Glycosyl phosphatidylinositols (GPIs), by contrast, are best known as anchors of membrane proteins, and free GPIs serve as intermediates along the path of GPI-anchor biosynthesis. By using in vivo cell surface biotinylation, we show that free GPIs: 1) can exit the rough endoplasmic reticulum and are present on the surface of a murine EL-4 T-lymphoma and a human carcinoma cell (HeLa), 2) arrive at the cell surface in a time and temperature-dependent fashion, and 3) are built on a base-labile glycerol backbone, unlike GPI anchors of surface proteins of the same cells. The free GPIs described in this study may serve as a source of hormone-sensitive phosphoinositol glycans. The absence of free GPIs from the cell surface may also account for the growth advantage of blood cells in paroxysmal nocturnal hemoglobinuria.

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Cell surface proteins and glycolipids mediate multiple essential interactions between cells and their surroundings. The diverse glycolipids that include ceramide are implicated as receptors for toxins, viruses, bacteria, and hormones, as differentiation and tumor antigens, and in cell-cell contact (1–4). Glycolipids, including those that have been attributed to ceramide-containing glycolipids and GPI-anchored proteins, might also serve as sources of hormone-sensitive mediators such as the insulin and interleukin-2-sensitive phosphoinositols.

MATERIALS AND METHODS

Cell Culture Conditions and Materials—The mouse lymphoma cell line EL-4.G.1.4 (Thy-1) was provided by Dr. R. Hyman (Salk Institute). Human cervical carcinoma cell line HeLa was obtained from ATCC (HeLa S3, catalog CCL 2.2). The cultures were maintained at 37 °C, 5% CO2 in Dulbecco’s modified Eagle’s medium (0.45% glucose) supplemented with 10% fetal bovine serum, penicillin, and streptomycin (Life Technologies, Inc.). 2-D-[3H]Mannose (specific activity 15 Ci/mmol) was from ARC (St. Louis, MO, catalog ART 120A). Bovine serum phosphatidylinositol-specific phospholipase D (GPI-PLD) was from K.-S. Huang (Hoffman La Roche, NJ). N. Singh, personal observations.

Glycolipid labeling, extraction, and analysis of [3H]Mannopols—Metabolic labeling of cells with [3H]Mannose was essentially as described (40). In a typical experiment, 1-h pulse-labeled cells (107) were washed with high glucose DMEM at 4 °C and divided into two parts. End of pulse samples were washed with cold PBS, biotinylated (see below), and extracted with 2 ml of chloroform/methanol/water (10:3:1, v/v) for 30 min at room temperature. Chase samples were reincubated in serum containing Dulbecco’s modified Eagle’s medium lacking tunicamycin (1 × 106 cells/ml) before surface biotinylation and extraction of the mannolipids. Extracts were clarified, dried, and phase-partitioned twice with water prior to analysis of butanol phase on a TLC plate (40). PI-PLD treatment on purified mannolipids was as described (40). Cell Surface Biotinylation—For cell surface biotinylation (42), NHS-SS-biotin (Pierce) was used at 3 mg/ml in ice-cold PBS at pH 8.0. In a typical experiment, 3–4 × 106 [3H]Mannose-labeled cells were washed four times with ice-cold PBS, pH 7.4, and (for HeLa) lifted off the culture dishes with warm EDTA. After one more wash with PBS at pH

**FREE GLYCOSYL PHOSPHATIDYLINOSITOLS**

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A Novel Class of Cell Surface Glycolipids of Mammalian Cells

Glycosyl phosphatidylinositol (GPI) lipids function as anchors of membrane proteins, and free GPI units serve as intermediates along the path of GPI-anchor biosynthesis. By using in vivo cell surface biotinylation, we show that free GPIs: 1) can exit the rough endoplasmic reticulum and are present on the surface of a murine EL-4 T-lymphoma and a human carcinoma cell (HeLa), 2) arrive at the cell surface in a time and temperature-dependent fashion, and 3) are built on a base-labile glycerol backbone, unlike GPI anchors of surface proteins of the same cells. The free GPIs described in this study may serve as a source of hormone-sensitive phosphoinositol glycans. The absence of free GPIs from the cell surface may also account for the growth advantage of blood cells in paroxysmal nocturnal hemoglobinuria.

