Identification of Triton X-100 Insoluble Membrane Domains in the Yeast *Saccharomyces cerevisiae*

**LIPID REQUIREMENTS FOR TARGETING OF HETEROTRIMERIC G-PROTEIN SUBUNITS**

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**Abstract**

Low density Triton X-100 insoluble (LDTI) membrane domains are found in most mammalian cell types. Previous biochemical and immunolocalization studies have revealed the presence of G-protein coupled receptors and heterotrimeric G-protein subunits (Gα and Gβγ subunits) within these structures, implicating mammalian LDTI membrane domains in G-protein coupled signaling. Here, we present biochemical evidence that similar LDTI structures exist in a genetically tractable organism, the yeast *Saccharomyces cerevisiae*. Yeast LDTI membranes were purified based on the known biochemical properties of mammalian LDTI membranes: (i) their Triton X-100 insolubility; and (ii) their discrete buoyant density in sucrose gradients. As with purified mammalian LDTI membranes, these yeast LDTI membranes harbor the subunits of the heterotrimeric G-proteins (Gα and Gβγ subunits). Other plasma membrane marker proteins (the plasma membrane H+-ATPase and a GPI-linked protein Gas1p) are preferentially excluded from these purified fractions. Mutational and genetic analyses were performed to define the requirements for the targeting of G-protein subunits to these yeast membrane domains. We find that the targeting of Gαi is independent of myristoylation, whereas targeting of Gβγ requires prenylation. Perhaps surprisingly, the targeting of Gβγ to this membrane domain did not require coexpression of Gαi. It should now be possible to dissect the function of LDTI membrane domains using yeast as a model genetic system.

Over the last 4 years, low density Triton X-100 insoluble (LDTI) membrane domains have been purified from a variety of different mammalian cell types and their protein composition determined. The Triton X-100 insolubility of these domains has been attributed to their high content of glycosphingolipids and cholesterol (1). At least two types of LDTI structures have been described: caveolar domains and non-caveolar domains. These two types of LDTI domains differ mainly in their caveolin content; noncaveolar LDTI structures lack caveolin. However, these two domains have many properties in common. These common characteristics include the enrichment of cytoplasmically oriented signaling molecules, with glycosphingolipids and cholesterol as their major lipid components. The biochemical isolation of the LDTI membrane domains is based on: (i) their discrete buoyant density in a sucrose gradient; and (ii) their Triton X-100 insolubility (2–7). Morphologically, the mammalian LDTI membrane domains are 50–200-nm vesicular structures, as observed by electron microscopy (8).

It has been proposed that LDTI membrane domains participate in vesicular trafficking (transcytosis and potocytosis (9, 10)) and/or signal transduction (2, 3, 11, 12). The latter function is supported by the observation that several different classes of signaling molecules have been localized to LDTI membrane domains. For example: (i) G-protein coupled receptors (the β-adrenergic receptor (13), the endothelin receptor (14), the muscarinic acetylcholine receptor (15)); and (ii) growth factor receptors (the epidermal growth factor receptor (6) and the insulin receptor (16)) have been morphologically and biochemically localized to LDTI membrane domains. We and other laboratories have shown recently the localization of several G-protein α-subunits within these domains (3, 5) and demonstrated that they directly interact with caveolin (17). Both the localization of Gαi subunits to LDTI complexes and their interaction with caveolin are dependent on the activation state of Gαi (17). *In vitro* reconstitution experiments directly show that caveolin can act as a scaffolding protein to recruit Gαi subunits onto membranes (18). These data suggest an important functional role for LDTI complexes in relaying extracellular messages to the interior of cells.

Due to the difficulties inherent in genetically manipulating mammalian cells, we have started to explore the use of a genetically tractable organism, the yeast *Saccharomyces cerevisiae*, to study the function of low density Triton X-100 insoluble membrane domains. Although a lower eukaryote, yeast has many characteristics in common with mammalian cells with respect to signal transduction. The two yeast pheromone receptors (α- and β-factor receptors, respectively, involved in the mating process) have a structure similar to the β-adrenergic receptor (seven-transmembrane domains), and they transduce the extracellular signal through tripartite G-proteins. In addition, plasma membrane invaginations similar to the mammalian LDTI plasma membrane invaginations have been observed in electron micrographs of whole yeast cells (19). Therefore, it is likely that yeast cells have a mechanism to organize incoming signals that resembles mammalian cells in that yeast may contain membrane domains similar to mammalian LDTI complexes.
Here, we report the identification of yeast LDTI membrane domains. These domains exhibit many of the known features of mammalian Triton X-100 insoluble membrane domains. As with purified mammalian LDTI membranes, purified yeast LDTI membrane domains: (i) appeared as 100–130-nm vesicular structures; (ii) were enriched in heterotrimeric G-proteins (Gα and Gβγ subunits); (iii) preferentially excluded other plasma membrane marker proteins; and (iv) were selectively solubilized by the detergent octyl-glucoside. Thus, yeast may serve as a model organism for genetically dissecting the function of low density Triton X-100 insoluble membrane domains.

