Segregation of Glycosylphosphatidylinositol Biosynthetic Reactions in a Subcompartment of the Endoplasmic Reticulum*

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Glycosylphosphatidylinositols (GPIs) are synthesized in the endoplasmic reticulum (ER) via the sequential addition of monosaccharides, fatty acid, and phosphoethanolamine(s) to phosphatidylinositol (PI). While attempting to establish a mammalian cell-free system for GPI biosynthesis, we found that the assembly of mannosylated GPI species was impaired when purified ER preparations were substituted for unfractonated cell lysates as the enzyme source. To explore this problem we analyzed the distribution of the various GPI biosynthetic reactions in subcellular fractions prepared from homogenates of mammalian cells. The results indicate the following: (i) the initial reaction of GPI assembly, i.e. the transfer of GlcNAc to PI to form GlcNAc-PI, is uniformly distributed in the ER; (ii) the second step of the pathway, i.e. de-N-acetylation of GlcNAc-PI to yield GlcN-PI, is largely confined to a subcompartment of the ER that appears to be associated with mitochondria; (iii) the mitochondria-associated ER subcompartment is enriched in enzymatic activities involved in the conversion of GlcN-PI to H5 (a singly mannosylated GPI structure containing one phosphoethanolamine side chain; and (iv) the mitochondria-associated ER subcompartment, unlike bulk ER, is capable of the de novo synthesis of H5 from UDP-GlcNAc and PI. The confinement of these GPI biosynthetic reactions to a domain of the ER provides another example of the compositional and functional heterogeneity of the ER. The implications of these findings for GPI assembly are discussed.

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† The abbreviations used are: GPI, glycosylphosphatidylinositol; BiP, binding protein (glucose-regulated protein 78, GRP78); ER, endoplasmic reticulum; EtN, ethanolamine; GPIMT-II, the second mannosyltransferase of the GPI biosynthetic pathway; Mito, mitochondria; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; CHO, Chinese hamster ovary; PAG, polycrylamide gel electrophoresis; DTT, dithiothreitol; TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanone; PLAP, placental alkaline phosphatase; H, homogenate; dol-P-Man, dolichol-P-mannoolose; Hsp, heat shock protein; GlcN-PI(dIC16), d-GlcNA1-6β-myo-inositol-1-P-(sn-1,2-dipalmitoylglycerol).
from “bulk” ER; these subfractions may sediment differently from conventionally isolated ER during the fractionation procedure, resulting in an apparent loss of activity. Growing evidence of the lateral compositional heterogeneity of the ER (10) and the recent isolation of functionally specialized subregions of the ER (27, 28) lend credence to the latter possibility. In order to explore these issues we undertook an analysis of the distribution of the various GPI biosynthetic reactions in subcellular fractions prepared from homogenates of mammalian cells. The results, described in this paper, indicate that post-initiation GPI biosynthetic reactions are most active in a subcompartment of the ER. This “GPI-active” subcompartment of the ER co-fractionates with mitochondria and probably corresponds to a previously described ER fraction shown to be active in phospholipid synthesis (27), and possibly also involved in phospholipid trafficking between the ER and mitochondria (29).

**EXPERIMENTAL PROCEDURES**

**Materials**—GDP-[3H]mannose (20 Ci/mmol) and translation grade (3S)methionine were purchased from NEN Life Science Products, and UDP-[3H]mannose (20 Ci/mmol) and [3H]uridine 5’-triphosphate (10 Ci/mmol) from American Radiolabeled Chemicals (St. Louis, MO). Protease inhibitor mixture and tunicamycin were obtained from Calbiochem. TlCl, leupeptin, and *Staphylococcus aureus* nuclease were from Roche Molecular Biochemicals. Rabbit reticulocyte lysate was obtained from Dr. Michael Ferguson (University of Dundee, Dundee, Scotland). The Thy-1 antibodies were purchased from Affinity Bioreagents, Inc. (Golden, CO). Horseradish peroxidase-conjugated anti-rabbit IgG was from Santa Cruz Biotechnology (Santa Cruz, CA). The ECL Western blotting detection system for chemiluminescent detection of Western blots was obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). Glass-backed silica 60 thin layer plates were from Merck (Darmstadt, Germany). GlcN1(4C)Ac-F-PldI(C16) and GlcN-F-PldI(C16) were gifts from Dr. Michael Ferguson (University of Dundee, Scotland).

**Cell Culture**—The Thy-1 mouse thymoma cell line BW5147.3 was maintained in suspension culture in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in an atmosphere of 5% CO2 at 37 °C. The CHO-K1 cell line G9PLAP (stably expressing human placental alkaline phosphatase (PLAP)) and its derivative G9PLAP-85 (a mutant deficient in the second step of GPI anchor assembly) was provided by Dr. Victoria Stevens (Emory University School of Medicine, Atlanta, GA) and described previously (30). CHO-K1 cells were cultured in 5-dm dishes in Ham’s F-12 medium supplemented with 10% fetal calf serum.

