Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 7817–7821, November 1987
Biochemistry

Differences in the glycolipid membrane anchors of bovine and human erythrocyte acetylcholinesterases

[phosphatidylinositol-specific phospholipase C/alkylacylglycerol/3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine/lipid radiolabeling]

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Communicated by Harland G. Wood, June 29, 1987

ABSTRACT **Acetylcholinesterases (AcChoEases; EC 3.1.1.7) from bovine (E^{bo}) and human (E^{hu}) erythrocytes were purified to apparent homogeneity by affinity chromatography. The hydrophobic portion of the glycolipid membrane anchor of each enzyme was radiolabeled with the photoactivated reagent 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine. Several cleavage procedures demonstrated that this radiolabel was highly selective for the fatty acid portion of the anchor in both enzymes. The labeled enzymes were digested with phosphatidylinositol (PtdIns)-specific phospholipase C (EC 3.1.4.10), and label release was assessed by polyacrylamide gel electrophoresis. About 85% of the radiolabel was cleaved from E^{bo} AcChoEase, whereas only 5% was released from E^{hu} AcChoEase. This finding agrees with a report that E^{bo} AcChoEase was quantitatively released from intact erythrocytes by PtdIns-specific phospholipase C but E^{hu} AcChoEase was not [Low, M. G. & Finean, J. B. (1977) *FEBS Lett.* 82, 143–146]. The two AcChoEases contained comparable amounts of the anchor components ethanolamine, glucosamine, and *myo*-inositol, but qualitative and quantitative differences were found in the fatty acids. Thin-layer chromatography of radiolabeled fragments generated from E^{bo} and E^{hu} AcChoEases by nitrous acid deamination revealed a major difference in the membrane anchors of the two enzymes. The fragment released from E^{bo} AcChoEase by this procedure comigrated with PtdIns, whereas the corresponding fragment from E^{hu} AcChoEase had a mobility much greater than that of PtdIns even though it contained *myo*-inositol and fatty acids. These studies show that 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine is useful for analysis of lipid-containing compounds and indicate that, whereas E^{bo} AcChoEase contains PtdIns in its glycolipid anchor, E^{hu} AcChoEase has a different anchor structure, which is resistant to PtdIns-specific phospholipase C. This observation suggests the existence of a class of glycolipid-anchored membrane proteins resistant to this phospholipase.**

Acetylcholinesterase (AcChoEase; acetylcholine acetylhydrolase; EC 3.1.1.7) is found in a variety of molecular forms whose distribution is tissue-specific (1, 2). While these forms have very similar catalytic and antigenic properties, they differ in their extents of oligomeric assembly and their membrane-attachment structures. Globular dimeric (G_2) AcChoEase forms predominate in mammalian erythrocytes and insect heads and are major components in *Torpedo* electric organ. Considerable evidence indicates that these G_2 AcChoEases are anchored in the plasma membrane exclusively by a covalently linked glycolipid (3–6). Similar anchors have been demonstrated in trypanosome variant surface glycoproteins (VSGs) (7), rat Thy-1 (8, 9), and human decay-accelerating factor (DAF) (10, 11). Chemical analyses have revealed that anchor components include fatty acids, inositol, glucosamine, an oligosaccharide, and ethanolamine in amide linkage to the polypeptide C-terminus.

One important characteristic of the anchors of most of these proteins is their susceptibility to cleavage by purified phosphatidylinositol (PtdIns)-specific phospholipase C [PL-C_{Ins}; 1-phosphatidyl-D-*myo*-inositol inositolphosphohydrolase (cyclic-phosphate-forming), EC 3.1.4.10] (12). The VSG anchor contains PtdIns linked through the inositol to glucosamine (7), thus providing a structural basis for the action of these enzymes. PL-C_{Ins}-induced release from the cell surface in fact has become a primary criterion for identification of proteins with these anchors. Identified proteins include mammalian alkaline phosphatase, 5′-nucleotidase, alkaline phosphodiesterase I, trehalase, rodent neural cell adhesion molecule NCAM-120, the murine T-cell-activating protein TAP, and surface proteins in *Leishmania* (see ref. 12 for review). Although confirmation of glycolipid anchors in these proteins by direct chemical analysis has not been reported, the available data suggest that PL-C_{Ins}-induced release is strong evidence of a glycolipid-anchored protein. However, the failure of PL-C_{Ins} to release two proteins with glycolipid anchors raises the question of whether this is a necessary criterion. Purified PL-C_{Ins} from *Staphylococcus aureus* releases only about 5% of the AcChoEase (13) and 10–15% of the DAF (10, 11) from human erythrocytes. Resistance to PL-C_{Ins} could result from inaccessibility of the cleavage site *in situ*, and clearly this does account for the failure of added *S. aureus* PL-C_{Ins} to release VSGs from intact trypanosomes (14). In contrast to VSGs, however, extracted and purified human erythrocyte (E^{hu}) AcChoEase remains resistant to PL-C_{Ins} (15). This observation led us to investigate the structural basis of the resistance of E^{hu} AcChoEase.

