Phosphatidylinositol Anchor of HeLa Cell Alkaline Phosphatase†

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ABSTRACT: Alkaline phosphatase from cancer cells, HeLa TCRC-1, was biosynthetically labeled with either ^{3}H-fatty acids or [^{3}H]ethanolamine as analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and fluorography of immunoprecipitated material. Phosphatidylinositol-specific phospholipase C (PI-PLC) released a substantial proportion of the ^{3}H-fatty acid label from immunoaffinity-purified alkaline phosphatase but had no effect on the radioactivity of [^{3}H]ethanolamine-labeled material. PI-PLC also liberated catalytically active alkaline phosphatase from viable cells, and this could be selectively blocked by monoclonal antibodies to alkaline phosphatase. However, the alkaline phosphatase released from ^{3}H-fatty acid labeled cells by PI-PLC was not radioactive. By contrast, treatment with bromelain removed both the ^{3}H-fatty acid and the [^{3}H]ethanolamine label from the purified alkaline phosphatase. Subtilisin was also able to remove the [^{3}H]ethanolamine label from purified alkaline phosphatase. The ^{3}H radioactivity in alkaline phosphatase purified from [^{3}H]ethanolamine-labeled cells comigrated with authentic [^{3}H]ethanolamine by anion-exchange chromatography after acid hydrolysis. The data suggest that the ^{3}H-fatty acid and [^{3}H]ethanolamine are covalently attached to the carboxyl-terminal segment since bromelain and subtilisin both release alkaline phosphatase from the membrane by cleavage at that end of the polypeptide chain. The data are consistent with findings for other proteins recently shown to be anchored in the membrane through a glycosylphosphatidylinositol structure and indicate that a similar structure contributes to the membrane anchoring of alkaline phosphatase.

Recently it has been shown that some proteins are anchored in cell membranes via a phosphatidylinositol molecule which is covalently attached to the carboxyl terminus through an ethanolamine- and glucosamine-containing glycan [reviewed by Low et al. (1986) and Low (1987)]. The evidence for this glycosylphosphatidylinositol structure is largely derived from chemical analyses of the membrane anchoring domains of the variant surface glycoprotein (VSG)[1] of *Trypanosoma brucei*, Thy-1 antigen, and human erythrocyte acetylcholinesterase which all contain fatty acids, inositol, glucosamine, and ethanolamine (Ferguson et al., 1985a,b; Tse et al., 1985; Roberts & Rosenberry, 1985, 1986; Haas et al., 1986). Several other proteins [see Low (1987)] are also believed to be anchored by a similar mechanism since they can be released from membranes by treatment with a phosphatidylinositol-specific phospholipase C (PI-PLC).

Alkaline phosphatase, a nonspecific phosphomonoesterase widely distributed in mammalian tissues, was in fact the first protein to show such behavior (Slein & Logan, 1965; Ikezawa et al., 1976; Low & Finean, 1977a), and it was proposed that this was due to a covalent linkage with phosphatidylinositol (Low & Zilversmit, 1980). However, until recently more direct evidence for this conclusion was not available since the relatively low amounts of this protein in most mammalian tissues precluded isolation and detailed chemical analysis of the membrane anchoring domain. The identification of *myo*-inositol in hydrolysates of purified human placental alkaline phosphatase has provided strong support for this proposal (Low et al., 1987), but the location and composition of the inositol-containing structure were not determined.

The cDNA sequences of the three major types of mammalian alkaline phosphatase have been determined, and these all predict the presence of a stretch of approximately 20 hydrophobic amino acid residues at the carboxyl terminus (Kam et al., 1985; Millan, 1986; Weiss et al., 1986; Henthorn et al., 1986; Ovitt et al., 1986; Berger et al., 1987); it was therefore proposed that this hydrophobic sequence is responsible for membrane anchoring. This claim was consistent with previous studies in which the proteases papain, bromelain, and subtilisin released alkaline phosphatase from the membrane by removing a 2-kDa region of the polypeptide located at the carboxyl terminus (Colbeau & Maroux, 1978; Abu-Hasan & Sutcliffe, 1984; Jemmerson et al., 1984). However, the proposition that alkaline phosphatase is anchored by a hydrophobic peptide has not been confirmed by direct amino acid sequencing studies; the carboxyl-terminal residues of the mature proteins have yet to be determined for any of the mammalian alkaline phosphatases.

In the present report, using biosynthetic labeling techniques we demonstrate that components of the phosphatidylinositol-containing lipid anchor are covalently attached to the carboxyl-terminal region of HeLa cell alkaline phosphatase. These data provide independent support for the proposal that mammalian alkaline phosphatases are posttranslationally modified by a phosphatidylinositol-containing lipid which

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[1] Abbreviations: IgG, immunoglobulin G; PBS, phosphate-buffered saline; PI-PLC, phosphatidylinositol-specific phospholipase C; SDS–PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate; TBS, Tris-buffered saline; VSG, variant surface glycoprotein of *Trypanosoma brucei*; Tris, tris(hydroxymethyl)aminomethane; kDa, kilodalton(s).