**Preparation of Subcellular Fractions**—Subcellular fractions were prepared from CHO cells or thymoma cells (see Fig. 2 for an outline of the fractionation procedure). For typical fractionation experiments, CHO-K1 G9PLAP or G9PLAP-85 cells were scraped from 30 dishes into 5 ml/dish of phosphate-buffered saline. BW5147.3 thymoma cells (1×10^7) were collected by centrifugation (1,000 × g for 5 min). The cells were washed twice with phosphate-buffered saline and resuspended in 10 ml of buffer A (25 mM sucrose, 10 mM Hepes/NaOH, pH 7.5, 1 mM EDTA) supplemented with protease inhibitor mixture. Cells were transferrred to a 15-ml nitrogen cavitation bomb (Kontes Glass Co., Vineland, NJ) and held at 400 pounds/square inch for 30 min. The cavitated cells were treated with S. aureus nuclease (30 units/ml) in the presence of 1 mM CaCl2 for 20 min on ice. The treatment was stopped by adding 1 mM EGTA. Cells were further disrupted by three passages through a 21-gauge needle. The lysate was clarified by centrifugation at 1,000 × g for 10 min at 4 °C to remove large debris and nuclei. The resulting supernatant was centrifuged at 10,000 × g for 10 min at 4 °C. The pellet (P10) was washed twice with buffer A and was resuspended in buffer A for enzyme activities measurement or in isolation buffer (250 mM mannitol, 25 mM Hepes/NaOH, pH 7.5, 0.5 mM EGTA) for further purification at a protein concentration of 10–15 mg/ml. The supernatant from the 10,000 × g spin (S10) was centrifuged at 100,000 × g for 1 h (Ti 50.2 rotor [Beckman Instruments Inc., Fullerton, CA]). The crude microsome pellet (P100) so obtained was resuspended in 2 ml of buffer A at a concentration of 5–10 mg/ml protein. In some experiments the supernatant was layered on top of a discontinuous sucrose gradient (2 ml of 30% and 4 ml of 38% sucrose prepared in 10 mM Hepes/NaOH, pH 7.5) and fractionated by centrifugation at 100,000 × g for 2 h. The pellet and the supernatant were collected from each of the fractions and then collected at each interface as previously reported (31). The ER proteins ribophorin I, protein disulfide isomerase, and BiP (heavy chain binding protein (GRP78), and the mitochondrial proteins Hsp60 and Hsp70 were assayed using previously described procedures. NADPH-cytochrome c reductase activity (ER marker) was assayed as described previously (29) using 10–30 μg of protein for P100 fraction and 30–100 μg of protein for the other fractions. The determination of succinate dehydrogenase (mitochondrial marker) activity was carried out as previously reported (31). The ER markers ribophorin I, protein disulfide isomerase, and BiP were also assayed using standard Western blotting procedures. After probing with primary antibodies (chicken anti-protein disulfide isomerase or rabbit anti-BiP, anti-Hsp60, and anti-Hsp70), blots were developed using peroxidase-conjugated second antibodies and the ECL Western blotting detection system (Amersham). Band intensities were determined by densitometry (at least two different amounts of protein (typically 1 and 2.5 μg) were analyzed and several different film exposures were obtained to verify linearity of the film response for densitometric analysis).

**Summary of Characteristics of Subcellular Fractions**—As shown in Table I and Fig. 2, mitochondrial markers (Hsp60 and succinate dehydrogenase) were recovered mainly in the P10 fraction. However, the ER markers NADPH cytochrome c reductase, ribophorin I, BiP, and protein disulfide isomerase were present in both the P10 and P100 fractions (Table I; data for protein disulfide isomerase are not shown), indicating that the P100 fraction contains a substantial amount of ER. Ribophorin I, BiP, and protein disulfide isomerase were only slightly (1.1–1.5-fold) enriched in P100 versus P10, whereas NADPH cytochrome c reductase activity (specific activity as well as total) was about 4 times higher in the P100 fraction than in the P10 fraction. A similar trend was also seen in ER fractions obtained from the P10 and P100 (see below). The results point to compositional heterogeneities within the ER, consistent with a variety of other reports (Ref. 10; see Ref. 27 for specific data on NADPH cytochrome c reductase activity). 

Cell division was monitored using conventional light microscopy. Approximately 45% of the total ER marker activities measured in the P10 fraction were recovered in MAM (Table I). The MAM, and Mitochondria fractions contained 20% and 7–8%, respectively (figure represents averages of the data obtained with the markers listed in Table I), of the total ER marker activities measured in the P10 fraction. Taking into account that recovery of the ER markers was ~70% after the fractionation procedure, this result indicates that the majority of the ER membranes...
present in P10 are recovered in the MAM fractions (MAM<sub>M</sub> + MAM<sub>P</sub>), specifically MAM<sub>P</sub>. The mitochondrial content of each of the three fractions was determined by measuring levels of succinate dehydrogenase, Hsp60, and Hsp70. Succinate dehydrogenase was recovered mainly in the MAM<sub>M</sub> and Mito fractions (−20% and −26% of the total P10 level, respectively) of a significant amount (~22% of the total P10 amount) was also recovered in MAM<sub>P</sub>. Broadly similar results were obtained with the Hsp70 and Hsp60 markers. The recovery of these proteins in Mito and MAM<sub>M</sub> was 39 and 20% of the amount in P10, respectively, with only 13% being recovered in MAM<sub>P</sub>. It is possible that anol, 1M ammonium hydroxide 10:10:3, v/v/v, as the solvent) and chromatography (silica gel 60 thin layer plates, using chloroform/meth-

The lipid containing chloroform-rich lower phase was washed several times with 0.5 ml of mock upper phase, dried, and dissolved in water-

and 1.5 ml of ice-cold chloroform/methanol (1:2, v/v). A two-phase mix-

was induced by adding 0.5 ml of chloroform and 0.5 ml of water. The lipid containing chloroform-rich lower phase was washed several times with 0.5 ml of mock upper phase, dried, and dissolved in water-

saturated n-butyl alcohol. The products were analyzed by thin layer chromatography (silica gel 60 thin layer plates, using chloroform/meth-

ol, 1 ammonium hydroxide 10:10:3, v/v/v, as the solvent) and detected with a TLC radioc密度ometer (Berthold Analytical Instruments, Inc., Nashua, NH). Incorporation of radioactivity into the individual species was determined using the integration software supplied with the scanner.

De-N-acetylase Assay Using Synthetic GlcN[<sup>14</sup>C]Ac-Pi Substrate—

Each subcellular fraction to be assayed was adjusted to contain 150 µg of protein suspended in 250 mM sucrose, 50 mM Hepes, pH 7.5, 10 mM EDTA in a reaction volume of 100 µl. All measurements were carried out in duplicate. The de-N-acetylation reaction was started by the addition of 1 µl of a 0.1% Nonidet P-40 solution of GlcN[<sup>14</sup>C]Ac-Pi (dCiC6) (containing 2500 cpn, ~80 pmol), and samples were incubated at 37 °C for 4 h. The reactions were terminated by removing 100 µl of the incubation mixture at the appropriate time points and adding it to 50 µl of 100% propan-1ol, vortexing, and snap-freezing. The samples were thawed, diluted with 100 µl of 1 M ammonium acetate and 750 µl of water (resulting in approximately 1 ml of 5% propan-1ol, 100 mM ammonium acetate per reaction), and passed through a pre-equili-

brated 100-ml Sep-Pak cartridge (Waters Corp., Milford, MA). The cartridge was then washed with a further 1 ml of 5% propan-1ol, 100 mM ammonium acetate. The combined washes (~2 ml, containing re-

lease of [14]Acetate) were taken for liquid scintillation counting. Control incubations carried out with boiled membrane preparations were proc-

essentially as described previously (10, 33, 34). Briefly, isolated fractions (typically 50 µg of protein) were incubated with UDP-[<sup>3</sup>H]GlcNAc (1 µCi) in buffer A in the presence of 5 mM EDTA, in a total volume of 50 µl at 37 °C. In some experiments 1 µl/mg PI (dissolved in 0.1% Nonidet P-40 (the final concentration of Nonidet P-40 in the reaction mixture was 0.01%)) was also added. The addition of exogenous PI equally stimulated the formation of GlcNAc-PI (about 1.5–2-fold) in all examined fractions, but no difference in the ratios of products was