Low and Finean (13) showed that AcChoEase is released by PL-C_{Ins} from bovine but not human erythrocytes, and we have purified bovine erythrocyte (E^{bo}) AcChoEase for comparison to E^{hu} AcChoEase. Because it is not feasible to label erythrocyte AcChoEases biosynthetically, the photoactivated reagent 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID) (16), which specifically labels the hydrophobic domain of the E^{hu} glycolipid anchor (15), was employed to radiolabel E^{bo} and E^{hu} AcChoEases. Chemical fragmentation techniques that have been applied to [^{3}H]myristate-labeled VSG anchors were used to demonstrate interesting differences in [^{125}I]TID-labeled E^{hu} and E^{bo} AcChoEase anchors that correlate with their sensitivity to PL-C_{Ins}.

METHODS

Protein Purification. E^{hu} AcChoEase was purified from outdated erythrocytes by affinity chromatography (17) with an extensive resin wash and elution procedure (6). E^{bo}

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Abbreviations: AcChoEase, acetylcholinesterase; E^{bo}, bovine erythrocyte; E^{hu}, human erythrocyte; DAF, decay-accelerating factor; [^{125}I]TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine; PtdIns, phosphatidylinositol; PL-C_{Ins}, PtdIns-specific phospholipase(s) C; VSG, variant surface glycoprotein.

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AcChoEase was prepared from fresh citrate-treated bovine blood obtained from a local slaughterhouse. Cell membranes from 20 liters of blood were washed and concentrated to 2 liters in a filtration apparatus (17). Triton X-100 was added [15% (vol/vol) in 5 mM sodium phosphate, pH 7; 400 ml] and E^{bo} AcChoEase was purified from the mixture by affinity chromatography (6). The resin (300 ml) was washed as for E^{hu} AcChoEase except that the high-salt wash contained 250 mM instead of 500 mM NaCl. $NaDodSO_4$/PAGE of the product from affinity chromatography revealed a faint contaminant of 96 kDa, which was separated from AcChoEase activity on Sepharose CL-4B equilibrated in 20 mM sodium phosphate, pH 7 (buffer 1) containing 0.1% Triton X-100.

Enzyme was depleted of Triton X-100 by a second cycle of affinity chromatography and eluted from the acridinium resin with buffer 1 containing 10 mM decamethonium bromide and 500 mM (E^{hu} AcChoEase) or 250 mM (E^{bo} AcChoEase) NaCl. The protein content was estimated from the enzyme activity in a modified Ellman spectrophotometric assay assuming a specific activity of 410 units/nmol of subunit for both E^{bo} and E^{hu} AcChoEases (17) except where noted. Stokes radii were estimated as outlined previously (17).

Photolabeling with [^{125}I]TID. AcChoEases were labeled with [^{125}I]TID (Amersham) as described (15). Liposomes generated by bath sonication of 250 μg of PtdIns in 250 μl of water (25°C, 10 min) were radiolabeled with [^{125}I]TID (14 μCi; 1 μCi = 37 kBq) in a similar manner. Water was removed *in vacuo* (Speed-Vac; Savant Instruments), and the samples were resuspended in 2-propanol/hexane/water (8:6:0.6) and chromatographed on a μ-Porasil HPLC column (Waters Associates) in the same solvent (flow rate, 1 ml/min). The radiolabeled PtdIns comigrated with unlabeled PtdIns.

Base Hydrolysis. Samples of [^{125}I]TID-labeled E^{bo} and E^{hu} AcChoEases were dried *in vacuo* and 200 μl of 100 mM aqueous NaOH was added. After incubating for 16 hr at 4°C, the mixture was acidified with acetic acid and the radiolabeled free fatty acids were extracted with three 100-μl portions of chloroform for TLC analysis.

Acid Methanolysis. Aliquots of 1 M anhydrous methanolic HCl (200 μl) were added to [^{125}I]TID-labeled samples that had been dried *in vacuo*. Heptadecanoic acid was added as an internal standard for GLC analysis (4). After heating at 65°C for 16 hr, the samples were extracted with three 100-μl portions of 2,2,4-trimethylpentane to remove fatty acid methyl esters for analysis by GLC and TLC.