MATERIALS AND METHODS

Reagents—Monoclonal antibody 12CA5 (directed against the HA epitope) was provided by the Harvard Monoclonal Antibody Facility (Cambridge, MA). Polyclonal anti-Gα-subunit antibodies were kindly provided by Drs. D. Jennis and J. Hirschman (University of Massachusetts Medical Center, Worcester, MA). Anti-Gβγ-subunit antibodies were as described (20). Anti-Fma1p antibodies were kindly provided by Dr. U. Neel and anti-Gas1p antibodies by Dr. H. Riezman. Triton X-100 was from American Bioanalytical, and octyl-glucoside was from Boehringer Mannheim.

Strains and Plasmids—Strain K1534 (Dr. K. Nasmith; Mata, his3-11, ura3-1 leu2-3, ade2-1 trp1-1 can1-100, bar1) was used as the wild-type strain. Plasmids pVT-HASTE18 and its derivatives (G2A) were provided by Dr. G. Romeo (Cambridge, MA). Polyclonal anti-Gα-subunit antibodies were kindly provided by Drs. D. Jenness and J. Hirschman (University of Massachusetts Medical Center, Worcester, MA). Anti-Gα-subunit antibodies (G3A1) were transformed into K1534 to express GPA1::hisG alleles. The GPA1 wild-type gene and the mutant allele gpa1 (G2A) were expressed from an alcohol dehydrogenase promoter in plasmid prRS316 in strain K1534 (22). The G-protein γ-deficient strain was L5578 (Dr. G. R. Fink; Mata, ste18::URA3, len2-3, hisG, ura3–52).

Cell Fractionation—Yeast cells (1.0 × 10^8) were grown in YPD (strain K1534) or in synthetic media lacking uracil (strain K1534 harboring a plasmid) to mid-log phase, washed once with H2O, and resuspended in 300 μl of ice-cold lysis buffer (20 mM triethanolamine, pH 7.2, 0.3 M sorbitol, 1 mM EDTA, 0.1 mM PMSF, 0.5 μg/ml leupeptin, and 0.7 μg/ml pepstatin). The cells were broken by vigorous vortexing in the presence of glass beads at 4 °C. Unbroken cells were removed by a 500 × g spin, and the resulting supernatant was referred to as the total cell lysate. The total cell lysate was then centrifuged for 1 h in an SW50 rotor at 100,000 × g to pellet cellular membranes. The pellet was resuspended in 1 ml Mes buffered saline (MBS; 25 mM Mes, pH 6.5, 150 mM NaCl) including 60% sucrose, and Triton X-100 was added to a final concentration of 1%. The membranes were then further disrupted with a 10-s burst of a Polytron tissue grinder (Kinematica GmbH; Brinkmann Instruments, Westbury, NY). After 30–60 min at 4 °C, the extracted membranes were placed at the bottom of an ultracentrifuge tube and overlaid with a sucrose step gradient from 5–50% sucrose in MBS (0.34 ml at 5%, 0.34 ml at 30%, 0.68 ml at 40%, 0.68 ml at 45%, and 1.35 ml at 50%). For the experiment in Fig. 7, the gradient was slightly different in that 0.68 ml was added of all sucrose concentrations. This places the fraction with the Triton X-100 insoluble membrane structures two fractions closer to the bottom of the gradient. Sucrose gradients were centrifuged 16–20 h in an SW60 rotor at 250,000 × g at 4 °C.

Electron Microscopy—Wild-type yeast cells were fractionated as described above, and the resulting Triton X-100 insoluble membranes were pelleted in an ultracentrifuge tube at the highest speed for 10 min. Samples were then fixed with glutaraldehyde, postfix with OsO4, and stained with uranyl acetate and lead citrate, as described (22).

Immunoblotting of Gradient Fractions—Thirteen gradient fractions were collected from the top, and their proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. After transfer, nitrocellulose sheets were stained with Ponceau S to visualize protein bands. Immunoblotting was performed using 12CA5 ascites (1:500) to detect the HA-tagged STE18 gene products; polyclonal antibodies against the plasma membrane H-ATPase, Gas1p, Gα-subunit, or the Gβγ-subunit were used to detect the respective proteins. Bound antibodies were visualized on radiographic films (Eastman Kodak Co.) using an ECL detection kit. Quantification of the autoradiographs was done using the MacBAS (version 2.2; Fuji Photo Film Co., Ltd.) program after scanning the radiographic films into the Adobe Photoshop 3.0 program. Protein quantitation was done using the BCA assay (Pierce).