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eliminated identically and used as assay background. Release of [14C]acetate (incubations carried out with boiled membrane preparations were proc-

essed at 37 °C for 4 h. The reactions were terminated by removing 100 µl of the incubation buffer (1 mM Tris/NaOH, pH 7.5, 1 mM EDTA in a reaction volume of 100 µl), and samples were incubated for 90 min at 37 °C. Reactions were stopped by chloro-

form/methanol/water extraction as described above, and samples were desalted and processed for thin layer chromatography. TLC analysis was on silica 60 HPTLC plates using chloroform/methanol/ammonia, 1 x NH(OAc)/water (180:140:9:2:3, by volume). Chromatograms were visualized by fluorography after spraying with En'Hance (NEN Life Science Products) and quantitated using a TLC scanner.

Translation-Translocation Assays—Preparomini-PLAP mRNA was prepared from plasmid pGem-4mimiPLAPos (Ser (35, 36) by HindIII linearization and SP6 RNA polymerase-mediated transcription. In vitro translation-translocation reactions were performed as described previously (35, 37). To initiate the translocation reaction, 1 µl (5 µg) of membranes was added to 11 µl of translation mixture. The reaction was allowed to proceed for 60 min at 25 °C, after which the products were either directly analyzed by SDS-PAGE (12.5% gels) and fluorography or first subjected to protein-

ase K treatment prior to SDS-PAGE. For proteinase K treatment, the translation-translocation reaction samples were placed on ice, diluted to 50 µl with 150 mM Koa, 50 mM Hepes/NaOH, pH 7.5, 2.5 mM Mg(OAc)<sub>2</sub>, 1 mM DTT, and proteinase K was added to a final concentration of 100 µg/ml. After 30 min incubation on ice, proteolysis was stopped by adding 500 µl of buffer A supplemented with 2 mM phenyl-

methylsulfonyl fluoride (prepared freshly from a 150 mM stock in eth-

anol). The membranes were collected by centrifugation at 100,200 g, 75,000 rpm, 45 min, 4 °C through a 100-µl sucrose cushion (0.5 x sucrose in 50 mM Hepes/NaOH, pH 7.5). The pellet was dissloled in SDS-PAGE sample buffer and analyzed as described above.

RESULTS

Synthesis of GlcNAc-Pi and GlcN-Pi in Subcellular Fractions—We investigated the synthesis of early GPI biosynthetic intermediates (GlcNAc-Pi and GlcN-Pi, Fig. 1) in subcellular fractions obtained from thymoma cells. Thymoma cell lysates were prepared by nitrogen cavitation and, following the remo-

val of nuclei and unbroken cell debris, were subjected to differential centrifugation to obtain fractions sedimenting at 10,000 x g (P10) and at 100,000 x g (P100) (Fig. 2). The P10 fraction is typically characterized as a crude mitochondrial preparation but has been shown previously to contain also a subfraction of ER tightly associated with the mitochondrial membrane (27, 38, 39). The P100 fraction consists primarily of bulk ER membranes but also contains other membranes, including those derived from the Golgi and plasma membrane. Both the P10 and P100 fractions were able to synthesize GlcNAc-Pi and GlcN-Pi; the biosynthetic activity for the combined synthesis of both lipids on a per mg of protein basis was slightly higher (~1.3-fold) in the P10 fraction compared with P100 (Fig. 2). In order to examine this result more closely, we isolated ER membranes from the S10 (Fig. 2) by sucrose gradient centrifugation, and subfractionated the P10 material on Percoll gradients (Fig. 3).

The S10-derived ER fraction was generally similar to the P10 in terms of marker enzyme distribution, except that all markers (ER markers and residual mitochondrial markers) were about 1.5–2-fold enriched relative to P100 (Fig. 2 and Table 1). Subfractionation of P10 yielded three distinct fractions (mitochondria (Mito), MAM, and MAM; see Fig. 2; the acronym MAM stands for mitochondria-associated membranes (27)) which we characterized in terms of ER and mitochondrial
GPI biosynthetic pathway in mammalian cells. GPI biosynthesis is initiated by GlcNac transfer from UDP-GlcNac to PI yielding GlcNac-PI. GlcNac-PI is then de-N-acetylated, inositol-acetylated, and further modified with mannose and phosphoethanolamine residues. CHO mutant cells (G9PLAP.85) defective in the PIG-L gene are unable to de-N-acetylate GlcNac-PI. The singly mannosylated GPI intermediates that are detectable in the cell-free GPI synthesis assays shown in this paper are H2 and H5. H5 is decorated by a phosphoethanolamine residue linked to the 2-position of mannose. The mature, triply mannosylated GPIs (H7 and H8) bear two or three phosphoethanolamine side chains as shown. Biosynthetic intermediates between H5 and H7 are not shown.

marker enzyme activities before testing for GPI biosynthetic capability. The overall analysis of marker enzyme distribution (see Fig. 2, Table I, and “Experimental Procedures” for details) is consistent with the following assignments for the P10-derived fractions. 1) The Mito fraction consists primarily of mitochondria with very little ER contamination. 2) MAMp contains most (>70%) of the ER membranes that are found in the P10, along with some mitochondrial contamination (in contrast ER membranes isolated from the S10 contain relatively little mitochondrial contamination; see Fig. 2 and Table I). Mitochondrial markers recovered in MAMp may be carried by mitochondrial fragments or damaged mitochondria since MAMp represents a relatively light membrane mixture ($\approx$1520) of the ER membranes that are found in the P10. P10 was resolved into two fractions (upper band and lower band) by centrifugation through a Percoll gradient. The bands were collected and respun at 10,000 × g. The pellets so obtained were resuspended resulting in mitochondrial (Mito) and MAMp fractions. The supernatants from the 10,000 × g spin were combined and respun at 100,000 × g to obtain MAMs, (pellet). Fractions were characterized by measuring marker enzyme activities. The panels in the lower part of the figure show the specific activities of early GPI biosynthetic enzymes (the data show the combined synthesis of GlcNac-PI and GlcN-PI; cpm × $10^{-3}$/min/mg protein; black bars), ribophorin I (ER marker; arbitrary units/mg protein; white bars), and Hsp60 (mitochondrial marker (M); arbitrary units/mg protein; gray bars) in subcellular fractions from thymoma cells. Data are presented as mean ± S.E.