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contributes to its anchoring in the membrane.

Experimental Procedures

Cell Culture. HeLa TCRC-1 cells [a subclone of the HeLa line cultured from cells passaged at Tufts Cancer Research Center by Singer and Fishman (1974)] were grown in 150 cm^2 tissue culture flasks in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 2 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. To increase the level of alkaline phosphatase expression by the cells, the culture medium was supplemented with 2 mM sodium butyrate and 50 mM sodium chloride for 48 h (Jemmerson et al., 1985a).

Biosynthetic Labeling. Cells (2×10^7/flask) were labeled with 100–200 μCi of [9,10-^{3}H]palmitic acid (45–50 Ci/mmol), [9,10-^{3}H]myristic acid (55 Ci/mmol), or [1-^{3}H]ethan-1-ol-2-amine hydrochloride (12 Ci/mmol, Amersham) in 15 mL of complete medium containing fetal bovine serum that had been dialyzed (3×) against PBS using Spectrapor membrane tubing, molecular weight cutoff 6000–8000. For incorporation of fatty acids, incubation was generally for 18 h; however, in some experiments, labeling of alkaline phosphatase was observed in as short a time as 4 h. For ethanolamine incorporation, incubation was for 8 h.

Extraction of Cellular Proteins. For most experiments, a detergent solution was used to extract proteins from the cells. Radiolabeled cells were washed twice in cold TBS (2 mM Tris-HCl and 150 mM sodium chloride, pH 7.4). Proteins were extracted in 5 mL of TBS containing 0.5% Triton X-100, 0.05% sodium dodecyl sulfate, 1 mM calcium chloride, 1 mM magnesium chloride, and 0.5 mM iodoacetamide for 30 min at 4 °C. Insoluble material was removed by centrifugation. In experiments to examine the release of fatty acid or ethanolamine from purified alkaline phosphatase by phospholipase C and by bromelain, cellular proteins were extracted in TBS and 0.5% deoxycholate, pH 8.5.

Immunoprecipitation of Alkaline Phosphatase. Detergent-extracted cellular proteins were incubated overnight at 4 °C with 50 μg of monoclonal antibody E5 which is specific for alkaline phosphatase. The antibody (IgG2a class) was prepared and purified from ascites fluid as previously described (Jemmerson et al., 1985b). In control experiments, nonspecific mouse IgG (Sigma) was used. Antigen–antibody complexes were precipitated by the addition of 50 μL of protein A–bacterial adsorbent (Miles Scientific) for 2 h with end-over-end mixing. The bacterial absorbent was centrifuged, and the pellet was washed (2×) in TBS.

Purification of Biosynthetically Labeled Alkaline Phosphatase. Alkaline phosphatase, extracted from HeLa TCRC-1 cells (see above), was immunoaffinity purified on a column of Sepharose 4B to which monoclonal antibody E5 was covalently attached at a density of 10 mg of antibody per 0.5 g of Sepharose 4B. Generally, the procedure followed was that described by Vockley and Harris (1984). Protein bound to the affinity column was eluted as a sharp peak in 4 M KSCN, 20 mM Tris-HCl, and 150 mM sodium chloride, pH 8.1. On a single passage, the affinity column adsorbed 90–95% of the total alkaline phosphatase catalytic activity present in the cell extract. Minor contaminants in the eluate were eliminated by preadsorbing the HeLa TCRC-1 cell extract on another affinity column containing normal IgG (7 mg/0.5 g of Sepharose 4B). Further purification of alkaline phosphatase was effected by gel filtration chromatography of affinity-purified alkaline phosphatase on Sephadex G-100 in 50 mM Tris-HCl and 0.1% deoxycholate, pH 7.4.

Phospholipase C Treatment. Phospholipase C (type XII) from *Clostridium perfringens* was purchased from Sigma Chemical Co. PI-PLC was purified from *Staphylococcus aureus* by a modification of the procedure of Low (1981) as described by Malik and Low (1986). In studies of purified alkaline phosphatase, PI-PLC was used at a concentration of 2 μg/mL in 50 mM Tris-HCl, pH 7.4, and 0.1% deoxycholate, and in studies of whole cells, it was used at a concentration between 12 and 20 μg/mL in TBS. In either situation, the reaction was allowed to proceed at 37 °C for 45 min. In some experiments, protein A purified monoclonal antibodies to alkaline phosphatase at a final concentration of 50 μg/mL were tested for their effects on PI-PLC cleavage.

Bromelain Proteolysis of Alkaline Phosphatase. Purified, biosynthetically labeled alkaline phosphatase was digested with bromelain (20 μg/mL, Sigma Chemical Co.) in 50 mM Tris-HCl and 0.1% deoxycholate, pH 7.4, for 1 h at 37 °C.

