results

The distribution of PI-PLC-resistant ALP among cultured cell lines. Our previous studies had shown that ALP in HeLa cells was relatively resistant to release by PI-PLC. To determine whether the expression of PI-PLC-resistant ALP was restricted to a particular HeLa subline (TCRC-1), several other human cell lines (including HeLa cells from a variety of sources) were screened for PI-PLC resistance. The sensitivity of ALP to release by PI-PLC varied considerably among the cell lines examined (Figure 1). Only 13.3% ± 2.3% (mean ± SD) of ALP activity was released from the WISH cell line, compared with 71.7% ± 2.1% from the SKG3a cell line. The four different HeLa cell lines analyzed exhibited PI-PLC sensitivities ranging from 14.0% ± 2.6% for HeLa R to 47.5% ± 4.3% for HeLa TCRC-1. The rat cell line ROS 17/2.8, which expresses the tissue-nonspecific type of ALP, showed 92.2% ± 0.8% anchor degradation, similar to previous results with this cell line (21). These results indicated that the phenomenon of PI-PLC resistance was not only widespread but also extremely variable in magnitude.

Most of the cell lines were chosen because they were previously shown to express the human placental or germ-cell ALP isoenzyme, which we confirmed by the determination of inhibitor sensitivity. Most of the ALP in these cells was resistant both to heating at 56 °C and to 1-homoarginine inhibition, but exhibited partial sensitivity to L-phenylalanine and L-leucine inhibition (Table 1). The major exception was D98Au2.19, which exhibited partial heat sensitivity, suggesting that significant amounts of the intestinal ALP isoenzyme are present in these cells. ROS cells, as expected, were very

Fig. 1. Sensitivity of ALP in cultured cell lines to degradation by PI-PLC

ALP derived from the cell lines indicated on the horizontal axis was treated with PI-PLC, and anchor degradation was monitored as described in Materials and Methods. Solid bars, intact cells cultured in 96-well plates treated with PI-PLC. Anchor degradation is the amount of ALP activity released into the supernate, expressed as a percentage of the total activity in supernate and cells. Values represent means of four experiments and have been corrected for control release in the absence of added PI-PLC (generally <10%). Hatched bars, butanol-extracted ALP treated with PI-PLC. Anchor degradation is the amount of ALP released into the detergent-poor phase of a Triton X-114 phase separation, expressed as a percentage of the total activity and corrected for control release. Values are the means of duplicate experiments, except for HeLa S3 and HeLa SSL, which represent single experiments. No experiments were performed with butanol extracts from JEG3L.
sensitive to heat treatment but were insensitive to 10 mmol/L L-phenylalanine, consistent with expression of the tissue-nonspecific ALP isoenzyme. These data demonstrate that the observed variability in PI-PLC sensitivity between the cell lines does not reflect variable coexpression of different ALP isoenzymes.

Partial sensitivity to PI-PLC might reflect heterogeneity of the cell populations. Thus, the cell cultures could consist of a mixture of two (or more) cell populations, each expressing only sensitive or resistant ALP molecules. Alternatively, individual cells might be expressing a mixture of sensitive and resistant ALP molecules. To address these possibilities, we treated HeLa R cells with PI-PLC and then stained the cells with antibodies to ALP and a fluorescein isothiocyanate-labeled second antibody. Examination with a fluorescence microscope did not reveal the existence of a substantial proportion of ALP cells, which would have resulted if the cell cultures consisted of two distinct populations of cells (data not shown).

**PI-PLC resistance of butanol-extracted ALP.** The PI-PLC resistance of ALP in intact cells could be attributable to location of ALP either at an intracellular site or at the cell membrane–substratum interface. Either of these possibilities might prevent access of PI-PLC to its cleavage site and would be difficult to distinguish from PI-PLC resistance attributable to modifications to the GPI anchor structure. To distinguish among these various possibilities, we determined the PI-PLC resistance of ALP extracted from the cultured cell with butanol. In these experiments, it was not possible to use centrifugation to separate degraded from intact ALP because butanol removes the majority of the phospholipids, and ALP forms aggregates that are not readily sedimented (3, 7). The Triton X-114 phase separation technique (23) was therefore used to monitor removal of the hydrophobic portion of the GPI anchor. The butanol extracts generally exhibited the same sensitivity to PI-PLC as the cells from which they were prepared (Figure 1). The major exceptions were SKG3a and HeLa TCRC-1, which showed increases in PI-PLC sensitivity of ~20% and 40%, respectively. Thus, a significant proportion of ALP in these cell lines appears to be present in a location that is inaccessible to PI-PLC; alternatively, butanol extraction may result in a conversion of resistant ALP molecules to a PI-PLC-sensitive form. To distinguish between these possibilities, we treated intact SKG3 cells with NHS-Biotin after PI-PLC treatment. Biotinylated (cell surface) ALP was identified by electrophoresis in the presence of streptavidin, followed by immunoblotting with antibodies to ALP (data not shown). The result indicated that all of the ALP molecules on the cell surface could be released by PI-PLC and therefore that the increased PI-PLC sensitivity of ALP after butanol extraction is a consequence of cell lysis rather than the generation of PI-PLC-sensitive ALP.

