Phosphatidylethanolamine Is the Donor of the Ethanolamine Residue Linking a Glycosylphosphatidylinositol Anchor to Protein*

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Numerous cell surface glycoproteins from eukaryotic organisms including African trypanosomes and budding yeast (Saccharomyces cerevisiae), are anchored to the lipid bilayer by a glycoprophospholipid, glycosylphosphatidylinositol, covalently linked to the carboxyl terminal of the protein via a phosphoethanolamine bridge. In this paper we describe metabolic labeling experiments aimed at identifying the biosynthetic origin of the ethanolamine residue in the phosphoethanolamine bridge. Using yeast mutants generated by disruption of the ethanolamine phosphotransferase (EPT1) and cholinephosphotransferase (CPT1) genes, we report data consistent with the proposal that the ethanolamine residue is derived from phosphatidylethanolamine.

A wide variety of cell surface glycoproteins are associated with the plasma membrane through a glycosylphosphatidylinositol (GPI) membrane anchor. This complex glycoprophospholipid is covalently linked to the carboxyl-terminal amino acid of the mature protein via a phosphoethanolamine bridge (1-4). GPI-anchored proteins have been found in numerous eukaryotic organisms, including trypanosomes, budding yeast (Saccharomyces cerevisiae) (5-9), and mammals. Current data suggest that GPI anchors are synthesized as precursor glycolipids which are then covalently coupled to target proteins immediately (within 1-5 min) after completion of protein synthesis. Candidate GPI anchor precursors have been identified in African trypanosomes (10-15) and in maninalian cells (16-19), and the transfer of structurally characterized precursors to protein has been demonstrated in vitro (20).

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1 The abbreviations used are: GPI, glycosylphosphatidylinositol; ER, endoplasmic reticulum; GlcN, glucosamine; Man, mannose; P, phosphate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI-PLC, phosphatidylinositol-specific phospholipase C; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Recent biochemical studies using trypanosome membrane preparations indicate that the GPI anchor precursor (ethanolamine-P-Man,GlcN-P) is assembled by sequential addition of the various components (glucosamine, mannose, phosphoethanolamine) to phosphatidylinositol (21, 22). N-Acetylglucosamine is incorporated directly from the sugar nucleotide (23), but mannose first combines with endogenous dolichol phosphate to form dolichol-P-mannose and is then transferred to the GPI anchor core (24) (Fig. 1). Phosphoethanolamine addition in vitro neither requires nor is affected by exogenously added CDP-ethanolamine (21), suggesting that this moiety is derived from an endogenous precursor. A major ethanolamine-containing compound in cells and a potential candidate for this donor is phosphatidylethanolamine (PE). Because the cell-free systems used to study GPI biosynthesis require the presence of cellular membranes, the dependence on this phospholipid has not been tested. Attempts to demonstrate that [3H]ethanolamine is transferred from PE to GPI with metabolic radiolabeling studies have been complicated by the fact that exogenously supplied ethanolamine is first converted to CDP-ethanolamine before being incorporated into PE. Although both GPI and PE are radiolabeled under these conditions, the presence of CDP-ethanolamine makes it difficult to rule out this compound as the ethanolamine donor. To circumvent this problem, we have performed metabolic labeling experiments using yeast mutants unable to synthesize PE from ethanolamine via CDP-ethanolamine because of disruptions in the ethanolamine phosphotransferase (EPT1) and cholinephosphotransferase (CPT1) genes (25-28) (Fig. 1). The mutant cells grow normally and have a normal phospholipid composition because of the redundancy in the pathways for the biosynthesis of phosphatidylethanolamine and phosphatidylcholine (PE can be synthesized by carboxylation of phosphatidylserine, and phosphatidylcholine can be synthesized by methylation of phosphatidylethanolamine) (29, 30).

MATERIALS AND METHODS

Yeast Strains—Yeast strains DBY746 (a his3-AI leu2-3 leu2-112 ura3-52 trpl-289) (referred to as wild-type, wt) and its derivatives HJ651 (ept1-Al::URA3) (referred to as eptI) and HJ000 (ept1-Al::URA3 epl::LEU2) (referred to as ept1, eptI) were obtained from Dr. Russell Hjelmstad (Duke University Medical Center). The strains are described in greater detail in Ref. 25-28.