SDS–Polyacrylamide Gel Electrophoresis and Fluorography. Immunoprecipitated alkaline phosphatase, purified alkaline phosphatase, and alkaline phosphatase treated with phospholipase C and bromelain were electrophoresed in 10% polyacrylamide gels by the procedure of Laemmli (1970). After being fixed, in some experiments the gels were incubated in 1 M hydroxylamine (Sigma), pH 6.6, for 1 h. The gels were soaked in Amplify (Amersham) for 30 min. Dried gels were exposed to Kodak XAR-5 film with intensifying screens for 1–4 weeks. Densitometer tracings of fluorographs were recorded on a Beckman DU-8 spectrophotometer.

Thin-Layer Chromatography. Following PI-PLC treatment, neutral lipids were extracted from purified, biosynthetically labeled alkaline phosphatase with 2 volumes of diethyl ether. They were then dried under nitrogen and analyzed by thin-layer chromatography on silica gel 60 (E. Merck) in the solvent petroleum ether:diethyl ether:acetic acid (70:30:2 by volume).

Measurement of Alkaline Phosphatase Catalytic Activity. The enzyme (50 μL of 500 μL total volume) in TBS, pH 7.4, was incubated with *p*-nitrophenyl phosphate (200 μL of 9.8 mg/mL) in 0.1 M 2-amino-2-methyl-1-propanol, pH 10.5, containing 2 mM magnesium chloride. After 15 min at 37 °C, the reaction was stopped with the addition of 1.0 mL of 1 N NaOH, and the optical density at 405 nm was determined.

Results

Release of Alkaline Phosphatase from HeLa TCRC-1 Cells by PI-PLC. It has been known for a decade that *S. aureus* PI-PLC effectively releases alkaline phosphatase from mammalian cell membranes (Low & Finean, 1977a). Results of experiments presented in Table I show that PI-PLC also releases alkaline phosphatase from human cancer cells, HeLa TCRC-1. In some experiments, as much as 67% of the alkaline phosphatase catalytic activity could be released from cells incubated with 20 μg/mL PI-PLC. Generally, with 12 μg/mL PI-PLC, about 46–49% of the alkaline phosphatase catalytic activity could be released from the cells in 45 min. These results are quantitatively comparable to those observed for the PI-PLC-mediated release of Thy-1 glycoprotein from intact mouse lymphocytes (Low & Kincade, 1985) and strongly suggest that alkaline phosphatase is anchored in the HeLa cell membrane through phosphatidylinositol. The relatively high concentration of PI-PLC required to release alkaline phosphatase is probably a result of the salts in the incubation medium. Isotonic sodium chloride is known to have a marked inhibitory effect on the release of alkaline phosphatase from placental membranes by *S. aureus* PI-PLC (Malik & Low, 1986). The broad specificity phospholipase C from *Clostridium perfringens* which has low activity against

Table I: Phosphatidylinositol-Specific Phospholipase C Releases Alkaline Phosphatase from HeLa Cells[a]

preincubation conditions	alkaline phosphatase (catalytic activity)	
	% released	% cell bound
no additions	0	100
PI-PLC	47 ± 4	53 ± 6
PI-PLC + mAb E5	5 ± 3	95 ± 8
PI-PLC + mAb B10	29 ± 3	71 ± 6
PI-PLC + mAb B2	22 ± 3	77 ± 2

[a] HeLa cells were grown to confluency on 24-well plates, incubated with or without monoclonal antibodies (mAbs) to alkaline phosphatase, and treated with PI-PLC. Alkaline phosphatase catalytic activity in the supernatants (released) and in detergent extracts of the cells (cell bound) was then measured (for details, see Experimental Procedures). Values are the averages of six determinations (±SD) and are expressed as a percentage of the total activity (cell bound + released). The antibodies had no effect on alkaline phosphatase catalytic activity; under the five experimental conditions, the optical density at 405 nm after 15-min incubation of the enzyme and substrate averaged 1.031 ± 0.243.

phosphatidylinositol did not effect detectable release of alkaline phosphatase from the cells (results not shown).

That the release of alkaline phosphatase is due to direct cleavage of the anchoring domain from the protein by PI-PLC and not through a secondary effect such as stimulation of some endogenous protease is supported by the finding that monoclonal antibodies to alkaline phosphatase block the PI-PLC-mediated release (Table I). Each of the three monoclonal antibodies has some inhibitory effect while no effect was observed with nonspecific mouse IgG (results not shown). Since the antibodies are known to bind to different epitopes (Jemmerson & Stigbrand, 1984; Jemmerson et al., 1985c) which do not overlap on the basis of antibody blocking experiments (unpublished results), the inhibitory effect observed here is probably due to steric hindrance about the cleavage site for PI-PLC. It is interesting that B10 which is the most effective in blocking the bromelain-mediated release of alkaline phosphatase from the same cancer cells (Jemmerson et al., 1985c) is the least effective in blocking PI-PLC. If a protease was responsible for releasing catalytically active alkaline phosphatase from the cells in response to PI-PLC, then B10 should have blocked it completely. On the other hand, E5 has no effect in blocking bromelain cleavage and is the most effective at blocking PI-PLC cleavage. Monoclonal antibody E5 also effectively blocks the major trypsin cleavage site on alkaline phosphatase (Jemmerson & Stigbrand, 1984) which occurs between residues 62 and 63 (Millan, 1986), but cleavage at that site does not mediate the release of catalytically active alkaline phosphatase from cells.