PL-C_{Ins} Digestion. [^{125}I]TID-labeled E^{bo} and E^{hu} AcChoEases were prepared in buffer 1 containing 0.1% sodium deoxycholate by affinity chromatography and dialysis. Purified PL-C_{Ins} from *S. aureus* (kindly provided by Martin Low, Oklahoma Medical Research Foundation, Oklahoma City, OK) was added to the desired concentration and the samples were incubated for 90 min at 37°C. After incubation the samples were either subjected to PAGE or extracted with chloroform. The extracts were dried for further analysis.

Nitrous Acid Deamination. Samples of [^{125}I]TID-labeled E^{bo} and E^{hu} AcChoEases were treated with 500 μl of 125 mM nitrous acid in 50 mM citrate buffer (pH 4) at 50°C for 4 hr (18). After nitrous acid treatment the samples were extracted with 1 ml of chloroform/methanol (2:1) and three 500-μl portions of chloroform. The chloroform-rich phases were combined and subjected to TLC and inositol analysis.

TLC. Thin-layer chromatography was performed on 10 × 20-cm silica gel GF plates (Fisher) without activation. Development was either with solvent A [hexane/diethyl ether/acetic acid (60:30:1)] or solvent B [chloroform/methanol/water (65:25:4)]. The positions of radioactive components were identified by autoradiography employing Kodak XAR-5 film. The positions of lipid standards were located by exposure of dried plates to iodine vapor.

Inositol Analysis by GLC. Purified samples of detergent-depleted E^{bo} and E^{hu} AcChoEase and the chloroform-rich phase after deamination of E^{hu} AcChoEase (1–8 nmol) were hydrolyzed for 12–16 hr at 115°C in 6 M HCl. *scyllo*-Inositol was added as an internal standard prior to hydrolysis along with Tris·HCl (0.5–4.0 μmol) to improve inositol recoveries. The samples were dried and then derivatized with *N,O*-bis(trimethylsilyl)trifluoroacetamide/trimethylchlorosilane/pyridine (10:1:10; 10–25 μl/nmol of *scyllo*-inositol) at 25°C for 1 hr. Aliquots of the mixture were analyzed on a Hewlett Packard model 5890A gas chromatograph equipped with a flame ionization detector, a splitless injector, a 15 m long, 0.32-mm i.d. DB-1 column (J&W Scientific, Rancho Cordova, CA), and a Hewlett Packard model 3392 integrator.

RESULTS

Comparison of Affinity-Purified AcChoEases by PAGE. Purified AcChoEases from human and bovine erythrocytes appeared free of detectable contaminants after analysis by $NaDodSO_4$/PAGE (Fig. 1), although the bovine enzyme required an additional chromatographic step on Sepharose CL-4B following affinity chromatography to remove a minor contaminant (see *Methods*). Nonreduced and reduced samples of E^{hu} AcChoEase had apparent molecular masses very similar to the values (130 and 72 kDa, respectively) previously reported for the dimeric and monomeric species (17). The nonreduced E^{bo} AcChoEase appeared to be 10 kDa larger, and the reduced bovine enzyme 5 kDa larger, than the corresponding forms of E^{hu} AcChoEase. Although reduction of E^{hu} AcChoEase resulted in complete conversion of dimeric to monomeric enzyme, a small amount of dimer remained after reduction of E^{bo} AcChoEase (Fig. 1, lane 5).

Release of [^{125}I]TID Label by PL-C_{Ins}. To monitor cleavage of the hydrophobic portion of the glycolipid membrane anchors of E^{bo} and E^{hu} AcChoEases, [^{125}I]TID was used as a selective marker and release of radiolabel was assessed by PAGE. A typical gel pattern for PL-C_{Ins}-treated E^{bo} AcChoEase is shown in Fig. 2*A*. Released radiolabel migrated near the dye front, but the untreated enzymes showed no radiolabel in this region (data not shown). A comparison of the release of ^{125}I label from E^{bo} and E^{hu} AcChoEases at various concentrations of PL-C_{Ins} is shown in Fig. 2*B*. A maximum of 85% of the radiolabel was released from the bovine enzyme at PL-C_{Ins} concentrations greater than 2 μg/ml. In contrast, a maximum of only 5% of the radiolabel was released from E^{hu} AcChoEase at PL-C_{Ins} concentrations as high as 9 μg/ml.

