Supporting Information

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SI Text

Carboxy-TEMPO NO\(^+\) Is a Topological Probe. To verify that carboxy-TEMPO NO\(^+\)-mediated oxidation of MPD in intact vesicles was restricted to MPD molecules located in the outer leaflet, we tested to see whether carboxy-2,2,6,6-tetramethylpiperidin-1-oxyl NO\(^+\) could oxidize a molecule that was trapped in the vesicle lumen. We included GDP-[\(^2\)H]mannose as a soluble intraluminal marker over a range of protein amounts in our reconstructed systems. We verified that in both liposome and proteoliposome preparations GDP-[\(^2\)H]mannose was protected from oxidation by carboxy-TEMPO NO\(^+\) (Fig. S1; lanes 2–4) as observed by thin layer chromatography (TLC); however, in vesicles the analyzer. Citronellyl phosphate was detected in the negative ion mode and 

Diethylpyrocarbonate (DEPC) Treatment. Triton extract (TE) (40 μL, 1.5 mg/mL) was diluted in 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1% (w/v) Triton X-100 to a total volume of 150 μL. 50 μL DEPC (from a freshly prepared stock of 40 mM in 10 mM Tris-HCl, pH 7.4) was then added and the sample was incubated for 10 min at room temperature before being taken for reconstruction. A mock-treated sample was processed in parallel. Fig. S2 shows that DEPC treatment of TE essentially eliminates MPD flippase activity. This result parallels a previous report (2) of decreased mannose-phosphate citronellol transport across proteoliposomes generated from a detergent extract of DEPC-treated rat liver microsomes.

ESI-MS Analysis of Isoprenyl Phosphates Used in Competition Assays. The structure of each of the isoprenyl monophosphates used in the competition experiments described in Fig. 3 was confirmed by mass spectrometry. Electrospray ionization mass spectra (ESI-MS) were acquired using a Waters Acuity SQD mass spectrometer; liquid chromatography (LC) was performed using a Waters Acuity Ultra Performance Liquid Chromatograph. Samples were dissolved in CH\(_3\)OH/0.1% HCOOH and injected directly into the analyzer. Citronellol phosphate was detected in the negative ion mode as [M – H\(^-\)]\(^-\) ion with an m/z of 235.11 (expected m/z for [C\(_{10}\)H\(_{18}\)O\(_5\)P]: 236.12), geranyl phosphate was detected as [M – H\(^-\)]\(^-\) ion with an m/z of 233.21 (expected m/z for [C\(_{10}\)H\(_{16}\)O\(_5\)P]: 234.10) and neryl phosphate was detected as [M – H\(^-\)]\(^-\) ion with an m/z of 233.10 (expected m/z for [C\(_{10}\)H\(_{16}\)O\(_5\)P]: 234.10). The mass spectral analyses established the correct elemental composition of all three isoprenyl phosphates.

Chemical Synthesis of Nonradiolabeled α-Mannose-P-dolichol (M(α)PD). M(α)PD (Fig. S3A) was synthesized in four steps. Mannose was peracetylated, then phosphorylated to yield 2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl phosphate and coupled to dolichol. The resulting product was deprotected to yield M(α)PD.

Peracetylation of mannose was achieved as described (3). Briefly, a solution of acetic anhydride (1.0 mL) anhydrous pyridine (1.3 mL) and mannose (120 mg) was stirred for 24 h and the product was crystallized from ice-cold water. Formation of the product was verified by ESI-MS. 1,2,3,4,6-penta-O-acetyl-α/β-D-mannopyranosyl was detected in the positive ion mode as a sodium adduct [M + Na\(^+\)] with an m/z of 413.33 (expected m/z for [C\(_{16}\)H\(_{32}\)O\(_9\)Na]: 413.12). The product yield was 65%.