Incorporation of ^{3}H-Fatty Acids into Alkaline Phosphatase. To confirm that phosphatidylinositol is the membrane anchoring domain of alkaline phosphatase, biosynthetic labeling experiments were carried out. If alkaline phosphatase does indeed contain phosphatidylinositol, fatty acids should be incorporated, and since PI-PLC cleaves 1,2-diacylglycerol from phosphatidylinositol, these fatty acids should be removed from alkaline phosphatase by PI-PLC.

Both [^{3}H]palmitic acid and [^{3}H]myristic acid were found to label alkaline phosphatase when HeLa TCRC-1 cells were grown in the presence of these fatty acids. An example of a fluorograph from SDS-PAGE of anti-alkaline phosphatase immunoprecipitates in such an experiment is shown in Figure 1. Precipitation of alkaline phosphatase was specific in that mouse IgG did not bind the enzyme (lanes 1–4) while the specific monoclonal antibody E5 did (lanes 5–8). Some low molecular weight ^{3}H-labeled materials apparently not related

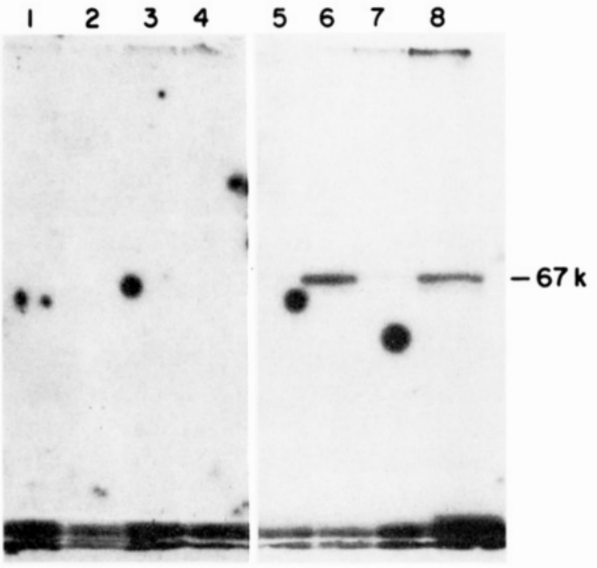

FIGURE 1: Fluorograph of an SDS–polyacrylamide gel demonstrating incorporation of [^{3}H]myristate and [^{3}H]palmitate into alkaline phosphatase. Protein from cells biosynthetically labeled for 18 h was immunoprecipitated with a monoclonal antibody, E5, and protein A bacterial adsorbent. Lanes 1–4, control mouse IgG; lanes 5–8, antibody E5; lanes 1 and 5, [^{3}H]myristate; lanes 2 and 6, [^{3}H]myristate, sodium butyrate, and hyperosmotic sodium chloride treated; lanes 3 and 7, [^{3}H]palmitate; lanes 4 and 8, [^{3}H]palmitate, sodium butyrate, and hyperosmotic sodium chloride treated. See Experimental Procedures for details. In this figure and Figures 2 and 4, the intense band at the bottom is probably lipid nonspecifically associated with the immune precipitate. In separate experiments (see text), it could be removed by gel filtration on Sephadex G-100 in the presence of detergent.

to alkaline phosphatase were nonspecifically precipitated in the presence of either antibody. These were probably lipids since they were also present in affinity-purified alkaline phosphatase but were effectively removed by gel filtration on Sephadex G-100 in the presence of detergent as judged by SDS–PAGE (results not shown). There did not appear to be a preference for incorporation of one fatty acid over the other as would be anticipated if the fatty acids were coupled directly to an amino acid side chain. A wide body of evidence indicates that palmitate and myristate are incorporated into distinct sets of proteins in cases where the fatty acids are covalently bound to amino acid residues (McIlhinney et al., 1985; Magee & Courtneidge, 1985; Olson et al., 1985). By contrast, a mixture of fatty acids is found in the phosphatidylinositol that is covalently attached to mammalian membrane proteins (Tse et al., 1985; Roberts & Rosenberry, 1985). However, caution must be exercised in interpretation of these data since in some cells a significant proportion of the added [^{3}H]myristate is elongated and then incorporated into the protein as [^{3}H]-palmitate (Olson et al., 1985). Treatment of polyacrylamide gels containing ^{3}H-fatty acid labeled alkaline phosphatase with hydroxylamine at neutral pH did not decrease the amount of radioactivity bound to the protein (see below). This indicates that the fatty acids are not bound to alkaline phosphatase through a thiol ester bond as is the case with many palmitylated proteins (Magee & Schlesinger, 1982).