To determine whether residual radiolabel on E^{bo} AcChoEase was due to incomplete digestion, samples of intact and PL-C_{Ins}-digested enzyme were chromatographed on Sepharose CL-4B in the absence of detergent. The enzyme activity and radioactivity profiles were superimposable, both for intact and for PL-C_{Ins}-digested E^{bo} AcChoEase, but the apparent Stokes radius for intact E^{bo} AcChoEase was 8.0 ±

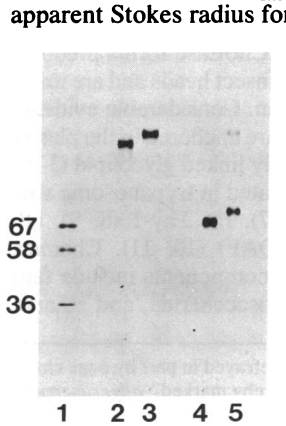

FIG. 1. $NaDodSO_4$/PAGE analysis of purified E^{bo} and E^{hu} AcChoEases before and after disulfide reduction. The slab gel (5–13% acrylamide) was prepared, and reduced (lanes 1, 4, and 5) or nonreduced (lanes 2 and 3) samples were run and stained as described (17). Lane 1, polypeptide standards: bovine serum albumin (67 kDa), bovine catalase (58 kDa), and bovine lactate dehydrogenase (36 kDa). Lanes 2 and 4, 0.5 μg of E^{hu} AcChoEase. Lanes 3 and 5, 0.5 μg of E^{bo} AcChoEase.

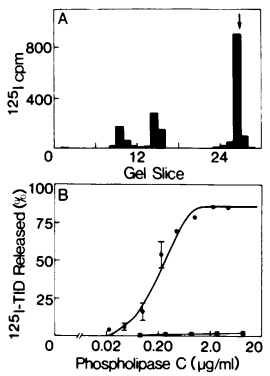

FIG. 2. PL-C_{Ins} concentration dependence of label release from [^{125}I]TID-labeled E^{bo} and E^{hu} AcChoEases measured by NaDodSO$_4$/PAGE. (*A*) Profile of ^{125}I cpm vs. gel slice number for labeled E^{bo} AcChoEase (3000 cpm) digested with PL-C_{Ins} (0.23 μg/ml). To the digested sample, which had been concentrated *in vacuo* to 40 μl, was added buffered 6% NaDodSO$_4$ (5 μl), and the mixture was reduced and subjected to PAGE as in Fig. 1. The unstained gel was sliced immediately (5-mm segments) as outlined in ref. 17. Arrow indicates dye front. ^{125}I in slices 9 and 15 corresponds to dimeric and monomeric enzyme, respectively. (*B*) Plot of percent ^{125}I release vs. logarithm of PL-C_{Ins} concentration for [^{125}I]TID-labeled E^{bo} (●) and E^{hu} (■) AcChoEases, generated from a series of gel-slice profiles as in *A*. Error bars indicate SD for duplicate determinations. Curve is a least-squares fit of the data assuming that the rate of radiolabel release is second-order with respect to concentrations of PL-C_{Ins} and labeled AcChoEase.

0.3 nm (n = 5), while that for PL-C_{Ins}-treated enzyme was 6.5 ± 0.3 nm (n = 3) (data not shown). This decrease in apparent Stokes radius after PL-C_{Ins} treatment of E^{bo} AcChoEase is qualitatively similar to that observed for E^{hu} AcChoEase after removal of the C-terminal glycolipid dipeptide by papain proteolysis and appears to result from loss of aggregation after removal of the hydrophobic membrane anchor (5, 19). The digestion was judged to be complete, since no residual enzyme activity or radiolabel corresponding to the elution of intact enzyme was seen in the PL-C_{Ins}-treated sample. Thus the portion of the initial radioactivity (5–15%) remaining associated with E^{bo} AcChoEase catalytic subunits after complete PL-C_{Ins} digestion apparently is attached covalently to a region of the enzyme other than the component shown below to be a lipid that is released by PL-C_{Ins}. This nonspecific label incorporation lowers the percentage of ^{125}I released by lipid-cleavage procedures presented below.

Compositional Analyses. Further analysis of E^{bo} AcChoEase was undertaken to investigate the presence of glycolipid anchor components previously reported for E^{hu} AcChoEase. The fatty acid compositions of the two enzymes are compared in Table 1. The total quantity of fatty acids is 1.4 mol per mol of E^{bo} AcChoEase monomer compared with 2.0 mol per mol found for E^{hu} AcChoEase (4). In addition, the fatty acid compositions of the two enzymes differ considerably. Stearate is the most abundant fatty acid in E^{bo} AcChoEase, in contrast to palmitate in E^{hu} AcChoEase. Docosatetraenoate (22:4) and docosapentaenoate (22:5), major components in the human enzyme, were not detected in the bovine enzyme. Most of the fatty acids in E^{bo} AcChoEase are saturated, in contrast to the equal amounts of saturated and unsaturated fatty acids in E^{hu} AcChoEase.