Octyl-glucoside Extractability—The fractionation procedure was as described above except that 60 mM octyl-glucoside was included in addition to Triton X-100 during the extraction period.

RESULTS

Purification of Yeast LDTI Membranes—On the basis of the biochemical properties of mammalian LDTI membrane domains, a very discrete buoyant density and Triton X-100 insolvibility, we purified similar structures from the yeast Saccharomyces cerevisiae. The purification procedure is outlined schematically in Fig. 1A. Briefly, yeast cells were disrupted by vigorous vortexing in the presence of glass beads. After a 500 × g spin, the resulting supernatant was clear of unbroken cells, as assessed microscopically, and was designated as the cell
lysatetoobtainatotalmembranepreparation devoidofsolubleproteins,thecelllysatewasthencentrifugedfor1 h at 100,000 \times g at 4°C. Theresulting pellet was resuspended in MBS (Mes buffered saline, pH 6.5) including 60% sucrose. Triton X-100 was added to a final concentration of 1%, and the suspension was further homogenized using a Polytron homogenizer. The extraction was performed on ice for 30–60 min. The mixture was then overlaid with a sucrose step gradient ranging from 5–50% sucrose and centrifuged for 16–20 h at 250,000 \times g at 4°C. A single light-scattering band was observed at a density corresponding to ~40% sucrose (fraction 5; designated yeast Triton X-100 insoluble membrane domains). Fig. 1B shows the distribution of total cellular proteinGas1p across the gradient, as visualized by Ponceau S staining, after transfer to nitrocellulose. Note that the detergent Triton X-100 solubilized most of the membrane proteins, which consequently remain within the bottom fractions or pellet of these sucrose density gradients (fractions 8–13).

Morphological Characterization of Yeast LDTI Membranes— Purified yeast Triton X-100 insoluble structures (fraction 5) were next subjected to morphological analysis by transmission electron microscopy. The isolated yeast membranes appeared as 100–130-nm closed circles and curved membrane fragments. Figure 2 shows the distribution of total cellular membrane proteins. The isolated yeast membranes appeared as 100–130-nm vesicular structures of varied shape (Fig. 2). Although only slightly larger than their mammalian counterparts, a similar variation in size and shape was observed during the study of mammalian LDTI membrane domains (23, 24). This may be related to the ability of these domains to remain totally flat or invaginated while still attached to the plasma membrane and within the Golgi complex.2

Distribution of Plasma Membrane Protein Markers Relative to Yeast LDTI Membranes—Western blot analysis was used to investigate the distribution of two well known plasma membrane proteins, the H+-ATPase (Pma1p) and the GPI-linked protein Gas1p, during the fractionation procedure. Fig. 3 shows that both Pma1p and Gas1p are predominantly detergent-soluble and remain mainly within the bottom fractions of these sucrose density gradients (fractions 8–13). Quantitation of the result indicates that Pma1p is partially excluded from fraction 5 (Fig. 7, A and F). This is in agreement with results obtained with mammalian cells, where another plasma membrane ATP-dependent ion transporter, the Na+/K+-ATPase, is excluded during the purification of LDTI (3). Similarly, several reports indicate that GPI-linked proteins are also predominantly excluded during the purification of mammalian LDTI membrane domains (7, 25, 26).

Targeting of G-Protein Subunits—G-protein coupled receptors, G-protein-modifying bacterial toxins, and G-proteins (α and βγ subunits) have all been localized to mammalian LDTI membrane domains (6, 27). In yeast cells, heterotrimeric G-protein subunits serve to regulate the pheromone response during yeast mating (28). The G\textsubscript{a} subunit (Gpa1p) acts as a negative regulator of the G\textsubscript{a} subcomplex (Ste4p and Ste18p). Thus, we chose to investigate the possible localization of these G-protein subunits within yeast LDTI membranes. In addition, we performed mutational and genetic analyses to define the lipid requirements for targeting of G-protein subunits to these yeast LDTI membrane domains.