GlcNac-PI in normal cells, the combined synthesis of both lipids (referred to as GlcNac/GlcN-PI) was measured. As shown in Fig. 2, all fractions except the Mito fraction were able to synthesize GlcNac/GlcN-PI. Although the ability to synthesize GlcNac/GlcN-PI correlated with the presence of ER marker enzymes in a particular fraction, the MAM fractions were significantly more active than the P10 and ER fractions. For example, the specific activity of GlcNac/GlcN-PI synthesis in MAMp was roughly 4-fold higher in comparison with P10 and 2.5 times higher in comparison with relatively pure ER (Fig. 2). Since the inclusion of exogenous PI had a similar stimulatory effect on all fractions (under our experimental conditions GlcNac/GlcN-PI synthesis was enhanced ~2-fold in samples with added PI relative to samples without added PI), the observed differences in the specific activity of GlcNac/GlcN-PI synthesis between the fractions cannot be explained by postulating that the fractions contain different amounts of one of the reaction substrates (PI). Also, differential enzyme lability in the various fractions was discounted as an explanation for
The table presents the protein content and specific marker enzyme activities (mean ± S.E.) of subcellular fractions from thymoma cells. Fractions were prepared from $10^8$ thymoma cells as described under “Experimental Procedures” and outlined in Fig. 2. Ribophorin, BiP, and NADPH-cytochrome $c$ reductase are ER markers. Hsp60 and succinate dehydrogenase are mitochondrial markers. Ribophorin, BiP, and Hsp60 were quantitated by Western blotting and densitometry, and the data are presented in arbitrary units. The units of NADPH:cytochrome $c$ reductase activity and succinate dehydrogenase activity are nmol of cytochrome $c$ reduced per min and nanomoles of $p$-nitroterazolium violet reduced per min/mg of protein, respectively. Total activities are presented in parentheses.

| Fraction* | Protein | Ribophorin I | BiP | NADPH-cytochrome $c$ reductase | Hsp60  | Succinate dehydrogenase |
|-----------|---------|--------------|-----|---------------------------------|--------|-------------------------|
| P10       | 15      | 92.5 ± 4.8   | 37.5 ± 3.6 | 5.7 ± 1                          | 185.5 ± 11.2 | 18.5 ± 4.2             |
|           |         |              |       |                                 |        |                         |
| P100      | 17.8    | 101.1 ± 5.8  | 47 ± 2.5 | 21 ± 1.3                        | 16.5 ± 5 | 2.5 ± 0.3               |
| ER        | 9.5     | 195 ± 16.5   | 79 ± 1.7 | 29.2 ± 2                        | 21.5 ± 4 | 3.1 ± 0.36              |
| MAM$_s$   | 4.2     | 138 ± 20.5   | 54.2 ± 2.2 | 11.8 ± 1.4                     | 88.75 ± 14 | 14.3 ± 2.5            |
| MAM$_p$   | 2.9     | 69.5 ± 6.0   | 30.8 ± 1.7 | 7.3 ± 0.8                      | 195 ± 24 | 29.4 ± 2.3             |
| Mito      | 4.5     | 20.5 ± 4.0   | 11.6 ± 2  | 0.9 ± 0.2                      | 243 ± 10 | 22.3 ± 4.9             |

*The distribution of marker enzyme activities for Golgi, lysosomes, and plasma membrane was as follows (specific activities are presented in arbitrary units per mg of protein, followed by total activities in parentheses): α-mannosidase II (Golgi), P10, 0.7 (10.5); P100, 3.5 (62.3); β-hexosaminidase (lysosomes), P10, 1.2 (18.5); P100, 0.2 (3.6); MAM$_s$, 0.3 (1.2); MAM$_p$, 0.7 (2); Mito, 2 (9); and alkaline phosphodiesterase (plasma membrane), P10, 27.2 (408); P100, 81.6 (1456); ER, 15.8 (151); MAM$_s$, 35.7 (150); MAM$_p$, 17.6 (79.2); Mito, 19.1 (55.4).

**Fig. 3. Recovery of GlcNAc-PI synthase + GlcNAc-PI de-N-acetylase activity in subcellular fractions from thymoma cells.** Subcellular fractions (50 μg) from thymoma cells were incubated with UDP-[^3]H]GlcNAc as described under “Experimental Procedures.” Lipids were extracted and analyzed by TLC. The chromatograms were scanned with a TLC scanner, and the yield of radiolabeled lipids was determined by integration of chromatogram peaks using software provided with the scanner in conjunction with liquid scintillation counting. A shows apparent specific activities (cpm × 10^{-3} min/mg; mean ± S.E.) for the synthesis of GlcNAc-PI and GlcN-PI in the various fractions: combined synthesis of the two lipids (gray bars), GlcNAc-PI (hatched bars), and GlcN-PI (black bars). The total apparent activities (cpm × 10^{-3} min; mean ± S.E.) for the synthesis of the lipids are shown in B.

**Subcellular Location of GPI Biosynthesis**

The differences in the specific activity of GlcNAc/GlcN-PI synthesis since recovery of activity in the subfractionation steps was excellent (see Fig. 3B, for example); ~93% of the total GlcNAc/GlcN-PI synthetic activity of P100 was recovered in the ER fraction and ~102% of the activity of P10 was recovered in the combined MAM fractions. These data suggest that ER membranes (MAM$_s$ and MAM$_p$) recovered from P10 are not only capable of initiating GPI biosynthesis but possess a somewhat higher specific activity for the formation of GlcNAc/GlcN-PI in comparison with ER isolated more conventionally from the S10 (Fig. 2).

**Further Analysis of GlcNAc/GlcN-PI Synthesis in Subcellular Fractions**—In the experiments described above, GPI biosynthesis in the various subcellular fractions was assessed as the combined synthesis of GlcNAc-PI and GlcN-PI. In order to determine the extent of synthesis of each of the two lipids, lipid extracts were resolved by thin layer chromatography (TLC), and the incorporation of radioactivity into GlcNAc-PI and GlcN-PI was quantitated. The results are shown in Fig. 3. Although the P10 and P100 fractions were able to synthesize comparable amounts of combined products (gray bars, Fig. 3B, P10, 1080 cpm × 10^{-3}/min; P100, 870 cpm × 10^{-3}/min; specific activity data are shown in A), the relative proportions of the two products differed significantly (hatched bars, GlcNAc-PI; black bars, GlcN-PI; Fig. 3). The yield of GlcN-PI was much higher in P10 versus P100, corresponding to 62 and 32% of the total lipid synthesized, respectively (Fig. 3B). The ER and MAM fractions appeared much like the parental P10 and P10 fractions, respectively, in terms of the yield of GlcN-PI relative to GlcNAc-PI. The yield of GlcN-PI (relative to GlcNAc-PI + GlcN-PI) was 24, 45, and 72% in the ER, MAM$_s$, and MAM$_p$ fractions, respectively (Fig. 3B). The apparent specific activity for GlcNAc-PI synthesis (cpm × 10^{-3} min/mg protein) was 15, 20.5, 43.4, 80, and 90.6 for P100, ER, P10, MAM$_s$, and MAM$_p$, respectively. These data suggest that the GlcNAc-PI de-N-acetylase activity responsible for GlcN-PI synthesis is more strongly represented in P10 than in P100 and concentrated in the P10-derived MAM fractions.