Both enzymes contained 1 mol of *myo*-inositol per mol of monomer (Table 2). Components with free amino groups that were detected and quantified in E^{hu} AcChoEase after reductive radiomethylation (5) also were present in E^{bo} AcChoEase. The N-terminal amino acid of the bovine enzyme is glutamate and, unlike the human enzyme, no N-terminal arginine is present. The lysine content of the two enzymes

Table 1. Fatty acid compositions of E^{bo} and E^{hu} AcChoEases

Fatty acid	mol/mol of AcChoEase monomer	
	E^{bo}*	E^{hu}†
14:0	0.03 ± 0.01	0.06 ± 0.02
16:0	0.08 ± 0.02	0.75 ± 0.04
16:1	0.04 ± 0.02	0.09 ± 0.03
18:0	1.13 ± 0.04	0.23 ± 0.03
18:1	0.13 ± 0.02	0.24 ± 0.04
18:2	ND	0.07 ± 0.05
20:4	ND	0.06 ± 0.01
22:4	ND	0.28 ± 0.02
22:5	ND	0.21 ± 0.01
22:6	ND	0.05 ± 0.01
Total	1.41 ± 0.01	2.02 ± 0.07

Compositions of purified AcChoEases were determined by GLC after acid methanolysis and are reported as mol of fatty acid per mol of enzyme monomer as previously described (4). ND, not detected (detection limit 0.02 mol/mol).
*Mean ± SEM of three determinations performed on three distinct enzyme preparations.
†From ref. 4; mean ± SEM of five enzyme preparations.

appeared similar, as did the content of the two glycolipid anchor constituents ethanolamine and glucosamine (Table 2).

TLC Analysis of [^{125}I]TID-Labeled Fragments. To investigate further the differences between the membrane anchors of E^{bo} and E^{hu} AcChoEases, samples of both ^{125}I-labeled enzymes were subjected to various cleavage procedures and the radiolabeled fragments were extracted and analyzed by TLC. Since both enzymes contained fatty acids as major hydrophobic components of the membrane anchor, we expected a large proportion of the radiolabel to be removed from each enzyme by treatments that remove fatty acids. Base hydrolysis was performed under conditions that should cleave ester bonds and release free fatty acids. The release of radioactivity into the organic phase was 48% and 60% for E^{bo} and E^{hu} AcChoEase, respectively. The TLC radiograph in Fig. 3*A* indicates that the major radioactive spots from both species migrated identically and slightly slower than free fatty acid standards. A companion procedure to base hydrolysis is acid methanolysis, which generates fatty acid methyl esters by cleavage of ester, thioester, and amide bonds. After acid methanolysis the amount of radioactivity in the nonpolar extracts was 43% for E^{bo} AcChoEase and 60% for E^{hu}

Table 2. Content of *myo*-inositol* and components with free amino groups†

Component	mol/mol of AcChoEase monomer	
	E^{bo}	E^{hu}
Anchor		
myo-Inositol	0.99 ± 0.06 (n = 3)	1.08 ± 0.09 (n = 5)
Glucosamine	0.97 ± 0.02 (n = 3)	0.98 ± 0.04 (n = 6)
Ethanolamine	1.03 ± 0.05 (n = 3)	1.35 ± 0.06 (n = 6)
Non-anchor		
Glutamate	0.87 ± 0.06 (n = 3)	0.66 ± 0.03 (n = 6)
Arginine	Not detected	0.34 ± 0.01 (n = 6)
Lysine	5.78 ± 0.23 (n = 3)	7.40 ± 0.20 (n = 6)

*Determined by GLC after hydrolysis and derivatization of samples whose protein content was estimated by AcChoEase activity (see *Methods*). Values are means ± SEM of n determinations.
†Samples of purified enzyme were reductively methylated with 10 mM H^{14}CHO and 50 mM NaCNBH$_3$, repurified by affinity chromatography, and hydrolyzed. Radiolabeled amines were identified as described (5). E^{bo} and E^{hu} values were calculated from the methyl-group specific activity and the amino acid content of selected amino acids (see ref. 5). The following mol percentages for these amino acids in E^{bo} AcChoEase were determined: Val, 7.5; Phe, 4.6; Arg, 6.9; Tyr, 3.1. Values for E^{hu} AcChoEase are from Haas *et al.* (5). Data presented are means ± SEM of n analyses.