Although the use of butanol extracts eliminates latency as a possible reason for PI-PLC resistance, it was conceivable that resistance was attributable to an inhibitory membrane protein that was not removed by butanol extraction. To reduce this possibility, we further purified ALP from butanol extracts of HeLa R cells by immunoaffinity chromatography. No increase in sensitivity was observed after this procedure (data not shown).

**PI-PLC resistance of ALP after induction.** Expression of ALP in many cell lines can be markedly increased by culture for several days in the presence of inducing agents. In three of the four HeLa cell lines studied, inducing agents produced substantial increases in ALP expression (Table 2). However, no major changes in the sensitivity to release by PI-PLC were observed (Table 2). Essentially the same result was obtained with butanol extracts of the induced cells (Table 2). In some of the experiments with HeLa R cells, two- to threefold increases in PI-PLC sensitivity were observed after induction, but these increases were not reproducible (Table 2). Furthermore, the inducing agents had little effect on the amount of ALP expressed in HeLa R cells.

**Is PI-PLC-resistant ALP also resistant to GPI-PLD?** The major anchor-degrading activity so far identified in human and other mammals is GPI-PLD. It was therefore of interest to determine whether the PI-PLC-resistant ALP in human cell lines was also resistant to GPI-PLD. Because the serum form of GPI-PLD has little or no activity on intact cells (21), these experiments were performed with butanol extracts in the presence of the detergent Nonidet-P40, and degradation was monitored by Triton X-114 phase separation. The amount of degradation produced by GPI-PLD was similar to or slightly greater than that obtained with PI-PLC (Figure 2), suggesting that the ALP molecules that were resistant to PI-PLC were also resistant to GPI-PLD. Support for this conclusion was obtained in experiments in

<table>
<thead>
<tr>
<th>Inducing agent</th>
<th>Cell line</th>
<th>Induction ratio</th>
<th>Intact cells</th>
<th>Butanol extract</th>
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<tr>
<td>Sodium butyrate</td>
<td>HeLa R</td>
<td>1.5</td>
<td>2.1 ± 0.83</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>HeLa 35L</td>
<td>4.0</td>
<td>1.0 ± 0.16</td>
<td>1.5</td>
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<tr>
<td></td>
<td>HeLa S3</td>
<td>11</td>
<td>0.8 ± 0.08</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>HeLa TCRC-1</td>
<td>8.0</td>
<td>0.8 ± 0.22</td>
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<td>Hydrocortisone</td>
<td>HeLa R</td>
<td>0.65</td>
<td>2.2 ± 0.7</td>
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<tr>
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<td>1.2±</td>
<td>1.1</td>
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<tr>
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<tr>
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<td>HeLa TCRC-1</td>
<td>2.4</td>
<td>1.3 ± 0.12</td>
<td>1.2*</td>
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<td>Prednisolone</td>
<td>HeLa R</td>
<td>0.78</td>
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<td>(1 μmol/L)</td>
<td>HeLa 35L</td>
<td>4.5</td>
<td>1.2±</td>
<td>1.1</td>
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<tr>
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<td>0.97 ± 0.09</td>
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<td></td>
<td>HeLa TCRC-1</td>
<td>3.2</td>
<td>1.2 ± 0.12</td>
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</table>

* (ALP activity in induced cells)/ALP activity in control cells; data are means of two independent experiments.

b (% Release from induced cells or extracts)/% release from control cells or extracts).

c Mean of three independent experiments ± SD.

d Mean of two independent experiments.

* Result of one experiment.