The results shown in Figure 1 are from an experiment in which cells were allowed to incorporate ^{3}H-fatty acid for 18 h. Similar patterns of incorporation, although lower in intensity, were observed when cells were labeled for 4 h. Whether incorporation was followed for 4 or 18 h, however, there was a marked enhancement of biosynthetic labeling of alkaline phosphatase in cells treated with sodium butyrate and sodium chloride (Figure 1, lanes 6 and 8). This is consistent with observations that these reagents increase the expression

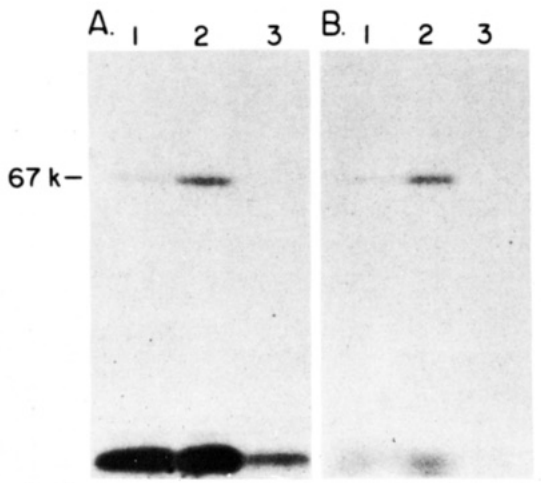

FIGURE 2: Fluorograph of an SDS–polyacrylamide gel showing that PI-PLC removes [^{3}H]palmitate from alkaline phosphatase at the same time it releases alkaline phosphatase from the cell surface. Panels A and B represent duplicate gels except in panel B the gel was treated with 1 N hydroxylamine, pH 6.6. The cells were treated with sodium butyrate and hyperosmotic sodium chloride prior to biosynthetic labeling. Alkaline phosphatase was immunoprecipitated as described in the legend to Figure 1. Lane 1, cells after treatment with PI-PLC; lane 2, cells not treated with PI-PLC; lane 3, supernatant from cells run in lane 1.

of cellular proteins, including alkaline phosphatase, causing elevation in the number of molecules 8-fold or more when used in combination (Ito & Chou, 1984; Jemmerson et al., 1985a). These reagents thus serve as tools to facilitate structural studies on alkaline phospatase of HeLa cells and are used here for that purpose.

Removal of [^{3}H]Palmitate from Alkaline Phosphatase by PI-PLC and by Bromelain. Two different types of experiments were carried out to examine whether PI-PLC cleaves fatty acids from alkaline phosphatase. In the first case, HeLa TCRC-1 cells were labeled with [^{3}H]palmitic acid as before. The cells were then incubated with PI-PLC. Both the supernatants and cells (extracted with detergent) were examined for anti-alkaline phosphatase immunoprecipitable radioactivity by SDS–PAGE. Results of such an experiment are shown in Figure 2A. Approximately 60–70% of the [^{3}H]palmitate in alkaline phosphatase was removed by PI-PLC as demonstrated by comparing alkaline phosphatase present on PI-PLC-treated (lane 1) and untreated (lane 2) cells by densitometer tracings (not shown) of the autoradiographs. As expected, the alkaline phosphatase released from the PI-PLC-treated cells into the supernatant did not retain the label (lane 3) since PI-PLC would cleave from the molecule any fatty acids incorporated into the 1,2-diacylglycerol of the phosphatidylinositol moiety. Hydroxylamine treatment of a duplicate gel (Figure 2B) did not release any detectable [^{3}H]palmitate from alkaline phosphatase, indicating that even the fatty acid remaining on alkaline phosphatase after PI-PLC treatment is not attached by a direct thiol ester linkage to the polypeptide.

The other type of experiment carried out involved the release of [^{3}H]palmitate from alkaline phosphatase purified by immunoaffinity chromatography by PI-PLC. Results of such an experiment are shown in Figure 3. PI-PLC was effective at removing 52–53% of [^{3}H]palmitate from the protein (Figure 3B,C). Since similar amounts of [^{3}H]palmitate were removed at the two PI-PLC concentrations used (i.e., 2 and 20 μg/mL), approximately half of the ^{3}H-labeled, purified alkaline phosphatase must be resistant to PI-PLC as was observed with the release of cell-bound alkaline phosphatase. The broad specificity phospholipase C from *Clostridium perfringens* did

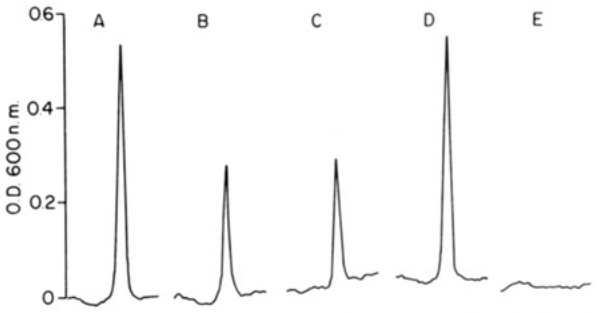

FIGURE 3: Densitometer tracing of a fluorograph of an SDS–polyacrylamide gel showing removal of [^{3}H]palmitate from purified alkaline phosphatase by PI-PLC and by bromelain. Alkaline phosphatase was incubated for 1 h at 37 °C alone (A) or with 2 μg/mL PI-PLC (B), 20 μg/mL PI-PLC (C), 20 μg/mL phospholipase C from *Clostridium perfringens* (D), or 20 μg/mL bromelain (E). Alkaline phosphatase was purified from sodium butyrate and hyperosmotic sodium chloride modulated HeLa TCRC-1 cells and treated with the enzymes as described under Experimental Procedures.

not release any ^{3}H-fatty acid from purified alkaline phosphatase (Figure 3D).