**Distribution of GlcNAc-PI De-N-acetylase Activity in Subcellular Fractions**—In order to test the hypothesis that the GlcNAc-PI de-N-acetylase activity is enriched in the P10 and MAM fractions relative to P100 and ER, we assayed the de-N-acetylase directly by incubating the fractions with chemically synthesized Glc[NI^{14}C][Ac-PI(diC16)] and measuring the release of radioactive acetyl coenzyme A (Fig. 4). Very low levels of de-N-acetylation were seen in the crude homogenate (H), P100, and mitochondrial (Mito) fractions. Although it is certain that the P100 possesses some de-N-acetylase activity (see Fig. 3A, for exam-
The data are presented as specific activities (cpm/min/mg; mean ± S.E.). Total recovery of activity (cpm/min; mean values) was 58, 160, 180, 84, and 6 for ER, P10, MAM, MAMp, and Mito, respectively. The total de-N-acetylation activity in MAM + MAMp (364 cpm/min) is ~1.7-fold greater than that in P10 (160 cpm/min). One explanation for this observation is that the de-N-acetylation assay (unlike other assays used in this paper) uses an exogenously added lipid substrate, GlcN\(^{[1^{14}C]}\)Ac-PI. Deposition of this lipid in biosynthetically inactive membranes such as the Mito component of the P10 would result in removing it from the assay pool. Since MAM + MAMp have fewer “GPI-inactive” membranes than P10, the total activity recovered in these fractions appears higher.

For GlcN-PI synthesis by P100, the activity is low and appears to be at the limit of detection of the GlcN\(^{[1^{14}C]}\)Ac-PI-based assay. The P10 fraction was active, as were the P10- and S10-derived ER fractions. The P10-derived ER fractions (MAM and MAMp) had 10.8- and 7.3-fold higher specific de-N-acetylation activity in MAMs and MAMp (264 cpm/min) is ~1.7-fold greater than that in P10 (160 cpm/min). One explanation for this observation is that the de-N-acetylation assay (unlike other assays used in this paper) uses an exogenously added lipid substrate, GlcN\(^{[1^{14}C]}\)Ac-PI. Deposition of this lipid in biosynthetically inactive membranes such as the Mito component of the P10 would result in removing it from the assay pool. Since MAM + MAMp have fewer “GPI-inactive” membranes than P10, the total activity recovered in these fractions appears higher.

Fig. 4. Distribution of GlcNAc-PI de-N-acetylation in subcellular fractions from thymoma cells. De-N-acetylation of GlcNAc-PI in thymoma cell fractions. Fractions (50 µg of protein) were incubated with GlcN\(^{[1^{14}C]}\)Ac-PI, and de-N-acetylation activity was monitored by release of \(^{[1^{14}C]}\)acetate as described under “Experimental Procedures.” The data are presented as specific activities (cpm/min/mg; mean ± S.E.). Total recovery of activity (cpm/min; mean values) was 58, 160, 180, 84, and 6 for ER, P10, MAM, MAMp, and Mito, respectively. The total de-N-acetylation activity in MAM + MAMp (364 cpm/min) is ~1.7-fold greater than that in P10 (160 cpm/min). One explanation for this observation is that the de-N-acetylation assay (unlike other assays used in this paper) uses an exogenously added lipid substrate, GlcN\(^{[1^{14}C]}\)Ac-PI. Deposition of this lipid in biosynthetically inactive membranes such as the Mito component of the P10 would result in removing it from the assay pool. Since MAM + MAMp have fewer “GPI-inactive” membranes than P10, the total activity recovered in these fractions appears higher.

Synthesis of GlcNAc-PI in Subcellular Fractions Derived from Wild-type CHO Cells and CHO Cells Defective in GlcNAc-PI De-N-acetylation—The results described above are consistent with the hypothesis that the enzymes responsible for the synthesis and de-N-acetylation of GlcNAc-PI are differently distributed in subcellular fractions. In order to explore this idea further we repeated the analysis with CHO cells to test whether the subcellular fractionation pattern of activities that we had obtained with thymoma cells also applied to CHO cells. The experiments showed that the pattern of GlcNAc/GlcN-PI synthesis in CHO cell fractions was identical to that seen in thymoma cell fractions (Figs. 2 and 3), and as with the thymoma cell fractions, the relative synthesis of GlcN-PI was consistent with the hypothesis that the enzymes responsible for the de-N-acetylation step and to measure the specific activity of GlcNAc-PI synthesis explicitly in various subcellular fractions. Subcellular fractions were prepared from the CHO G9PLAP.85 cells and assayed for GlcNAc-PI synthesis. The results, shown in Fig. 5, indicate that all the fractions were generally efficient at synthesizing GlcNAc-PI. In the particular experiment shown, the P10 fraction was slightly more active in synthesizing GlcNAc-PI (~1.4-fold) than the P100, and the P10-derived ER (MAM) was only marginally more active (~1.2-fold) than the S10-derived ER. The results indicate that, unlike GlcNAc-PI de-N-acetylation activity, GlcNAc-PI synthetic activity is similarly distributed among the ER subfractions isolated from P10 and P100.