± 2 mmol/L, except for HeLa TCRC-1 (10 mmol/L).
which ALP was treated with both PI-PLC and GPI-PLD. The combined treatment produced approximately the same amount of degradation as the individual enzymes (Figure 2).

**Partial anchor degradation of "resistant" ALP by phospholipases.** The observation that PI-PLC-resistant ALP was also resistant to GPI-PLD was unexpected because several previous studies indicated that PI-PLC-resistant GPls are sensitive to degradation by GPI-PLD. It is believed that this differential sensitivity is attributable to acylation of the inositol ring by a palmitate group (see Discussion). The acyl group is not removed from the inositol ring by the action of GPI-PLD. The GPI-PLD degradation product of PI-PLC-resistant ALP might therefore escape detection because it is sufficiently hydrophobic to be retained in the Triton X-114 phase. Polyacrylamide gel electrophoresis in the presence of Triton X-100 has also been used in previous studies to monitor anchor degradation by phospholipases (15, 16). Removal of the GPI anchor reduces the ability of ALP to interact with the Triton X-100 micelles and therefore produces a large increase in mobility. However, because of its continuous nature (analogous to chromatographic separation), electrophoretic analysis might allow detection of small changes of hydrophobicity not feasible with a single Triton X-114 phase separation.

When this technique was applied to butanol extracts of HeLa R and WISH cells, most of the ALP remained as a slow (S) band at the top of the gel after PI-PLC treatment (Figure 3). A similar proportion of the ALP remained as a slow band after treatment with GPI-PLD. In contrast, all of the ALP is present in a fast (F) band after PI-PLC or GPI-PLD treatment of ROS cell extracts, whereas in extracts prepared from SKG3a cells most of the ALP is converted to an F band, with a small amount of activity remaining in the S band. For each extract, the relative amounts of ALP activity in the S and F bands are the same for treatment with each phospholipase. At a superficial level, therefore, the electrophoretic analysis is consistent with the results obtained with Triton X-114 phase separation (Figures 1 and 2). This consistency was not unexpected, because both techniques detect a similar property, the affinity of ALP molecules of Triton micelles. Thus, when the two phases of a Triton X-114 phase separation were individually analyzed by gel electrophoresis, the detergent-rich phase contained predominantly the S band, whereas the detergent-poor phase contained mostly the F band (data not shown).

On closer examination of the electrophoretic patterns (Figure 3), it was apparent that the relative mobilities of both the F and S bands were different for the two enzyme treatments (illustrated schematically in Figure 4A). The F band produced by GPI-PLD (F1) had a significantly lower mobility than that produced by PI-PLC (F2) (Figures 3 and 4A), probably because of the loss of the phosphate on the inositol ring when the phosphatidate moiety is removed by GPI-PLD. The phosphate would be retained by PI-PLC-cleaved ALP as inositol 1,2-cyclic phosphate, and at the gel pH of 8.6 it would contribute an additional negative charge not present in the GPI-PLD-degraded species. When the extracts were treated sequentially with PI-PLC and GPI-PLD, the mobility of the product was characteristic of the first phospholipase used. Thus, treatment with PI-PLC before GPI-PLD resulted in a more mobile F species than did treatment with GPI-PLD before PI-PLC. The results of the combined treatments demonstrate that the F bands are derived from a single population of ALP molecules that is sensitive to both PI-PLC and GPI-PLD. They also confirm that the mobility difference between F1 and F2 is attributable to differences at the phosphodiester linkage on inositol rather than elsewhere in the ALP molecule.

With the S bands, the situation is more complicated,
major effect of PI-PLC is to alter the distribution of activity between these two bands, so that $S_0$ is now the major species. In contrast, the product of GPI-PLD action is a doublet of $S_1$ and $S_3$, with the majority of activity in $S_3$. After treatment with both PI-PLC and GPI-PLD, only $S_1$ and $S_3$ are produced, regardless of the order of phospholipase treatment. Thus, these two species must result from the degradation of a population of ALP molecules that is PI-PLC resistant and GPI-PLD sensitive. However, the data also suggest that the GPI-PLD degradation product still has relatively high affinity for the Triton micelles, and therefore low mobility, as a result of a hydrophobic modification of the GPI anchor.