Yeast Synthetic Medium—Complete synthetic medium for growth and for [3H]ethanolamine labeling of yeast was as follows: Yeast Nitrogen Base (Difco Laboratories, 1.2 g/liter), sodium succinate (10 g/liter succinic acid, 6 g/liter NaOH), ammonium sulfate (5 g/liter), glucose (20 g/liter), amino acids (Lys, Tyr, Ile (0.3 g/liter each), Asp, Thr (2 g/liter), Val (1.5 g/liter), His (0.02 g/liter), Leu (0.12 g/liter), Thr (2 g/liter), Val (1.5 g/liter), His (0.02 g/liter), Leu (0.12 g/liter), Trp (0.08 g/liter), Arg (0.04 g/liter), uracil (0.02 g/liter), adenine (hemisulfate salt, 0.05 g/liter). Vitamin-free synthetic medium (Difco Laboratories, 1.7 g/liter) was used and ammonium sulfate (included in the Vitamin-free Nitrogen Base) was omitted.

Metabolic Labeling—Single yeast colonies were transferred to synthetic medium and incubated overnight (with shaking) in a water bath at 30 °C. Growth was monitored by measurement of absorbance at 600 nm using disposable polystyrene cuvettes (1-cm path) and a CARY 219 spectrophotometer. Aliquots of dense suspensions were

* S. Morash, personal communication.
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FIG. 1. Outline of the GPI biosynthetic pathway. The GPI anchor precursor (ethanolamine-P-Man,GlCN-PI) is assembled by sequential addition of the various components (glucosamine, mannose, phosphatidylcholine) to phosphatidylinositol. The glucosamine residue is derived from UDP-GlcNAc, and the three mannose residues are derived from dolichol-phosphomannose. The data presented in this paper are consistent with the proposal that the ethanolamine residue is derived from phosphatidylethanolamine (PE). The reaction catalyzed by the EPT1 gene product is indicated (the evidence to support this idea is shown in the legend to Fig. 2 below). GDP-Man is converted to dol-P-Man via CDP-Mann (CDP-ethanolamine) (Fig. 2). CDP-ethanolamine is synthesized via the CDP-ethanolamine pathway (Fig. 1). CDP-ethanolamine is transferred from CDP-ethanolamine to form PE.

Phosphatidylethanolamine is transferred from CDP-ethanolamine to form PE.

RESULTS

The major water-soluble metabolites produced by yeast biosynthetically labeled with [3H]ethanolamine were ethanolamine phosphate and CDP-ethanolamine (Fig. 2A, wt). These compounds were also efficiently synthesized by yeast mutants with single (ept1) or double (ept1, cpi1) gene disruptions (Fig. 2A). The major phospholipids radiolabeled in wild-type yeast co-migrated with standards for phosphatidylethanolamine (PE) and phosphatidylcholine (PC) when analyzed by thin layer chromatography (Fig. 2B, wt). The ept1 null mutant produced reduced levels of these two phospholipids (Fig. 2B, ept1). Despite the disruption of the EPT1 gene, phosphoethanolamine is transferred from CDP-ethanolamine to form PE.

FIG. 2. Analyses of lipids and water-soluble metabolites from yeast strains metabolically labeled with [3H]ethanolamine. Yeast strains DBY746 (a his3Δ1 leu2-3,112 ura3-52 trpl-289) (referred to as wild-type, wt) and its derivatives JH051 (ept1Δ1::URA3) (referred to as ept1) and JH000 (ept1Δ1::URA3 cpi1Δ1::LEU2) (referred to as ept1, cpi1) were metabolically labeled for 2 h at 30 °C, and lipids were extracted as described under "Materials and Methods." Identical aliquots of each upper phase were applied to silica 60 thin layer plates and chromatographed using solvent system B (panel A). Different aliquots of each lower phase (wt, 7.5 μl; ept1, 30 μl; cpi1, 60 μl, out of a total of 300 μl in each case) were chromatographed on silica 60 using solvent system A (panel B). Radiolabeled standards were analyzed alongside; radioactivity was detected by spraying the plates with EN'HANCE (Du Pont-New England Nuclear) and exposing them to Kodak XAR-5 film at −70 °C.
in these cells because the reaction is catalyzed, albeit inefficiently, by the CPT1 gene product in vivo (26). A double null mutant (ept1, ept1) showed enormously reduced activity (~1%) compared with wild-type (Fig. 2B, middle lane), and did not synthesize radiolabeled PE from [3H]ethanolamine (the faint residual labeling probably represents the contribution of the base-exchange pathway to PE biosynthesis (33), although such an activity has not been previously identified in yeast).