Bromelain, by contrast, completely removes the fatty acid from purified alkaline phosphatase (Figure 3E). This protease is known to remove a 2-kDa segment from the polypeptide which presumably contains the membrane anchoring domain since alkaline phosphatase is released from the membrane by this treatment (Kottel & Hanford, 1980; Hanford & Fishman, 1983; Jemmerson et al., 1985c). Tandem digests of alkaline phosphatase by trypsin and bromelain indicate that these proteases cleave at opposite ends of the polypeptide chain (Jemmerson et al., 1984), consistent with the finding that each site is blocked by a different set of monoclonal antibodies (Jemmerson & Stigbrand, 1984; Jemmerson et al., 1985c). Since trypsin is now known to cleave near the amino terminus (Millan, 1986), the segment cleaved by bromelain must be toward the carboxyl-terminal end. The carboxyl-terminal location of the membrane anchoring domain of alkaline phosphatase has been demonstrated independently by Abu-Hasan and Sutcliffe (1984) using the protease subtilisin. Therefore, the results in Figure 3 suggest that the fatty acid is localized to the 2-kDa carboxyl-terminal segment of alkaline phosphatase and that little, if any, of the ^{3}H label in alkaline phosphatase is likely to be due to conversion to amino acids as observed by Olson et al. (1985) in 3T3 cells.

Several attempts were made to demonstrate that the ^{3}H radioactivity which was removed from purified alkaline phosphatase by PI-PLC (Figure 3) was part of a 1,2-diacylglycerol moiety. However, these experiments were complicated by the observation that most of the ^{3}H radioactivity that had copurified with ^{3}H-labeled alkaline phosphatase on the immunoaffinity and gel filtration columns could be extracted with diethyl ether and was thus not covalently attached to the protein. Gel filtration chromatography did remove substantial contaminating ^{3}H radioactivity often observed at the bottom of the SDS–PAGE gels (e.g., see Figure 2). However, this procedure was apparently ineffective at removing the ^{3}H radioactivity extractable by diethyl ether. Although the major ^{3}H-labeled contaminant was not precisely identified, it had a mobility in thin-layer chromatography consistent with triacylglycerol; no ^{3}H-fatty acid was detected, but significant amounts of ^{3}H-labeled 1,2- and 1,3-diacylglycerol were present as contaminants. In one experiment, PI-PLC treatment did produce a 4-fold increase over control values of ^{3}H radioactivity in the 1,2- and 1,3-diacylglycerol region of the thin-layer chromatography plate. However, since this amounted to less than 10% of the total radioactivity (i.e., approximately 100

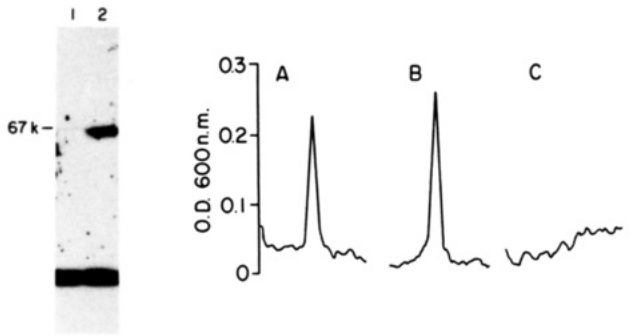

FIGURE 4: Fluorograph of one SDS–polyacrylamide gel demonstrating the incorporation of [^{3}H]ethanolamine into alkaline phosphatase and a densitometer tracing of another fluorograph showing the removal of [^{3}H]ethanolamine from alkaline phosphatase by bromelain but not PI-PLC. In the left panel, HeLa cells treated with sodium butyrate and hyperosmotic sodium chloride (lane 2) or not treated (lane 1) were incubated with [^{3}H]ethanolamine for 8 h, and alkaline phosphatase was immunoprecipitated as described in the legend in Figure 1. In the right panel, purified alkaline phosphatase from sodium butyrate and sodium chloride treated HeLa TCRC-1 cells (A) was treated with 20 μg/mL PI-PLC (B) or with 20 μg/mL bromelain (C).