Location and nature of the hydrophobic modification in phospholipase-resistant ALP. Our results are consistent with the presence of inositol-acylated GPI anchors on the ALP molecules. As mentioned previously, inositol acylation has been shown to prevent degradation by PI-PLC but not by GPI-PLD (13). The acyl group on the inositol ring in the GPI anchor is not affected by GPI-PLD-mediated degradation of the phosphodiester linkage and therefore could account for the hydrophobicity of the $S_1$ and $S_3$ species. To characterize further the location and nature of this modification, we performed two additional sets of experiments with the butanol extracts.

The nonspecific protease bromelain cleaves the polypeptide chain of ALP close to the carboxyl terminus, releasing the protein from its GPI anchor with full retention of enzyme activity. Bromelain-treated butanol extracts were analyzed by polyacrylamide gel electrophoresis. The majority of the ALP migrated as a fast band with a mobility similar to $F_1$ and $F_2$, with a small amount remaining at $S_0$ (data not shown). This result was obtained not only with extracts from SKG3a and ROS cells (ALP mainly converted to the F forms by phospholipase treatment) but also with extracts from HeLa R, WISH R, and WISH cells (ALP mostly remains as S forms after phospholipase treatment). Thus, the residual affinity for Triton X-100 micelles that remains after phospholipase treatment appears to be attributable to a hydrophobic modification located in the GPI anchoring region, not in the polypeptide chain.

Additional evidence for inositol acylation was obtained from experiments in which the butanol extracts (from HeLa R, WISH R, WISH, SKG3a, and ROS cells) were treated with hydroxylamine (which nonspecifically deacylates carboxylic esters) before phospholipase treatment. Hydroxylamine produced a substantial increase in the amount of ALP converted to the F bands by subsequent phospholipase treatment, although some ALP remained in the S bands (Figure 3; only HeLa R data are shown). The reason for incomplete deacylation is not clear, but it is possible that the pH was below the optimum for hydroxylamine-mediated deacylation. Unfortunately, use of a higher pH for hydroxylamine treatment resulted in an irreversible loss of ALP activity. In previous studies with human erythrocyte proteins, the extent of deacylation under similar conditions was variable (15, 16). The observation that hydroxyl-
amine alone produces relatively little ALP migrating in the F band is of interest. Hydroxylamine is capable of removing both fatty acids from a diacylglycerol structure to produce a hydrophilic species with no affinity for Triton X-100 micelles. Its failure to do so in our experiments suggests that the GPI anchors on ALP contain predominantly alkylacylglycerols rather than diacylglycerols.

**Discussion**

An important feature of proteins that, like ALP, are attached to the plasma membrane by a GPI anchor is their sensitivity to release from the membrane by PI-PLC. However, in previous studies, it was observed that treatment of HeLa TCRC-1 cells or human placental microsomes with PI-PLC resulted in a maximum of 50–60% release of ALP activity into the supernate (7, 20). By contrast, under similar conditions, PI-PLC could release 95–100% of ALP from the rat osteogenic sarcoma cell line ROS 17/2.8 (M. G. Low, unpublished data). Because we are interested in the role that phospholipase-mediated GPI anchor degradation might play in the release of ALP from human tissues in vivo, we decided to examine this phenomenon more closely.

In these studies, we had three main goals: (a) to confirm that ALP can occur in a PI-PLC-resistant form; (b) to determine whether PI-PLC-resistant ALP is also resistant to degradation by GPI-PLD; and (c) to determine the mechanism of PI-PLC resistance. Although we used cultured cell lines for our experiments, we believe our results have implications for the mechanism of ALP release into human serum in vivo.

We showed that most of the cell lines examined expressed a substantial proportion of ALP in a form that could not be released from the membrane by PI-PLC. Resistance to PI-PLC was not a consequence of an internal location for the ALP, because similar results were generally obtained with butanol extracts prepared from the same cells. The proportion of ALP that was PI-PLC resistant varied markedly between the different cell lines, but we were unable to determine the reason for this variability. PI-PLC resistance among the cell lines did not correlate with their relative ALP expression, their sensitivity to ALP induction, or coexpression of different ALP isoenzymes. In one of the more resistant cell lines (HeLa R), PI-PLC resistance did not correlate with cellular heterogeneity of the cultures or with the cell cycle (cells were synchronized with the use of a mitotic-shakeoff technique) (Y. W. Wong and M. G. Low, unpublished data).