AcChoEase, and acid methanolysis of the isolated labeled product released by PL-C_{Ins} from E^{bo} AcChoEase resulted in partitioning of 58% of the label into the nonpolar extract. Fig. 3*B* shows the TLC patterns obtained after this procedure. The major spots for each sample were similar in mobility and migrated to a position slightly below the unlabeled fatty acid methyl ester standard. Methanolysis of control [^{125}I]TID-labeled PtdIns released 76% of the radiolabel into the nonpolar phase as a product with TLC mobility identical to that observed for the AcChoEase samples (data not shown).

The removal of 1,2-dimyristoylglycerol from VSGs by PL-C_{Ins} has been demonstrated (7). To determine whether PL-C_{Ins} cleaves 1,2-diacylglycerol from E^{bo} AcChoEase, a sample of the [^{125}I]TID-labeled enzyme was digested with PL-C_{Ins} with consequent release of 95% of the radiolabel into the organic phase after chloroform extraction. The mobility of the major extractable [^{125}I]TID-labeled fragment was closer to that of the diacylglycerol standard 1,3-diolein than to that of the 1,2-diolein standard (Fig. 3*C*). In contrast, diacylglycerol released from [^{125}I]TID-labeled PtdIns by PL-C_{Ins} migrated at the position of the 1,2-diolein standard (data not shown). This observation prompted us to examine more closely the lipid component released from E^{bo} AcChoEase by PL-C_{Ins}, and we have recently identified this diradylglycerol.†

Nitrous acid deamination cleaves the glycosidic bond at the 1 position of glucosamine (18) and has been shown to release PtdIns from glycolipid-anchored proteins (7). Treatment of [^{125}I]TID-labeled E^{bo} and E^{hu} AcChoEases with nitrous acid resulted in release of 40% and 48% of the radiolabel into the chloroform-rich phase, respectively. These relatively low percentages largely reflected a poor efficiency of extraction, as analysis of deamination samples by NaDodSO$_4$/PAGE revealed radiolabel release of 65–80% from both enzymes (data not shown; also ref. 10). The results of TLC analysis in Fig. 3*D* show that the deamination product of E^{bo} AcChoEase migrated near the position of PtdIns as predicted, while the product from E^{hu} AcChoEase had a much larger R_f. The control, [^{125}I]TID-labeled PtdIns, comigrated with unlabeled PtdIns on TLC with solvent B (Fig. 3*D*). Because of the anomalous TLC mobility of the [^{125}I]TID-labeled deamination fragment from E^{hu} AcChoEase, the chloroform-rich phase following deamination and extraction of the human enzyme was analyzed for *myo*-inositol. Nearly quantitative release of *myo*-inositol into this phase was observed. Based on the ^{125}I specific activity of labeled E^{hu} AcChoEase and the radioactivity in the organic phase, 0.84 ± 0.07 residue of *myo*-inositol per E^{hu} AcChoEase subunit was released (n = 5). Thus, even though the [^{125}I]TID-labeled deamination fragment from E^{hu} AcChoEase does not correspond to PtdIns on TLC, it does contain *myo*-inositol as well as fatty acids.

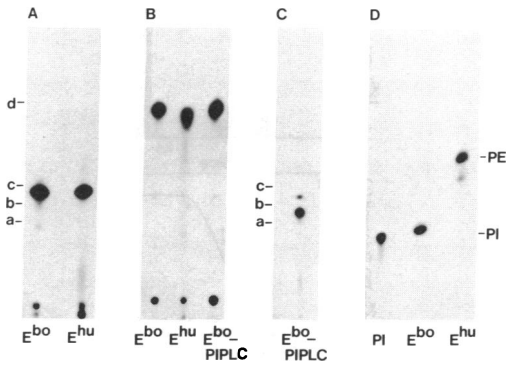

FIG. 3. Silica TLC analysis of [^{125}I]TID-labeled fragments generated from E^{bo} and E^{hu} AcChoEases. Samples were subjected to base hydrolysis (*A*), acid methanolysis (*B*), PL-C_{Ins} digestion (*C*), or nitrous acid deamination (*D*), and material extracted into the organic phase was analyzed by TLC and autoradiography as described in *Methods*. Solvent A was used in *A–C* and solvent B was used in *D*. In *A–C* the standards were 1,2-diolein (a), 1,3-diolein (b), palmitic acid (c), and methyl palmitate (d). In *D*, standards were phosphatidylethanolamine (PE) and PtdIns (PI). The starting material for the cleavage reaction is indicated below each lane: E^{bo} and E^{hu} designate [^{125}I]TID-labeled and purified enzyme samples; E^{bo}-PIPLC indicates the isolated labeled fragment released from E^{bo} AcChoEase by PL-C_{Ins} digestion; and PI (*D*) represents [^{125}I]TID-labeled PtdIns spotted directly on the plate without nitrous acid treatment.