G\textsubscript{a} Subunit—The yeast G\textsubscript{a} subunit, Gpa1p, was overexpressed from a plasmid, and its distribution was followed during cellular fractionation using polyclonal antibodies. In striking contrast to the plasma membrane H+–ATPase and Gas1p, the G\textsubscript{a} subunit showed a bimodal distribution in the sucrose density gradients (Fig. 4), and quantitation revealed that about 15% of it was present within the detergent-insoluble membrane structures in fraction 5 (Fig. 7B) and that it is more than 2-fold enriched relative to the H+–ATPase (Fig. 7F). The remainder was solubilized by Triton X-100 and was restricted to the bottom fractions of the sucrose gradient or the pellet (fractions 8–13). This bimodal distribution of Gpa1p agrees well with the recent finding that Gpa1p is localized both at the plasma membrane and within the Golgi complex.2

As the G-protein α subunit (Gpa1p) undergoes myristoylation upon addition of pheromone (20), this lipid modification could serve to regulate the localization of Gpa1p within a specific domain of the plasma membrane. To investigate a requirement for myristoylation, we expressed a point mutant G2A of Gpa1p that fails to undergo myristoylation. Figs. 4 and 7B show that G2A Gpa1p behaved as the wild-type protein with respect to its localization to LDTI membranes (fraction 5). This indicates that myristoylation is not required for the targeting of Gpa1p to yeast LDTI membrane domains.

G\textsubscript{γ} Subunit—In mammalian cells, prenylation of the G\textsubscript{γ} subunit at its C-terminal CAAX motif is important for its function and membrane localization (29). To examine if the yeast G\textsubscript{γ-}
subunit also resides in LDTI structures, we followed an HA-tagged version of the STE18 gene product expressed from a plasmid (21). As observed with the yeast Gα subunit (Gpa1p), the yeast Gγ subunit (Ste18p) also showed a similar bimodal distribution during fractionation, with ~35% confined to the yeast LDTI membrane fraction in fraction 5 (Fig. 5) and an enrichment factor relative to the plasma membrane H^+-ATPase of almost 5 (Fig. 7F).

To evaluate a possible requirement for prenylation in the targeting of Ste18p to LDTI membranes, we next expressed two mutants of ste18 (Q94stop and C107Y) that no longer undergo prenylation because their C-terminal C107AAX motif has been removed. Q94stop contains a premature termination codon and, therefore, lacks the C-terminal 16 amino acids of ste18, including the CAAX motif. C107Y converts the C-terminal CAAX motif to YAAX, thereby preventing prenylation (21). A Western blot experiment (Fig. 5) and its quantification show that both of these mutant proteins were still targeted to the total membrane fraction but failed to localize to LDTI membrane structures. Therefore, in contrast to myristoylation of the Gα subunit, prenylation of the Gγ subunit is required for its localization to yeast LDTI membranes.

Gβ Subunit—The Gβ subunit interacts tightly with the Gγ subunit in mammalian cells and yeast (30). Not surprisingly, the yeast Gβ subunit (encoded by the STE4 gene) colocalized with the yeast Gγ subunit in our fractionation protocol (see below). However, the yeast Gβ subunit (Ste4p) does not undergo fatty acylation and does not contain a transmembrane domain. As such, membrane localization of the Gββ subunit must depend on its interaction with a membrane bound protein, such as the Gγ subunit.

Thus, we next examined if the targeting of the Gβ subunit to LDTI membranes is strictly dependent on the coexpression of the Gγ subunit. Using a polyclonal antibody directed against the Gγ subunit (Ste4p), we evaluated its targeting in a yeast strain deleted for the Gγ subunit gene (ste18Δ). Figs. 6 and 7D show that targeting of the Gβ subunit to LDTI membranes was not affected and that it is highly enriched within these structures (~5–7 fold; Fig. 7F) in the yeast strain lacking the Gγ subunit. This suggests that a protein other than the Gγ subunit (Ste18p) may be the membrane carrier protein for the Gβ subunit.

Selective Detergent Solubilization of Yeast LDTI Membranes—LDTI membranes purified from mammalian cells are selectively solubilized by the detergent octyl-glucoside (2, 4, 31). This finding has been attributed to the resemblance of the detergent to glycolipids, a major lipid component of LDTI membranes (6, 27, 32), thus making it possible to effectively disrupt the glycolipid-rich microenvironment of LDTI membranes.

Yeast cells also synthesize glycosphingolipids, and these glycolipids are targeted to the plasma membrane (33). As a first step toward comparing the lipid components of yeast LDTI membranes to those of mammalian LDTI membranes, we extracted total yeast membranes with octyl-glucoside and Triton

**Fig. 4.** Subcellular fractionation of yeast cells expressing different alleles of the G-protein α subunit (Gpa1p). Fractionation and Western blot analysis of yeast cells expressing either wild-type or a nonmyristoylated mutant (G2A) of Gpa1p from a plasmid. Fractionation is as described in Fig. 1. Note that ~15% of the Gα subunit localizes to the LDTI membrane domains (fraction 5); this association is not affected by a mutation that prevents myristoylation of Gpa1p. In addition, the fractionation behavior of Gpa1p is very similar to that observed with Gαα expressed in mammalian cells (17).