To gain some insight into the mechanism of PI-PLC resistance, we also treated butanol extracts from the cell lines with GPI-PLD. PI-PLC-resistant GPI-anchored proteins (for example, human erythrocyte acetylcholinesterase) have been characterized in a number of cell types, and it is believed that in all such GPIs the inositol ring is acylated by palmitate or some other fatty acid residue (13–15, 17–19, 24, 25). Although such a modification renders the GPI resistant to PI-PLC, sensitivity to GPI-PLD is retained. In our Triton X-114 phase separation studies, little difference was observed in the sensitivity of butanol-extracted ALP to PI-PLC or GPI-PLD. We believe that our failure to detect anchor removal by GPI-PLD was due to the inability of the Triton X-114 phase separation technique to separate the GPI-PLD degradation products from the substrate. The technique relies on the hydrophobic interactions between the hydrocarbon chains in the GPI anchor of the substrate and the Triton micelles. However, if the ALP molecules were acylated on inositol, the GPI-PLD degradation product might retain sufficient hydrophobicity to associate with Triton micelles and therefore escape detection. Electrophoresis in the presence of Triton X-100 provided additional resolution and allowed us to detect ALP species of intermediate hydrophobicity produced as a result of phospholipase-mediated degradation.

With the electrophoretic technique, we observed that both PI-PLC and GPI-PLD produced the expected high-mobility F band at the bottom of the gel, migrating just behind the dye front. However, GPI-PLD also converted the remainder of the ALP (the majority in HeLa R and WISH cells) into two low-mobility S bands instead of one. Even more surprising was the observation that PI-PLC also converted most of the remaining ALP to an S band of higher mobility. These results are inconsistent with a simple model in which the GPI anchor of each ALP molecule is either unacylated (and therefore sensitive to both phospholipases) or acylated (and therefore PI-PLC resistant but GPI-PLD sensitive). The simplest explanation is that some of the ALP molecules are heterodimers consisting of subunits that differ with respect to the absence or presence of the acylated anchor (Figure 4). Triton X-100 would not be expected to dissociate the dimers, so the ALP dimer population could contain at least three distinct species, with 0, 1, or 2 acylinositol groups. Such a scheme would account for the observed production of two new species after PI-PLC treatment and three after GPI-PLD treatment (Figure 4, bottom). From the distribution of ALP activity between bands (and Triton X-114 phases) after GPI-PLD treatment, we can make some rough estimates of the relative proportion of each species in the starting material. For WISH and HeLa R cells, ~60% of ALP would be monacylated, with 20% each of the nonacylated and diacylated species. For SKG3a cells, only 10% of ALP would be monoaacylated, 90% would be nonacylated, and none would be diacylated. Finally, for ROS cells, all ALP molecules would be nonacylated.

The relative electrophoretic mobilities of some of these species fit a predictable pattern on the basis of the decrease in total number of hydrocarbon chains (F<sub>2</sub>→S<sub>3</sub>→S<sub>1</sub> or F<sub>1</sub>→S<sub>1</sub>→S<sub>j</sub>) or negative charge on the inositol ring (F<sub>2</sub>→F<sub>j</sub>). However, one cannot predict with any certainty the relative mobilities of species that differ in the distribution of hydrocarbon chains between acylinositol and phosphatidyl groups. Prediction becomes even more difficult when the hydrophobicity and electrical charge are changed simultaneously. S<sub>j</sub> may have a lower mobility than S<sub>1</sub>, either because of the loss of two negative charges or because two hydrocarbon...
chains provide a more effective anchor than three if one is present on each ALP subunit. The origin of the activity in the S₉ region with untreated ALP is not known. It could represent the product of an endogenous GPI-PLC acting on monoacylated ALP. However, it is also possible that both GPI anchors are intact and that differences in glycosylation of the polypeptide yield two species that are distinguishable by electrophoresis. This latter possibility could also account for the appearance of minor bands (visible on the original gels but not in Figure 3) that migrated slightly faster than S₉ and S₈ in the phospholipase-treated samples (Figure 4, top).

The origin of inositol acylation on GPI-anchored proteins is not understood, but one hypothesis that fits many of the available data is that the GPI precursors themselves undergo inositol acylation early in their biosynthesis, before they are attached to protein (19, 24, 25). The acyl group may be subsequently removed from the inositol ring by an inositol "deacylase" after GPI anchor attachment. If this model is correct, then the PI-PLC resistance we have observed may be a result of insufficient inositol deacylase. Why different cell lines should vary so markedly in the expression of an inositol deacylase is not clear, but such variability could simply result from the absence of any selection against the PI-PLC-resistant phenotype. However, it is unlikely that this question can be answered until more is known about the mechanism of inositol acylation–deacylation and the physiological function of ALP and its GPI anchor.