DISCUSSION

Two important points are documented in this paper. (*i*) The photoactivated reagent [^{125}I]TID can be used to label selectively the hydrophobic portion of the glycolipid-anchored AcChoEases in human and bovine erythrocytes, and labeled fragments derived from the anchor can be identified by TLC. (*ii*) Analyses of labeled fragments from E^{hu} AcChoEase indicate that its glycolipid anchor is resistant to PL-C_{Ins} because it contains a structural modification of PtdIns. (A third point of interest, the presence of 1-alkyl-2-acylglycerol in the E^{bo} AcChoEase anchor, will be developed elsewhere.†) The localization of most of the covalently linked [^{125}I]TID to lipid groups in the glycolipid anchor is evidenced by an 85–95% release of radiolabel by PL-C_{Ins} from E^{bo} AcChoEase and a 50–60% release associated with fatty acids by base hydrolysis from both E^{bo} and E^{hu} AcChoEases. Anchor fragments generated following [^{125}I]TID labeling can be analyzed by extraction and TLC techniques similar to those used (7) to investigate trypanosome VSG anchors labeled biosynthetically with ^{3}H-labeled fatty acids. TLC mobilities of [^{125}I]TID-labeled PtdIns and its methanolysis products were similar to those of the corresponding unlabeled stocks, an indication that [^{125}I]TID can be used as a marker of fatty acid-containing components with little or no effect on their chromatographic mobility. Thus exogenous labeling with [^{125}I]TID appears to be an attractive alternative when biosynthetic labeling of anchored proteins with radioactive fatty acids is impractical.

AcChoEases from bovine and human erythrocytes are globular dimeric (G_2) forms that can be purified to apparent homogeneity and appear similar in size by PAGE before and after disulfide reduction. The amounts of the glycolipid anchor components ethanolamine, glucosamine, and *myo*-inositol in these two enzymes were similar. However, differences were found in both the type and the quantity of the fatty acid species that were present. E^{hu} AcChoEase contained 2 mol of fatty acids per mol of monomeric catalytic subunits, with equal amounts of saturated and unsaturated species and a large amount of polyunsaturated fatty acids. E^{bo} AcChoEase, in contrast, contained slightly >1 mol of fatty acids, most of which were saturated, per mol of catalytic subunits. In addition, [^{125}I]TID-labeled E^{hu} AcChoEase was largely resistant to PL-C_{Ins} under conditions that quantitatively released diradylglycerol from [^{125}I]TID-labeled E^{bo} AcChoEase. This finding agrees with earlier results that compared AcChoEase release from human and bovine erythrocytes (13) and also confirms that E^{bo} AcChoEase is anchored in the cell membrane by covalently attached diradylglycerol. Since our experiments were conducted with highly purified E^{bo} and E^{hu} AcChoEases under identical digestion conditions where any influence of the *in situ* membrane environment was eliminated, the differential resistance to

†Diradylglycerol is glycerol with two O-linked acyl, alkyl, or alkenyl substitutions. The TLC conditions of Fig. 3*C* would result in comigration of 1,2-alkylacylglycerols with the 1,3-diolein standard (alkylglycerol standards are not commercially available). A sensitive GLC procedure has confirmed that PL-C_{Ins} does release a 1-alkyl-2-acylglycerol from E^{bo} AcChoEase (W.L.R., J. J. Myher, A. Kuksis, and T.L.R., unpublished data).

PL-C_{Ins} appeared to arise from structural differences in their glycolipid anchors. One factor that could contribute to this differential resistance is the marked dissimilarity of the fatty acid compositions of E^{bo} and E^{hu} AcChoEases. The most abundant fatty acid in E^{hu} AcChoEase is palmitate and in E^{bo} AcChoEase, stearate, whereas VSG fatty acids are exclusively myristate (20). That both E^{bo} AcChoEase and VSGs are susceptible to complete digestion at PL-C_{Ins} concentrations of 2–3 μg/ml despite their dissimilar fatty acids suggests that the type of fatty acids present has little effect on the rate of digestion by PL-C_{Ins}. However, the effect of the large amount of polyunsaturated fatty acids in E^{hu} AcChoEase on susceptibility to PL-C_{Ins} digestion is unknown.