Synthesis of 2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl phosphate was achieved as described (4). Briefly, dried crystalline phosphoric acid was added to a solution of mannose pentaacetate (100 mg) in dry diethyl ether, heated to 65°C (oil bath) and stirred for 2 h. To the resulting solution (dissolved in anhydrous tetrahydrofuran) concentrated ammonium hydroxide was added. TLC analysis revealed that the major product had an R\(_f\) of 0.40 (solvent: CH\(_2\)Cl\(_2\)/CH\(_3\)OH/H\(_2\)O, 60/35/6 (v/v/v)); a minor contaminant had an R\(_f\) of 0.3. The major product was purified and its molecular identity verified by ESI-MS and NMR (\(^1\)H and 31P). 2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl phosphate was detected as [M – H\(^-\)]\(^-\) ion at m/z of 427.27 (expected m/z for [C\(_{14}\)H\(_{28}\)O\(_8\)P]: 428.07) in the negative ion mode and an acetate 

Chemical Synthesis of [\(^3\)H]M(α)PD. Radiolabeled M(α)PD was synthesized as described above for nonradiolabeled M(α)PD except that [\(^3\)H]mannose (specific activity; 20 Ci/mmol) was used and reaction products were TLC purified at every step. The final product was radiochemically pure as seen by the appearance of a single peak on TLC (Fig. S3B).

Carboxy-TEMPO NO\(^+\)-Mediated Oxidation of [\(^3\)H]M(β)PD. To verify carboxy-TEMPO NO\(^+\)-mediated oxidation of MPD to its corresponding mannuronic acid, we oxidized nonradiolabeled M(α)PD under our standard assay conditions. M(α)PD was dissolved in 0.5% (w/v) Triton X-100 and treated with carboxy-TEMPO NO\(^+\) for 30 min on ice; a sample without carboxy-TEMPO was mock treated in parallel. Lipids were extracted into chloroform/methanol 2/1 (v/v) and analyzed by LC/ESI-MS in the positive ion mode. An increase of 14 mass units in the carboxy-TEMPO-treated sample confirmed oxidation of the primary hydroxyl on mannose to the carboxylic acid group. To establish that both [\(^3\)H]M(α)PD and [\(^3\)H]M(β)PD were oxidized with similar kinetics in liposomes, the lipids were separately reconstituted into liposomes. [\(^3\)H]M(α)PD (or [\(^3\)H]M(β)PD)-containing liposomes, as well as Triton X-100-disrupted liposomes, were treated with carboxy-TEMPO NO\(^+\) for 0–50 min on ice and processed to determine the extent of MPD oxidation. As shown in Fig. S4, the kinetics of oxidation of [\(^3\)H]M(α)PD and [\(^3\)H]M(β)PD were similar in intact liposomes, as well as in Triton X-100-disrupted vesicles (pseudo first order time constant τ = 6–9 min in all cases). Oxidation of liposome samples and Triton X-100-disrupted vesicles yielded the expected maximum of ~50% oxidation (indicating protection of 50% of the [\(^3\)H]MPD molecules in the inner leaflet) and 100% oxidation, respectively.
Con A-Sepharose Chromatography. Chromatography was done at 4 °C. TE (1.2 mg/mL) was adjusted to 10 mM Hepes-NaOH, pH 7.4, 50 mM NaCl, 0.5% (w/v) TX-100, then incubated with Con A-Sepharose (500 μL bed volume; preequilibrated with 10 column volumes of 10 mM Hepes-NaOH, pH 7.4, 50 mM NaCl, 0.5% (w/v) TX-100) by rotating end-over-end for 2 h. The supernatant was removed and the resin was washed with 10 column volumes of equilibration buffer. The first wash and supernatant were combined (flow-through fraction). Bound proteins were eluted over a 3 × 24 hr period with 3 × 500 μL of 0.5 M α-methylmannoside supplemented with 10 mM Hepes-NaOH, pH 7.4, and 0.5% (w/v) TX-100. The eluted fractions were combined. The load, flow-through, and salt-eluted fractions were adjusted to 10 mM Hepes-NaOH, pH 7.4, 100 mM NaCl and 1% (w/v) TX-100 on a Biogel-P6 desalting column. Aliquots of each sample were taken for SDS-PAGE immunoblotting (Fig. S5) while the remainder was reconstituted into proteoliposomes and tested for MPD and M5-DLO flippase activities.