The results of our study may have relevance for the release of ALP into circulation. Both the amount and the physicochemical properties of this enzyme have been used as an aid to clinical diagnosis (26). ALP isoenzymes are encoded by a multigene family and exhibit diverse tissue distributions. At present, it is believed that all ALP isoenzymes are expressed in a GPI-anchored form. Thus, it is reasonable to speculate that one mechanism for release of ALP from tissues into the circulation is the degradation of its GPI anchor by an endogenous GPI-specific phospholipase. Butanol- or detergent-activated anchor-degrading activities that could act on ALP were first identified more than a decade ago (3-6), and these are now believed to result from the action of GPI-PLD (8, 9, 27). The enzyme is abundant in serum and plasma, and because it is present in unperfused tissues it may contaminate homogenates and possibly membrane fractions prepared from them. In addition to plasma, GPI-PLD has been identified in a diverse group of cell types (mast cells, sensory and motor neurons, pancreatic α and β cells, and keratinocytes in the upper gastrointestinal tract and skin) (28-31). However, none of these cells contain significant amounts of ALP, and at present it is difficult to assess the role that cellular GPI-PLD might play in the release of ALP from tissues in vivo.

GPI-PLD-mediated degradation could, however, have a major influence on the disposition of ALP after it reaches the blood. Normally, GPI-PLD, in common with many other mammalian phospholipases, is inactive against substrates located in membranes (21). However, it can be activated by agents that perturb membrane structure (such as detergents or butanol). If similar membrane-perturbing conditions were present in vivo, the effective GPI-PLD activity in plasma would become so high that it could remove most of the GPI-anchored protein from an exposed cell in a few seconds. Three different situations can be envisaged in which membrane structure may be sufficiently perturbed to promote in vivo degradation of ALP: (a) Physical injury to the membrane may produce small membrane vesicles or fragments that could represent a more favorable surface for GPI-PLD action, either because of the loss of cytoskeletal structures or because of the high curvature of the membrane. (b) The membrane may become perturbed by chemical agents that accumulate during pathological processes such as inflammation and biliary obstruction. (c) In some situations, ALP circulates in high molecular mass complexes. Although the precise physical and chemical nature of these complexes is unknown, they could provide a favorable substrate for GPI-PLD action (32-34).

The possibility of in vitro degradation may also be of practical importance for electrophoretic analysis of ALP isoenzymes in serum or tissue samples. The ability of butanol to promote GPI-PLD-mediated degradation of ALP has already been noted and is likely to be a problem in butanol extraction of ALP, particularly from unperfused tissues or cultured cells. This problem can be reduced if tissues or cells are washed before extraction or if extraction is performed at an alkaline pH. A different problem can occur if Triton X-114 is used to analyze the hydrophobicity of ALP in serum samples. The GPI-PLD in serum will be activated by Triton X-114 during the phase separation, and any hydrophobic species will be rapidly degraded—possibly creating the mistaken impression that the serum sample contained only hydrophilic forms of ALP. In this instance, it is obviously impractical to remove the contaminating GPI-PLD from the sample; however, it may be possible to reduce its action by performing the phase separation at alkaline pH.

Previously, it was assumed that after the GPI anchor was degraded by GPI-PLD, ALP would necessarily be released from the membrane. However, the results of our study suggest that this may not always be the case. GPI-PLD-mediated degradation of inositol-acylated ALP produces forms of ALP with intermediate hydrophobicity, which might remain in the membrane unchanged in their properties, or they might show an increased tendency to transfer between membranes or to lipoprotein particles, or to form micellar complexes with other hydrophobic proteins. For similar reasons, inositol acylation may have major effects on the rate of clearance of ALP from the circulation. Thus, inositol acylation could affect not only the isoenzyme pattern but also the amount of ALP in the plasma. The potential contribution of ALP forms with intermediate hydrophobicity to the serum pool is uncertain at present.
because of the limited information regarding PI-PLC sensitivity of GPI anchors in human tissues. However, we believe that the predictive value of serum ALP determination might be enhanced by further studies in this area.

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References