cpm), the significance of this observation is difficult to assess. These results are not inconsistent with the observtions made above that the purified ^{3}H-labeled alkaline phosphatase radioactivity was substantially (i.e., approximately 50%) decreased by PI-PLC treatment when analyzed by SDS–PAGE and fluorography (Figure 3). In this case, denaturation would most likely have removed these noncovalently bound ^{3}H-lipid contaminants from the polypeptide. Presumably, they were not detected by fluorography since they would have been spread diffusely throughout the gel or removed during the fixation process. The results of these experiments indicated that the amount of ^{3}H radioactivity covalently attached to the purified alkaline phosphatase was only a small proportion of the total that copurified with the protein and the release of [^{3}H]diacylglycerol by PI-PLC was generally obscured by the noncovalently bound contaminants. This problem was exacerbated by the fact that at most only approximately 50% removal of the covalently attached [^{3}H]palmitate was anticipated (see Figure 3). In this regard, it is relevant to note that the VSG of *T. brucei* (an abundant protein that is readily labeled in the covalently attached phosphatidylinositol moiety by [^{3}H]myristate) contains significant amounts of ^{3}H-lipid as noncovalently bound contaminants (Ferguson et al., 1985b).

Incorporation of [^{3}H]Ethanolamine into Alkaline Phosphatase. Studies of the VSG of *T. brucei* showed that the phosphatidylinositol moiety is covalently linked to the polypeptide chain through an intervening carbohydrate sequence consisting of ethanolamine, glycan, and glucosamine (Ferguson et al., 1985b). The ethanolamine residue is attached to the carboxyl terminus via an amide bond (Holder, 1983). Various reports suggest that this may be the general structure for the carboxyl-terminal end of proteins linked to phosphatidylinositol [see Low et al. (1986) and Low (1987)].

The finding that [^{3}H]ethanolamine is incorporated biosynthetically into HeLa cell alkaline phosphatase is also consistent with this proposal (Figure 4). As was the situation with the fatty acid incorporation, a combination of sodium butyrate and sodium chloride enhanced the incorporation of [^{3}H]ethanolamine (Figure 4, left panel, lane 2). The amount of [^{3}H]ethanolamine incorporated appears to be greater than that observed with the ^{3}H-fatty acids. Bromelain released [^{3}H]ethanolamine from purified alkaline phosphatase (Figure 4, right panel, scan C), while PI-PLC had no effect (Figure 4, right panel, scan B).

Alkaline phosphatase was released from the [^{3}H]ethanolamine-labeled cells by PI-PLC and purified by immunoaffinity chromatography (see Experimental Procedures) and anion-exchange chromatography (Low et al., 1987). The ^{3}H radioactivity in this purified alkaline phosphatase was released by acid hydrolysis (6 M HCl, 110 °C, 24 h) and analyzed by cation-exchange chromatography (Pharmacia Mono-S HR5/5 column eluted with 20 mM sodium citrate, pH 3.25). Most of the eluted radioactivity (i.e., 91–93%) was found in a peak that had a similar mobility to authentic [^{3}H]ethanolamine; approximately 6% was eluted with a retention time about twice that of the [^{3}H]ethanolamine. Treatment of the immunoaffinity/anion-exchange-purified alkaline phosphatase with bromelain or subtilisin (50 μg/mL; 60 min, 37 °C) released 84–90% and 90–100%, respectively, of the ^{3}H radioactivity in a form which passed through an ultrafiltration membrane with a 10000 molecular weight cutoff. However, these ^{3}H-labeled proteolytic fragments were eluted from a Bio-Gel P-6 column in single peaks (V_e = 41 and 44 mL, respectively) between the void volume (V_0 = approximately 30 mL) and the total bed volume (V_e = approximately 68 mL). They were also retained by an anion-exchange column (Pharmacia Mono-Q HR5/5 in 20 mM NH_4HCO_3) from which they could be eluted by 200–250 mM NH_4HCO_3. These properties distinguish the ^{3}H radioactivity released by the proteases from free [^{3}H]ethanolamine and indicate that the ^{3}H radioactivity in the alkaline phosphatase purified from [^{3}H]ethanolamine-labeled HeLa cells is covalently attached to a structure in the protein as a [^{3}H]ethanolamine residue.

The ability of bromelain and subtilisin to remove the ^{3}H radioactivity from the alkaline phosphatase in these experiments indicates that [^{3}H]ethanolamine is not converted into amino acids and then incorporated throughout the polypeptide chain since both of these proteases have been shown to remove small segments from the carboxyl terminus (see above). Thus, it seems likely that ethanolamine is incorporated toward the carboxyl-terminal end of the polypeptide chain and in the membrane anchoring domain. The failure of PI-PLC to remove any [^{3}H]ethanolamine label from alkaline phosphatase (Figure 4) is consistent with these conclusions.