In Vitro Biosynthesis of Mannosylated GPI Species from UDP-GlcNAc—Elaboration of GlcN-acetyl-PI in mammalian cells occurs via the addition of mannose and phosphoethanolamine residues (Fig. 1). These biosynthetic steps have not been studied individually thus far since the various GPI reaction substrates (GlcN-acetyl-PI and partially mannosylated GPI structures) are not generally available, and cell-free assay conditions remain to be worked out. Efficient delivery of lipid substrates is perhaps the greatest hurdle to be overcome in assaying these later biosynthetic steps in vitro since the reactions are known to be detergent-labile. Nevertheless, it is possible to derive information on the elaboration of GlcN-acetyl-PI by incubating subcellular fractions with more distant substrates, such as radioactive sugar nucleotides, and tracking the incorporation of radioactivity into mannosylated GPI structures. In order to extend our analyses of the subcellular distribution of GPI biosynthesis to include these “later” biosynthetic reactions, we therefore incubated the various subcellular fractions (Fig. 2) with GDP-[\(^{3H}\)Man, in the presence or absence of nonradioactive UDP-GlcNAc, and we analyzed the radiolabeled lipids formed by organic solvent extraction and TLC (Fig. 6). Related experiments in which fractions were incubated with GDP-[\(^{3H}\)Man in the presence or absence of nonradioactive GlcN-PI are described in the legend to Fig. 7.

Fig. 6, A and B, shows representative chromatograms of radiolabeled lipids synthesized from GDP-[\(^{3H}\)Man in the MAM + MAMp fraction. The major radiolabeled lipid synthesized in the absence of UDP-GlcNAc was dolichol-P-mannose (dol-P-Man) (Fig. 6A). Minor but detectable amounts of singly and triply mannosylated GPs (H5, H7, and H8, see Fig. 1) were also synthesized. The extent of incorporation of radiolabel into GPI species (H8) under these conditions (no added UDP-GlcNAc) was roughly similar in all P100- and P10-derived ER fractions examined, i.e. ER, MAM, and MAMp.

When the reaction was supplemented with nonradioactive UDP-GlcNAc, biosynthesis of the GPI intermediate H5 (EtN-P-2-Man)–4GlcN-acetyl-PI increased 3–4-fold in the MAM and MAMp fractions (Fig. 6, C and D; see Fig. 6, A and B, for TLC scans showing H5 synthesis in MAMp). In contrast, no increase in the formation of mature GPI species (H7 and H8) was observed in any of the fractions (Fig. 6, C and D) indicating that the synthesis of mature [\(^{3H}\)mannose-labeled GPI species occurs predominantly via [\(^{3H}\)mannose transfer to endogenous acceptors (GlcN-acetyl-PI and partially mannosylated GPs) rather than to de novo synthesized GlcN-acetyl-PI species. Al-
though all fractions tested were able to synthesize mannosylated GPI species (H5, H7, and H8) to some extent, the UDP-GlcNAc-dependent stimulation of H5 biosynthesis was only seen in the MAM fractions. Variations in synthesis of dolichol-P-mannose, the mannose donor, are unlikely to account for this observation since dolichol-P-mannose synthesis differed by no more than 1.7-fold between the various fractions and was lower in the MAM fractions relative to ER (see Fig. 6 legend). The results presented above indicate the following: (i) that the MAM fractions, unlike the S10-derived ER, are capable of the de novo synthesis of H5, and (ii) de novo synthesis of more elaborate GPI structures (e.g. H7 and H8) does not occur in either the S10-derived ER or the MAM fractions. Possible reasons for the latter result are detailed under “Discussion.” The de novo (UDP-GlcNAc-dependent) synthesis of H5 in the MAM fractions may be a result of the concentration of GlcNAc-PI de-N-acetylase activity in these membranes (Fig. 4B) rather than a consequence of correspondingly high levels of the relevant inositol acyl-, mannosyl-, and phosphoethanolaminotransferases. However, results presented below indicate that the MAM is also enriched in these latter enzymes.

Post-GlcNAc-PI De-N-acetylation Reactions Leading to the Biosynthesis of H5 Are Concentrated in the MAM Fractions—

The synthesis of H5 from GlcNAc-PI involves four reactions yielding the biosynthetic intermediates GlcN-PI, GlcN-acylPI, and H2; phosphoethanolamine transfer to the mannose residue in H2 yields H5 (Fig. 1). We were interested in discovering whether the ability of the MAMs to synthesize H5 de novo is due solely to their enhanced ability to synthesize GlcN-PI or whether all reactions involved in the conversion of GlcNAc-PI to H5 are enriched in the MAMs. To test these possibilities, we assayed the conversion of exogenously added GlcN-PI to [3H]Man-labeled H5. This approach allowed us to bypass the GlcNAc-PI de-N-acetylase and directly assess the cumulative activity of the following “H5 biosynthetic enzymes”: GlcN-PI inositol acyltransferase, mannosyltransferase, and phosphoethanolaminotransferase (Fig. 1).

When subcellular fractions were incubated with GDP-[3H]Man in the presence of GlcN-PI, GlcN-acylPI, and H2; phosphoethanolamine transfer to the mannose residue in H2 yields H5 (Fig. 1). We were interested in discovering whether the ability of the MAMs to synthesize H5 de novo is due solely to their enhanced ability to synthesize GlcN-PI or whether all reactions involved in the conversion of GlcNAc-PI to H5 are enriched in the MAMs. To test these possibilities, we assayed the conversion of exogenously added GlcN-PI to [3H]Man-labeled H5. This approach allowed us to bypass the GlcNAc-PI de-N-acetylase and directly assess the cumulative activity of the following “H5 biosynthetic enzymes”: GlcN-PI inositol acyltransferase, mannosyltransferase, and phosphoethanolaminotransferase (Fig. 1).
Subcellular Location of GPI Biosynthesis

The results described in this paper point to an unusual segregation of GPI biosynthetic reaction(s) in a subcompartment of the ER (Fig. 8). The cell-free assembly of GPI intermediates subsequent to the initial GlcNAc transfer reaction and up to the formation of the singly mannosylated species H5 is substantially enhanced in this ER subcompartment compared with conventionally isolated ER. This ER subcompartment appears to be similar to the one previously identified by a number of investigators as a region of ER tightly associated with mitochondria (27, 28, 38–42). The association between mitochondria and regions of the ER was demonstrated morphologically and biochemically in these earlier studies and shown to be a general feature of many cell types (e.g. rat hepatocytes, CHO cells, S. cerevisiae). Cell homogenization apparently disrupts the connection between this mitochondria-associated ER subcompartment (termed mitochondria associated membrane or MAM (27)) and the rest of the ER resulting in its isolation, together with mitochondria, in a 10,000 × g pellet (P10, Fig. 2).