DE-52 Chromatography. Chromatography was done at 4 °C. TE (1.2 mg/mL) was adjusted to 20 mM Tris-HCl, pH 8.5, 50 mM NaCl, 0.25% (w/v) TX-100, then incubated with DE-52 resin (500 μL bed volume; preequilibrated with 10 column volumes of 20 mM Tris-HCl, pH 8.5, 50 mM NaCl, and 0.25% (w/v) TX-100) by rotating end-over-end for 2 h. The supernatant was removed and the resin was washed with 10 column volumes of equilibration buffer. The first wash and supernatant were combined (flow-through fraction). Bound proteins were eluted with 1.0 M NaCl supplemented with 20 mM Tris-HCl, pH 8.5, and 0.25% (w/v) TX-100. The load, flow-through, and salt-eluted fractions were adjusted to 10 mM Hepes-NaOH, pH 7.4, 100 mM NaCl, and 1% (w/v) TX-100 by desalting over Biogel P-6, reconstituted into proteoliposomes and tested for MPD, M5-DLO, and PL flippase activities.


**Fig. S1.** Intravesicular GDP-[2-3H]mannose is not oxidized by carboxy-TEMPO NO•. Proteoliposomes and liposomes were reconstituted in the presence of GDP-[2-3H]mannose (~106 cpm). Vesicles were collected by centrifugation (~200,000 × g, 30 min, 4 °C), washed extensively to remove unincorporated GDP-[2-3H]mannose and incubated with carboxy-TEMPO NO• for 30 min on ice. Parallel samples were treated with 0.2% (v/v) TX-100 to disrupt the vesicle membrane before adding carboxy-TEMPO NO•. Reactions were quenched and samples were subjected to lipid extraction as described for the MPD flippase assay. The aqueous phase containing GDP-[2-3H]mannose and/or its oxidized product was dried in a Speedvac; the residue was dissolved in EtOH and resolved by thin layer chromatography on silica-60 (solvent: BuOH/EtOH/0.1 NHCl:1/10/6 (v/v/v)). The chromatogram was exposed to a tritium screen and visualized with a phosphorimager. Lane 1, GDP-[2-3H]mannose (standard); Lanes 2–4, GDP-[2-3H]mannose-containing vesicles with PPR 0, 10, and 50 mmol, respectively, treated with carboxy-TEMPO NO• for 30 min on ice; Lane 5, GDP-[2-3H]mannose-containing vesicles disrupted with Triton X-100 before being treated with carboxy-TEMPO NO• for 30 min on ice.

**Fig. S2.** MPD-flippase activity is largely abolished by DEPC treatment. TE was treated with either 10 mM DEPC, or mock treated prior to reconstitution and assay of MPD-flippase activity. Data represent mean ± range from two independent experiments.
Fig. S3. M(α)PD. (A) Chemical structure of M(α)PD. (B) Thin layer chromatogram of [$^3$H]M(α)PD demonstrating radiochemical purity of the chemically synthesized material. Analysis was done on a silica 60 thin layer plate using CHCl$_3$/CH$_3$OH 5:1 (v/v) as the solvent system. The chromatogram was visualized using a Berthold LB2842 TLC scanner.

Fig. S4. Kinetics of oxidation of [$^3$H]M(α)PD in intact and Triton X-100-disrupted liposomes. Intact and Triton X-100-disrupted (+TX-100) liposomes containing [$^3$H]M(α)PD were incubated with carboxy-TEMPO NO$^+$ for 0–50 min on ice. Lipids were extracted and the extent of [$^3$H]M(α)PD oxidation was determined by TLC and peak integration using software provided with the TLC scanner. [$^3$H]M(β)PD-containing samples were analyzed alongside. The extent of oxidation in the liposome samples was 50%, whereas that in the Triton X-100-disrupted liposomes was 100%. The pseudo first order time constant for oxidation was $\tau \sim 7.1$ and 6.3 min in liposomes containing M(α)PD and M(β)PD, respectively, and $\tau \sim 8.8$ and 9.4 min for Triton X-100-disrupted liposomes containing M(α)PD and M(β)PD, respectively.

Fig. S5. Separation of glycoproteins from nonglycoproteins by Con A-Sepharose chromatography. TE was fractionated on Con A-Sepharose. Fractions ("flo" (unbound) and "elut" (bound to Con A-Sepharose and eluted with α-methylmannoside)) as well as TE were analyzed by SDS-PAGE and immunoblotting to probe the distribution of the nonglycosylated SRP receptor α-subunit (Srα) and the glycoproteins ribophorin I (RpnI) and signal sequence receptor α-subunit (Ssrα). The data show that RpnI and Ssrα are recovered in the bound and eluted material whereas Srα does not bind to the resin, confirming separation of glycoproteins from nonglycoproteins.