Cell surface proteins and glycolipids mediate multiple essential interactions between cells and their surroundings. The diverse glycolipids that include ceramide are implicated as receptors for toxins, viruses, bacteria, and hormones, as differentiation and tumor antigens, and in cell-cell contact (1–4). Glycosyl phosphatidylinositol (GPI) lipids function as anchors of membrane proteins, and free GPI units serve as intermediates along the path of GPI-anchor biosynthesis. By using in vivo cell surface biotinylation, we show that free GPIs: 1) can exit the rough endoplasmic reticulum and are present on the surface of a murine EL-4 T-lymphoma and a human carcinoma cell (HeLa), 2) arrive at the cell surface in a time and temperature-dependent fashion, and 3) are built on a base-labile glycerol backbone, unlike GPI anchors of surface proteins of the same cells. The free GPIs described in this study may serve as a source of hormone-sensitive phosphoinositol glycans. The absence of free GPIs from the cell surface may also account for the growth advantage of blood cells in paroxysmal nocturnal hemoglobinuria.

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periments show that the biotinylation reagent does not penetrate the plasma membrane of intact cells under the conditions used (see below), these reagents should derivatize only molecules with a free amino group on the outer leaflet of the plasma membrane, such as the ethanolamine of GPIs (42). After biotinylation, polar lipids were extracted and fractionated by thin layer chromatography (TLC) under conditions in which the species running most slowly are known GPIs (9, 43).

When EL-4 cells are pulse-labeled with \(^{3}H\)mannose and surface biotinylated for 1 h, the disappearance of H6–H8 is more extensive, and greater quantities of the same products appear in both end of pulse and chase samples (Fig. 2A, lanes 3 and 6). Control experiments show that the total counts/min in peak b exceed the reduction in labeled H6–H8 due to differential loss of non-biotinylated H8 (panel C versus panel B). To further confirm that the biotinylation reagent does not penetrate the plasma membrane, HeLa cells were either biotinylated, washed, methanol-fixed and permeabilized with Triton X-100, or methanol-fixed and permeabilized prior to biotinylaton on ice. Both samples were stained with fluorescein-conjugated streptavidin, washed, and examined. Only the cell surface is stained unless the plasma membrane is permeabilized prior to biotinylaton (data not shown). By contrast, when surface-biotinylated cells are incubated with avidin before extraction (panel C), the biotinylated product b is eliminated, with apparently no effect on non-biotinylated H8 (panel C versus panel B). To further confirm that the biotinylation reagent does not penetrate the plasma membrane, HeLa cells were either biotinylated, washed, methanol-fixed and permeabilized with Triton X-100, or methanol-fixed and permeabilized prior to biotinylaton on ice. Both samples were stained with fluorescein-conjugated streptavidin, washed, and examined. Only the cell surface is stained unless the plasma membrane is permeabilized prior to biotinylaton (data not shown).

To confirm the identity of \(^{3}H\)mannose-labeled lipids of EL-4 cells, the lipids were treated with GPI-PLD (see Fig. 1). As shown in Fig. 3A (panel A versus B), GPI species H5–H8 and some more rapidly moving early intermediates in the GPI biosynthetic pathway "i" are cleaved by the enzyme treatment. Lipid species "r" and dolichol phosphoryl mannose (DPM) are insensitive to cleavage by GPI-PLD (panels A and B). Partial partitioning of cleaved GPI-lipids into the butanol phase (panel B, peak *) is expected because of the remaining inositol-linked acyl chain (see Fig. 1, A and B).

To demonstrate directly that the change in mobility of man-
controls were suspended in PBS (pulse (0 h) and chase samples (9 h) were divided into three parts. Were pulsed with [3H]mannose for 1 h and chased for 0 and 9 h. End of more rapidly migrating (less mature) mannolipids lack ethanolamine chromatographed.

lanes 0.1% TritonX-100 (and were biotinylated on ice in the absence (lanes 1 and 3) bind avidin and are retained by the column, causing them to be absent from lanes 2 and 4 (+ avidin). Additionally, the surface-biotinylated mannolipids are sensitive to nitrous acid deamination (Fig. 3C, panel A versus panel B), consistent with their being derived from GPI lipids and retaining glucosamine with a free amino group (44).