Analysis of [^{125}I]TID-labeled fragments by TLC indicated some similarities between the E^{bo} and E^{hu} AcChoEase membrane anchors. Acid methanolysis, as well as base hydrolysis, released radioactivity associated with fatty acids from both enzymes, and the products released by either procedure comigrate, since this TLC system does not distinguish among different fatty acids. Release from E^{bo} AcChoEase of roughly 50% of the radiolabel after base hydrolysis was significantly less than the >85% release found after PL-C_{Ins} digestion and suggests that species other than fatty acids are labeled. Some of the label may be associated with glycerol due to its close proximity to the hydrophobic environment of the fatty acids and to the high reactivity of the carbene intermediate produced by irradiation of [^{125}I]-TID. However, the identification of the product released from E^{bo} AcChoEase by PL-C_{Ins} as a 1-alkyl-2-acylglycerol† indicates a second reason for only partial ^{125}I release after alkaline hydrolysis or acid methanolysis. Some of the radiolabel is presumably attached to the ether-linked alkyl group, which is resistant to alkaline hydrolysis or acid methanolysis. Mato *et al.* (21) recently reported an apparent alkylacylglycerol in a glycolipid that resembles the PL-C_{Ins}-sensitive glycolipid protein anchors described here. These observations contrast with reports of 1,2-diacylglycerol in the membrane anchor of VSGs (7) and indicate another area of structural diversity in glycolipid anchors.

Major differences between E^{bo} and E^{hu} AcChoEases were seen when fragments generated by nitrous acid deamination were compared. TLC of deamination fragments showed that PtdIns is released from E^{bo} AcChoEase, as expected, whereas a unique fragment with a TLC mobility distinct from that of PtdIns, but containing *myo*-inositol and fatty acids, is released from the human enzyme. These observations clearly indicate a novel but as yet poorly defined modification of the PtdIns structure in E^{hu} AcChoEase. One possibility is that a long-chain acyl or alkyl substituent on the 2-hydroxyl of inositol would prevent formation of a cyclic 1,2-phosphodiester intermediate (7) and confer resistance to digestion by PL-C_{Ins}. Such a substituent would also yield a deamination fragment more hydrophobic than PtdIns with consequent higher TLC mobility. Note that triacylated PtdIns has been isolated from *Corynebacterium xeroxis* (22).

The extent to which PL-C_{Ins}-resistant glycolipid anchors similar to the E^{hu} AcChoEase anchor are present on other membrane proteins is unknown. One other example has been reported, the DAF protein on human erythrocytes (10). Nitrous acid deamination of [^{125}I]TID-labeled DAF releases a predominant fragment with a TLC mobility identical to that observed for the E^{hu} AcChoEase fragment in Fig. 3*D* (23). PL-C_{Ins} releases 10–15% of the DAF from human erythrocytes (10, 11) and somewhat higher percentages of DAF from other cells (11). Furthermore, PL-C_{Ins} releases considerably less than 100% of the total cell surface amount of several other anchored proteins including alkaline phosphatase (24), Thy-1 (25), trehalase (26), and alkaline phosphodiesterase (27). These observations are consistent with the hypothesis that precursor pools for both PL-C_{Ins}-sensitive and PL-C_{Ins}-resistant anchors exist in a number of cells and that the proportion of attached glycolipid anchors arising from these two pools is both tissue- and protein-specific. A glycolipid precursor pool in trypanosomes for VSG anchors has been reported (28), and analysis of similar pools in a variety of cells should permit closer examination of this hypothesis.

The results reported here also raise the question of the functional significance of both sensitivity and resistance to PL-C_{Ins} in glycolipid-anchored proteins. Saltiel *et al.* (29) reported that insulin stimulates an endogenous PL-C_{Ins} to hydrolyze a glycolipid that resembles the PL-C_{Ins}-sensitive glycolipid protein anchors described here. These workers also suggested that the glycoconjugate cleaved by PL-C_{Ins} may act as a second messenger in mediating the actions of insulin. If activation of endogenous PL-C_{Ins} toward glycolipid substrates indeed serves to mediate metabolic regulation, then attachment of either PL-C_{Ins}-sensitive or PL-C_{Ins}-resistant protein anchors may be a cellular response coordinated with PL-C_{Ins} activity.

We thank Todd Marshall for excellent technical assistance and Dr. Martin Low for helpful discussions. This investigation was supported by Grants NS16577 and GM07250 from the National Institutes of Health and by grants from the Muscular Dystrophy Association. W.L.R. is a Medical Scientist Predoctoral Trainee supported by Grant T32 GM07250 from the National Institutes of Health.

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