We fractionated the P10 material to generate two MAM fractions, MAMp and MAMm (Fig. 2), which probably represent operational variants of the same membrane structure. Most of the ER content and all of the GlcNAc/GlcN-PI biosynthetic activity of the P10 were recovered in the MAM fractions (Table I and Fig. 2). The MAM fractions contain mitochondrial marker proteins, a likely consequence of the close association between the MAM and mitochondria that is readily apparent in electron microscopic images of intact cells and isolated MAM fractions (31, 38–40). The mitochondrial inner membrane marker succinate dehydrogenase was found to be preferentially enriched in the MAM relative to the mitochondrial matrix marker, Hsp60 (Table I), indicating that the MAM fractions are probably contaminated with mitochondrial membrane fragments (possibly contact site membranes (31)) or damaged mitochondria. However, the absence of GlcNAc/GlcN-PI biosynthetic activity in mitochondria (Figs. 2 and 4) indicates that the GPI biosynthetic activity detected in the MAM can be directly attributed to ER membranes rather than to mitochondria.

**DISCUSSION**

De novo Incorporation of Radioactivity into DolICHOL-P-Mannose—Dolichol-P-mannose was comparable in all fractions but higher in P100 and ER relative to P10 and the MAM fractions, respectively. Thus reduced levels of dolichol-P-mannose synthesis cannot account for low H5 synthesis in P100 and ER. These results show clearly that the inositol acyltransferase, manno-acyltransferase, and phosphoethanolaminetransferase required to convert GlcN-PI to H5 are, in combination, enriched in the P10-derived ER membranes relative to S10-derived ER. The relative enrichment of these activities in the MAM versus ER (−6-fold) is less than that obtained for the GlcNAc-PI de-N-acetylase (−9-fold, Fig. 4), but as pointed out above, this figure is likely to be an underestimate.

Translocation and Processing of Prepromini-PLAP in Isolated Fractions—The final step in the assembly of GPI-anchored proteins is the transamidase-catalyzed replacement of a carboxyl-terminal signal sequence by a pre-formed GPI anchor, following protein translocation across the ER membrane. A model protein, prepromini-PLAP (derived from placental alkaline phosphatase; PLAP), has been extensively used to study conversion of proproteins to the mature GPI-anchored form in cell-free assays (7). Upon membrane translocation and aminoterminal signal sequence cleavage, prepromini-PLAP is converted to a lower molecular weight, protease-protected form, promini-PLAP. Promini-PLAP is subsequently converted to GPI-anchored mini-PLAP which also resists protease treatment of intact vesicles. We tested the ability of P10, P100, ER, and MAM to translocate and convert prepromini-PLAP into GPI-anchored form (mini-PLAP). Although it is impossible to deduce specific activities for the translocation and GPI-anchoring steps using this assay, all four fractions were able to translocate and GPI anchor preproteins—promini-PLAP (data not shown). Protease-protected products corresponding to promini-PLAP and mini-PLAP were generated in every case at approximately similar levels, and the amount of mini-PLAP synthesized relative to the total amount of protease-protected material was also similar in all fractions (data not shown).

**FIG. 8. A model for the compartmentation of GPI biosynthetic steps in the ER.** The circle depicts the ER membrane as a continuum of bulk ER shown in dark gray and a GPI biosynthesis-active, mitochondria-associated membrane domain shown in light gray (MAM, recovered as MAM and MAMp, in the fractionation procedure outlined in Fig. 2). The MAM and bulk ER are separated on cell homogenization and are recovered separately from the P10 and S10 fractions, respectively. GlcNAc-PI is synthesized equally efficiently in the ER and MAM fractions, but de-N-acetylation of GlcNAc-PI occurs preferentially in the MAM (the specific activity of the de-N-acetylated form is ~9-fold higher than in the ER and the total activity recovered in the MAM (MAM, + MAMp) is ~7-fold higher than in the ER). It is unclear whether GlcNAc-PI can diffuse between the two membrane domains and whether GlcNAc-PI synthesized in the bulk ER can access the de-N-acetylated form in the MAM fraction (dashed line). De novo biosynthesis of the singly mannosylated GPI intermediate H5 occurs in the MAM but not in the ER. The location(s) at which H5 is elaborated to the mature structures H7 and H8 is unknown. A potential complication is the topological distribution of the mannosylation steps in the ER and/or MAM; trans-bilayer movement (double-headed dashed arrow) of lipid intermediates (H5, dolichol-P-mannose) may be required to allow synthesis of mature GPls, but it is unknown whether such movement occurs in the MAM. Dolichol-P-mannose, the mannose donor for GPI mannosylation reactions, is synthesized equally well in the ER and MAM fractions and is known to undergo trans-bilayer movement in the ER. For further details, see under “Discussion.”

The relative enrichment of these biosynthetic activities in the P10 and MAMs is likely to be higher than indicated since the assay readout was not linear over the time periods required to obtain detectable levels of [3H]Man-labeled H5. The lack of linearity is likely due to the complexity of the incubation mixtures, suboptimal assay conditions, and the fact that the assay measures the cumulative output of three enzymes. Fig. 7B also shows the incorporation of radioactivity into dolichol-P-mannose, the donor of the mannose residue in H5; synthesis of dolichol-P-mannose was comparable in all fractions but higher in P100 and ER relative to P10 and the MAM fractions, respectively. Thus reduced levels of dolichol-P-mannose synthesis cannot account for low H5 synthesis in P100 and ER. These results show clearly that the inositol acyltransferase, manno-acyltransferase, and phosphoethanolaminetransferase required to convert GlcN-PI to H5 are, in combination, enriched in the P10-derived ER membranes relative to S10-derived ER. The relative enrichment of these activities in the MAM versus ER (−6-fold) is less than that obtained for the GlcNAc-PI de-N-acetylase (−9-fold, Fig. 4), but as pointed out above, this figure is likely to be an underestimate.
alization of the distribution of the PIG-L protein, a candidate GlcNAc-PI de-N-acetylase, showed a homogeneous reticular staining pattern very similar to that of the ER resident protein disulfide isomerase (43). The absence of heterogeneity in the immunofluorescence distribution of PIG-L (an epitope-tagged version of PIG-L was used for these studies) may be attributed to the relatively low resolution of the technique or to the possibility that the localization of PIG-L may not provide an accurate representation of the membrane distribution of the de-N-acetylase activity; for example, the active form of the de-N-acetylase may be a complex between PIG-L and another, hitherto unidentified, heterogeneously distributed (MAM-localized) protein (9).

The enzymes responsible for inositol acylation, mannosylation, and phosphoethanolamine modification of GlcN-PI are also concentrated in the MAM. Owing to difficulties in assaying these activities explicitly, we measured these enzymes in combination by monitoring the conversion of exogenously added GlcN-PI to H5. The combined activity was enriched (~6-fold) in the P10 and MAM fractions relative to P100 and ER.