**Fig. 5.** Subcellular fractionation of yeast cells expressing different HA-tagged alleles of the G-protein γ subunit (Ste18p). A, fractionation and Western blot analysis of yeast cells expressing either the HA-tagged wild-type allele of STE18 or prenylation mutants (Q94stop or C107Y). A significant amount (~35%) of the wild-type STE18 gene product is targeted to LDTI structures (fraction 5), while the remainder is found within the pellet fraction. In striking contrast, note that two nonprenylated mutants of Ste18p are excluded from the LDTI fraction (fraction 5) and are exclusively found within the pellet fraction. B, schematic diagram summarizing the targeting to LDTI membrane structures of wild-type and mutant alleles of HA-tagged STE18. In addition to the targeting of wild-type and nonprenylated mutants of Ste18p, the targeting of two other dominant-negative alleles of the STE18 gene (R48H, E49K, and R34K) are shown.
X-100 prior to sucrose gradient fractionation. Fig. 8 shows that, as expected, octyl-glucoside extracts most membrane proteins from the yeast LDTI fraction as compared with Triton X-100 alone. In addition, inclusion of octyl-glucoside led to the disappearance of the light-scattering membraneous band that normally corresponds to the LDTI membrane fraction. Thus, in terms of their differential detergent solubility, yeast LDTI membranes behave as expected from experiments with purified mammalian LDTI membranes.
concentrated in plasma membrane LTDI structures, it appears that these structures are well suited to act as organizing centers for transducing external signals.

Here, we show that the similar membranous structures may exist in the yeast *Saccharomyces cerevisiae* for the compartmentalization of heterotrimeric G-proteins. As with purified mammalian caveolae, purified yeast LTDI membrane domains: (i) appeared as 100–130-nm vesicular structures; (ii) were enriched in heterotrimeric G-proteins (*G*α and *G*βγ subunits); (iii) preferentially excluded other plasma membrane marker proteins; and (iv) were selectively solubilized by the detergent octyl-glucoside. Our current biochemical observations are directly supported by the earlier morphological observations of Mulholland *et al.* (19), who observed invaginations at the plasma membrane of intact yeast cells by electron microscopic techniques that are similar to the mammalian caveolae plasma membrane invaginations.

**Targeting of Heterotrimeric G-Proteins—**During sucrose density gradient fractionation, the distribution of *G*α and *G*γ subunits was completely overlapping, whereas the *G*β subunit demonstrated a much broader distribution within the same sucrose gradients (compare Figs. 4–6 or see Fig. 7, B–D). This difference in distribution can be attributed to the recent finding that the *G*β subunit is also found within the Golgi apparatus, where it may function in regulating yeast vesicular transport.2 The enrichment factors of the G protein subunits range from 2.2 (for *G*α), 4.6 (for *G*γ), and 7 (for *G*β). However, due to overexpression of the *G*α and the *G*β subunits leading to possible mistargeting, these proteins may be underrepresented within LTDI membrane domains in our experiments.

Interestingly, two nonprenylated mutants of *G*α (Q94stop; C107T) failed to be targeted to the LTDI membrane fraction in yeast, despite the fact that they remained membrane-bound (Fig. 5). This agrees well with the recent finding that prenylation of the Ras protein is required for its correct targeting to caveolae in mammalian cells (7). We also found that targeting of the *G*β subunit to LTDI structures is independent of the coexpression of the *G*α subunit (Fig. 6). This is surprising, because in mammalian cells, the expression and membrane targeting of the *G*β and *G*γ subunits are interdependent (35). This suggests that a second as yet unidentified G-protein γ subunit may exist in yeast that fulfills a role similar to Ste18p. Alternatively, a scaffolding protein may exist in yeast for organizing and concentrating G-protein subunits within these LTDI membrane domains.

**Do Yeast Cells Have Caveolae?—**In mammalian cells, the LTDI membrane structures that contain the protein caveolin are thought to represent caveolae. There, caveolin is suggested to have an important role as a scaffolding protein to organize and concentrate G-proteins within these structures (36). Are the yeast LTDI membrane domains caveola-like structures? Although these structures have many characteristics in common with mammalian caveolae, a definite answer must await the identification of a functional yeast homologue of mamma-

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