Metabolic labeling with [3H]inositol showed that GPI-anchor biosynthesis was unimpaired in the double mutant (Fig. 3). Both wild-type and mutant showed identical patterns of labeled proteins (analyzed after detergent phase separation using Triton X-114; Fig. 3, lanes 2 and 5), consisting of a characteristic smear of high molecular weight material as well as a ladder of lower molecular weight bands. Treatment with phosphatidylinositol-specific phospholipase C (PI-PLC) reduced the intensities of all the labeled bands in the detergent phases (Fig. 3, compare lanes 3 and 7 with lanes 2 and 5), and some of these were recovered in the aqueous phases (Fig. 3, lanes 4 and 8). The most prominent of these was a band at ~125 kDa that probably corresponds to the GPI-anchored GAS1 gene product (5, 7, 34); a fainter band at ~110 kDa could also be visualized (Fig. 3, lanes 4 and 8; see also Fig. 4, lanes 2 and 4). The inefficient recovery of the other cleaved proteins has been noted previously (5, 7, 32) but is yet to be explained.

If the ethanolamine bridge in the GPI anchor of proteins is derived from PE, then yeast cells deficient in the EPT1 and CPT1 gene products will not be able to incorporate [3H]ethanolamine into GPI. Wild-type and mutant yeast strains were grown in [3H]ethanolamine and labeled proteins were extracted, Triton X-114-selected, treated with or without PI-PLC, and analyzed by SDS-PAGE and fluorography. Fig. 4 (lanes 5–8) shows an autoradiogram of an SDS-PAGE analysis of aqueous phases obtained from such an experiment. The ~125- and ~110-kDa bands released by PI-PLC treatment are clearly visible in the [3H]ethanolamine-labeled wt sample (Fig. 4, lane 6) and correspond exactly to the bands obtained in [3H]inositol-labeling experiments (Fig. 4, lanes 2 and 4; also Fig. 3, lanes 4 and 8). No [3H]ethanolamine-labeling was seen in the double mutant (Fig. 4, lane 8), although the ~125- and ~110-kDa bands could be detected via Coomassie staining in the same gel lane. The observation that [3H]ethanolamine incorporation into GPI-anchored proteins is abolished in a mutant strain unable to synthesize labeled PE from [3H]ethanolamine indicates that PE is an obligatory intermediate in the transfer of [3H]ethanolamine to GPI.

**DISCUSSION**

A set of yeast proteins, including a major 125-kDa membrane glycoprotein, that can be metabolically labeled with [3H]ethanolamine, [3H]palmitic acid, and [3H]inositol have been shown to be GPI-anchored by a number of criteria including susceptibility to hydrolysis by phosphatidylinositol-specific phospholipase C and the presence of the cross-reacting determinant found in other GPI anchors (this paper, and Refs. 5–9). That these proteins are not labeled by [3H]ethanolamine in mutant yeast unable to transfer this compound from CDP-ethanolamine to PE argues strongly that the phospholipid is the immediate donor of the ethanolamine residue linking a GPI anchor to protein, rather than CDP-ethanolamine.

Additional evidence that PE is the donor of the ethanolamine bridge in GPI can be obtained by taking advantage of the redundancy in the biosynthetic pathways for this phospholipid. Because PE can be produced by decarboxylation of phosphatidylserine, growth of cells in [3H]serine should result in the metabolic labeling of PS, PE, and, if derived directly from PE, GPI. Unfortunately, such an experiment is not technically possible in yeast. To date, ethanolamine-containing GPI precursors generated either in vivo or in vitro from yeast have not been identified and characterized. Therefore, protein-associated GPI would have to be analyzed for radio-labeled ethanolamine derived from [3H]serine. To do this, a GPI-modified peptide free of serine must first be isolated. Then, the GPI anchor must be acid-hydrolyzed and the components analyzed to verify that the radiolabel is in ethanolamine. Although we have attempted this experiment with yeast, we have been unable to isolate sufficient amounts of radioactive material to complete the analyses. This is probably because the efficiency of uptake and utilization of exogenously supplied compounds by yeast is extremely low. Such

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3. A. K. Menon, unpublished data.
4 A. K. Menon, M. Eppinger, S. Mayor, and R. T. Schwarz, manuscript in preparation.
5 T. Kamitani, A. K. Menon, Y. Hallaq, C. D. Warren, and E. T. H. Yeh, manuscript in preparation.

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