Our data show that the MAM fractions are uniquely able to carry out the de novo synthesis of H5 (Fig. 6, C and D). It is clear from the results summarized in this section and shown in Figs. 3, 4, and 7 that this unique capability is due to the enrichment of GlcNAc-PI de-N-acetylase as well as the H5 biosynthetic enzymes in the MAM.

**Bioynthesis of Mature GPs** —The de novo synthesis of GPs proceeds up to the formation of the intermediate H5 in the MAM fraction. Further mannosylation and phosphoethanolamine addition reactions appear to be so severely attenuated that it is impossible to detect de novo synthesized mature GPs (H7 and H8). The MAM fraction undoubtedly has the ability to synthesize these structures since low levels of radiolabeled H7 and H8 can be detected, but synthesis appears to occur primarily via mannone transfer to endogenous acceptors rather than via de novo synthesis from UDP-GlcNAc and PI (Fig. 5).

The inability of de novo synthesized H5 to progress further along the GPI assembly pathway may be explained in a number of ways. We enumerate a few possibilities as follows. (i) Dolichol-P-[3H]mannose synthesized in the mammalian is present only in radiochemical amounts and is possibly insufficient to supply the second and third GPI mannosyltransferases at reasonable rates leading to detectable H7 and H8. However, in vitro biosynthesis of mature [3H]mannose-labeled GPs proceeds readily, albeit at optimal rates, in other cell-free systems primed with GDP-[3H]mannose (23, 24, 51), suggesting that other factors must also contribute to the elaboration of H5. (ii) The second mannosyltransferase (GPIMT-II) is highly unstable and consequently non-functional in the subcellular fractions. (iii) The efficient synthesis of H5 is a result of substrate channeling through a metabolon (44) composed of the de-N-acetylase, inositol acyltransferase, and the first GPI mannosyltransferase; discharge of H5 from the metabolon and consequent dilution into the membrane milieu would result then in the inefficient, essentially undetectable synthesis of more elaborate GPs. Substrate channeling in this segment of the GPI biosynthetic pathway has been suggested previously on the basis of results obtained with trypanosomals and Leishmania cell-free systems for GPI biosynthesis (45) although other data (46) suggest that this may not occur in mammalian systems. (iv) GPIMT-II and its substrates are topologically separated (Fig. 7). The substrates for GPIMT-II are H5 and dolichol-P-mannose (the mannone donor in the GPIMT-II catalyzed mannosyl transfer reaction in trypanosomes and presumably also in mammalian cells (47)), both of which are synthesized in the MAM (Fig. 5 and legend). The prevailing evidence (6, 8, 48) suggests that the two lipids are synthesized on the cytoplasmic face of the endoplasmic reticum membrane and, in the absence of lipid translocators (flipases), may well be confined to this membrane leaflet. Although GPIMT-II has yet to be identified, its active site may face the ER lumen, similar to the PIG-B gene product that probably encodes the third GPI mannosyltransferase (49). Trans-bilayer movement of H5 and dolichol-P-mannose would overcome such a topological barrier. Protein-mediated flipping of dolichol-P-mannose has been described in “microsomal” ER preparations (50), and it remains to be seen whether such an activity also exists in the MAM. There is no information on trans-bilayer movement of GPs except that such movement is likely to occur (6, 8, 51, 52). At this point, none of the possibilities discussed above can be ruled out, and further investigations are clearly necessary in order to understand the membrane localization and regulation of the later steps of GPI biosynthesis.

**ER Heterogeneity and MAM Function** —The confinement of GPI biosynthetic reactions to a domain of the ER provides yet another example of the compositional and functional heterogeneity of the ER. The functional diversity of ER membranes and the existence of functionally specialized and morphologically distinct subregions of the ER have been the subjects of several recent reviews (26, 53–55). The existence and functional uniqueness of a mitochondria-associated ER domain have been documented in a number of studies using biochemical and ultrastructural methods. For example, Wassler and Fries (28) showed that a protease involved in post-translational processing of the secretory protein haptoglobin is localized to ER membranes that sediment similarly to mitochondria after cell homogenization. Connections between this membrane fraction and the remaining of the ER could be re-established through GTP-dependent membrane fusion, resulting in the effective processing of haptoglobin. It is not clear why the protease is confined to a mitochondria-associated ER domain and whether mitochondria contribute in any way to the control of the proteolytic event. In another example, the ER protein calreticulin and the inositol 1,4,5-triphosphate receptor were shown to be clustered in regions of the ER that are closely associated with mitochondria and that appear to define sites of enhanced Ca2+ release from ER stores (56–58). Here the functional significance of a mitochondria-associated ER subcompartment is somewhat clearer since mitochondrial activity was shown to be required to preserve cytosolic Ca2+ responses (59).

Mitochondria-associated ER domains have a clearly defined function in the transfer of lipids between the ER and mitochondria (29, 60, 61). Phosphatidylserine (PS) which is synthesized in the ER must be transported to the mitochondrial inner membrane in order to be decarboxylated to phosphatidylethanolamine (PE) (62). PE, in turn, must be exported from the mitochondria to participate in ER-localized N-methylation reactions (63) involved in the synthesis of phosphatidylcholine. In rat liver, the PE N-methyltransferase, PEMT-2, is uniquely localized to the MAM (63), and the activity of PS synthase in liver and CHO cells is about 2.5-fold higher in the MAM than in microsomal ER fractions (29, 64).

At this point it is difficult to explain the precise physiological significance of our finding that post-initiation GPI biosynthetic reactions are localized to the MAM. It is possible that the phosphoethanolamine residues in the GPI structure are preferentially derived from PE synthesized via the mitochondrial PS decarboxylation pathway rather than PE generated via the ER-localized CDP-ethanolamine pathway (65). Phosphoethanolamine transfer reactions in a MAM-localized GPI biosynthetic pathway would thus be adequately primed with PE since PE is exported from mitochondria to the MAM (29). Alterna-
tively, the differential distribution of GlcNAc-PI de-N-acetylase (restricted to the MAM) versus GlcNAc-PI synthase (uniformly distributed in the ER) may serve to generate two functionally distinct pools of GlcNAc-PI. Although it is conceivable that GlcNAc-PI synthesized in other regions of the ER in intact cells diffuses into the MAM to be de-N-acetylated and subsequently elaborated, it is tempting to speculate that GlcNAc-PI serves an independent function in the cell and that non-MAM pools of GlcNAc-PI are preserved for this purpose by restricting their access to the de-N-acetylase.

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