Discussion

The cDNA sequences of the major types of mammalian alkaline phosphatases have recently been reported by several laboratories (Kam et al., 1985; Millan, 1986; Weiss et al., 1986; Henthorn et al., 1986; Ovitt et al., 1986; Berger et al., 1987). From the predicted amino acid sequence, a hydrophobic stretch of about 20 amino acids was indicated at the carboxyl terminus, and it was suggested that the portion of the polypeptide chain serves as the membrane anchoring domain. However, this prediction is not consistent with previous observations that mammalian alkaline phosphatases, including the type of alkaline phosphatase expressed by the HeLa cells, can be released from membranes by PI-PLC (Low et al., 1986; Malik & Low, 1986). In this report, we provide biosynthetic labeling evidence supporting the earlier claim (Low & Zilversmit, 1980; Low et al., 1987) that alkaline phosphatase is anchored in the membrane via covalent linkage with a phosphatidylinositol structure similar to that found in VSG. Since we have no specific labeling technique for the hydrophobic peptide predicted to be present at the carboxyl terminus, we cannot preclude its presence in the mature protein. However, the present data indicate that it is unlikely to serve a membrane anchoring function. It is probable that this hydrophobic stretch of amino acids at the carboxyl terminus is cleaved from the polypeptide prior to the attachment of phosphatidylinositol and

the intervening carbohydrate groups as is known to be the case for the VSG of *T. brucei* and for the Thy-1 glycoprotein (Bangs et al., 1985; Tse et al., 1985; Ferguson et al., 1986).

The observation that [^{3}H]ethanolamine can be incorporated into the membrane anchoring domain of HeLa cell alkaline phosphatase is of considerable interest since recent biosynthetic labeling studies with two other proteins anchored by glycosylphosphatidylinositol (i.e., decay accelerating factor and Thy-1) have yielded similar results (Medof et al., 1986; Fatemi et al., 1987). This supports the proposal that one or more ethanolamine residues is a general feature of the anchoring domain of proteins anchored by glycosylphosphatidylinositol (Low et al., 1986; Haas et al., 1986; Medof et al., 1986; Low, 1987). The recent observation that human placental alkaline phosphatase (the type expressed by HeLa cells) contains *myo*-inositol covalently attached to it by a nitrous acid sensitive linkage (Low et al., 1987) has indicated that alkaline phosphatase exhibits other conserved features of this novel and complex anchoring structure.

Although a substantial proportion of alkaline phosphatase in HeLa cells was susceptible to the action of PI-PLC, some was clearly resistant, even when the experiments were done under conditions where excess PI-PLC was present. This result was obtained in three different types of experiment. First, only 50–70% of enzyme activity was releasable from intact cells; second, only 60–70% of the [^{3}H]palmitate-labeled alkaline phosphatase could be removed from intact cells; and finally, a maximum of approximately 50% of the [^{3}H]palmitate could be removed from detergent-solubilized affinity-purified alkaline phosphatase. This compares with a previous report that at most only 65% of the alkaline phosphatase in a human placental particulate fraction was releasable by PI-PLC (Malik & Low, 1986). The reason for this resistance is presently unknown, but it could occur as the result of chemical modification of the attached phosphatidylinositol rendering it resistant to PI-PLC. Resistance to release by PI-PLC of membrane proteins believed to be anchored by phosphatidylinositol, or a derivative thereof, has been described previously (Low & Finean, 1977b; Futerman et al., 1985; Roberts & Rosenberry, 1985, 1986; Davitz et al., 1986; Haas et al., 1986; Medof et al., 1986). It is also possible that the alkaline phosphatase which is resistant to release from the membrane by PI-PLC is a distinct form of this protein in which the hydrophobic carboxyl-terminal "anchoring" peptide was not removed from the newly synthesized polypeptide and replaced by the phosphatidylinositol-containing glycolipid anchor. However, such a model would not account for the resistance of some of the [^{3}H]palmitate-labeled alkaline phosphatase to hydrolysis by PI-PLC.

The present findings will allow for further structural analysis of the membrane anchoring domain of alkaline phosphatase by biosynthetic labeling and proteolytic cleavage. That portion of the molecule can now be biosynthetically labeled in sodium butyrate and sodium chloride modulated HeLa TCRC-1 cells with [^{3}H]ethanolamine or ^{3}H-fatty acids. The labeled protein could serve as a tracer in the isolation of a bromelain- or subtilisin-released glycosylphosphatidylinositol peptide suitable for structural studies. For instance, amino acid composition and sequence analysis of such a fragment would identify the site of proteolytic posttranslational processing at the carboxyl terminus predicted to occur before or simultaneous with attachment of the phosphatidylinositol (Low et al., 1986).

Since the biological function of alkaline phosphatase itself is not known, the physiological significance of its phosphatidylinositol anchor cannot at present be assessed, but it is possible that the mechanism by which this enzyme is anchored in the membrane is important in regulating that function. An endogenous phospholipase activity has been identified in placental tissue (Malik & Low, 1986) which, below neutral pH, is effective at removing the membrane anchor from alkaline phosphatase during butanol extraction. If this or a related phospholipase was activated in response to physiological stimuli, it could serve to regulate the level of expression of alkaline phosphatase as its functional sites on the cell surface. The ability of monoclonal antibodies to block the exogenous PI-PLC that we have demonstrated suggests a strategy to explore the possible regulation of alkaline phosphatase function by endogenous phospholipases.

While this paper was being prepared for publication, we became aware of studies showing that the human placental type of alkaline phosphatase expressed in the WISH amniotic cell line could also be labeled with ^{3}H-fatty acids, ethanolamine, and *myo*-inositol (Howard et al., 1987).

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