Free GPIs have various proportions of diacylglycerol versus base-resistant alkyl-acyl species (30, 40), whereas characterized mammalian GPI anchors of HeLa and other cells have a 1-O-alkyl, 2-Oacylceramide backbone (40, 45, 46). A lipid remodeling reaction, either during or soon after transfer to protein, has therefore been suggested to account for the base resistance of protein anchors (40). Alternatively, the transamidase responsible for anchor addition may accept only alkyl, acyl substrates, despite the presence of a pool of diacyl GPIs. GPI lipid remodeling also occurs in Trypanosoma brucei (48, 49). To learn whether cell surface free GPIs are base-sensitive, [3H]mannose pulse-labeled HeLa cells were chased for 20 h and surface-biotinylated. Control and biotinylated lipids were extracted and chromatographed. A prominent peak b appears after surface biotinylation (Fig. 3D, panel BIO), with a decrease in the amount of H8 (panel CONT versus panel BIO). To check for base sensitivity, biotinylated lipid extracts were treated with alkylamine monomethylamine (+MMA) or received only buffer (panel BIO) (40). Lipid extracts from each sample were subsequently partitioned with butanol/water and both phases were analyzed. For simplicity, only the butanol phases are illustrated. As shown in Fig. 3D, HeLa cell surface-biotinylated lipid species b are fully sensitive to mild base hydrolysis, indicating a diacylglycerophosphoinositol structure for the cell surface free GPIs (+MMA versus BIO). Similar results were obtained for EL-4 cells (data not shown).

Thus, the glycerol backbone of free GPIs on the cell surface is distinct from GPI-protein anchors of the same cells and does not undergo the lipid-remodeling reaction characteristic of protein-linked anchors. Incidentally, pulse-labeled GPIs include a significant proportion (15–20%) of base-resistant species (data not shown).

At 15–20 °C, the vesicular transport of proteins and glyco-
FIG. 3. A, polar mannolipids H5-H8 are sensitive to cleavage by GPI-PLD. Polar lipids from HeLa cells labeled with [3H]mannose for 1 h were treated with GPI-PLD, re-extracted with butanol, and chromatographed. Note that polar lipids H5-H8 and some immature GPIs (i) are sensitive to cleavage by GPI-PLD. As expected, enzyme-treated lipids still partition partially into the butanol phase because of the remaining inositol-linked acyl chain (panel B, peak *). The identity of the resistant species (r) is not known. DPM, dolichol phosphoryl mannose; O, origin; F, solvent front. B, biotinylated GPIs bind to avidin-agarose. [3H]Mannose-labeled HeLa cells (lanes 1 and 2) and EL-4 cells (lanes 3 and 4) were chased for 20 h and surface-biotinylated. Free biotin was quenched followed by extensive washing with cold PBS. The cells were then lysed, and half of each lysate was passed over an avidin-agarose column. The samples that were not chromatographed (lanes 1 and 3), and flow-through fractions (Av, lanes 2 and 4), were extracted with chloroform/methanol/water (10/10/3; v/v/v) and analyzed by TLC. Note that the majority of biotinylated species b (present in lanes 1 and 3) bind to avidin and are absent in lanes 2 and 4 in both HeLa and EL-4 cell extracts. The heavily labeled bands above band b are non-GPI mannolipids. Their identity is under investigation. Dolichol phosphoryl mannose is absent due to the prolonged chase. Origin is at the bottom of the chromatograph. C, biotinylated GPIs are sensitive to nitrous acid deamination. HeLa cells were labeled with [3H]mannose for
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1 h and biotinylated in the presence of detergent, following which the lipids were extracted and treated with nitrous acid. After re-extraction with butanol, the butanol phases were chromatographed. The biotinylated products (panel A) are extensively sensitive to nitrous acid (panel B). O, origin; F, solvent front. D, surface-biotinylated GPIs are base-sensitive. [3H]Mannose-labeled HeLa cells were chase incubated for 20 h and set aside (CONT) or surface-biotinylated (BIO). Labeled mannosides were extracted from both samples and chromatographed. The predominant GPI species after chase is H8 (CONT). Following surface biotinylation, prominent species b appear (BIO), which are absent in non-biotinylated control cells (CONT). When the surface-biotinylated sample is treated with alkaline monomethyamine, phase partitioned with butanol/water, and then analyzed by TLC, all of the labeled species (including b) are eliminated (panel +MMA). Species s is a non-GPI, base-sensitive mannoside whose identity is not known. Less mature GPI species H5–H7 are absent due to the prolonged chase. O, origin.

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