Flip-flop of glycosylphosphatidylinositols (GPI's) across the ER†

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The transbilayer flip-flop of early intermediates in the glycosylphosphatidylinositol (GPI) biosynthetic pathway has been demonstrated using novel fluorescent GPI probes and a biochemical reconstitution approach.

Flip-flop of lipids across biomembranes is a fundamental feature of membrane biogenesis.[1] Glycerophospholipids, the building blocks of membrane bilayers, are made on the cytoplasmic face of biogenic (self-synthesizing) membranes like the endoplasmic reticulum (ER) and must be flipped to the opposite face (lumen) for bilayer expansion. Flipping is not spontaneous and requires specific proteins (flippases) that facilitate ATP-independent, bi-directional lipid flip-flop. Glycerophospholipid flippases (distinct from plasma membrane lipid translocators like the ABC transporters) have yet to be identified in biogenic membranes and the mechanism of lipid flipping remains a mystery.[1]

The issue of lipid flipping acquires an additional dimension when one notes that major pathways of protein and lipid glycosylation in eukaryotes and prokaryotes require flipping of biosynthetic lipid intermediates.[1] A prominent example is the ER-localized synthesis of glycosylphosphatidylinositol (GPI) anchored proteins, a family of cell surface proteins that plays key roles in signaling, cell adhesion and immunology, as well as in the biology of parasitic protozoa. GPI-proteins are synthesized from a precursor GPI lipid and an ER-translocated protein bearing a GPI signal sequence. A number of years ago we discovered[2] that GPI synthesis begins on the cytoplasmic side of the ER (Fig. 1). Since GPI addition to proteins occurs in the lumen[3] this implies that a GPI intermediate flips across the ER during GPI assembly; although indirect data suggest that this happens at the GlcN-PI or GlcN-acyl-PI stage (Fig. 1),[2,3] there has been no direct demonstration of this critical flipping step. It is also unclear whether GPI flipping, like that of glycerophospholipids, occurs *via* an ATP-independent process mediated by specific proteins. As part of ongoing efforts to elucidate the molecular details of GPI biosynthesis, we now report the synthesis of novel, functional GPI probes and use these to demonstrate, for the first time, ATP-independent, protein-mediated flip-flop of GPI lipids.

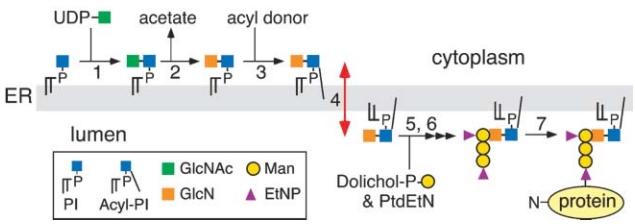

Fig. 1 Topology of GPI biosynthesis in the ER. The first GPI biosynthetic intermediate, GlcNAc-PI, is synthesized (step 1) from PI, then de-*N*-acetylated (2) to GlcN-PI on the cytoplasmic face of the ER. GlcN-PI or an inositol-acylated derivative (as shown) is proposed to flip across the ER membrane (4). Subsequent elaboration of the structure (5, 6) occurs in the ER lumen where GPI is attached to protein (step 7).

We recently used biochemical reconstitution to show that glycerophospholipid flipping in the ER requires specific proteins.[4] We did this by making proteoliposomes from a Triton X-100 extract (TE) of ER and using phospholipid analogs as well as natural phospholipids to report on flippase activity.[4] In the simplest procedure, trace amounts of fluorescent acyl-NBD-labeled phospholipids were included during reconstitution and flipping of the NBD-lipids in reconstituted vesicles was assayed with dithionite, a membrane-impermeant dianion that destroys the NBD moiety of lipids in the outer leaflet of vesicles.[5] We note that although acyl-NBD-phospholipids display a non-natural looping back of the NBD-bearing acyl-chain such that the polar NBD group is positioned at the membrane–water interface,[6] they are nevertheless excellent reporters of flippase activity. Where comparisons have been made, acyl-NBD lipid reporters yield data that are qualitatively—if not quantitatively—similar to those obtained with other analogs as well as natural phospholipids.[4,6,7] Because of the convenience of a fluorescence-based assay and the usefulness of acyl-NBD lipids as reporters of lipid translocation, we developed acyl-NBD-labeled GPI probes to investigate GPI flipping in the ER. Building on our previous work[8] we synthesized NBD-GlcNAc-PI and NBD-GlcN-PI (Fig. 2). Although approaches for GPI synthesis have been described,[9] including one for placing a label in the glycan domain, synthesis of fluorescent GPI probes labeled in the glycerolipid domain required a new strategy (Scheme 1).

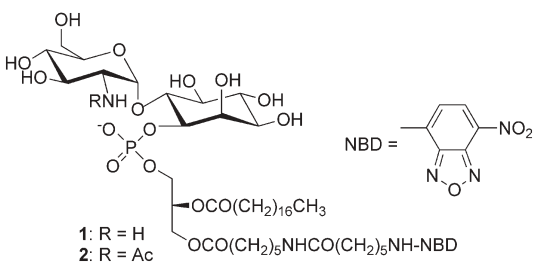

Fig. 2 Structures of NBD-GlcNAc-PI and NBD-GlcN-PI.

† Electronic supplementary information (ESI) available: Details of vesicle preparation, flippase assay and synthesis of **1** and **2**. See http://www.rsc.org/suppdata/cc/b4/b413196g/

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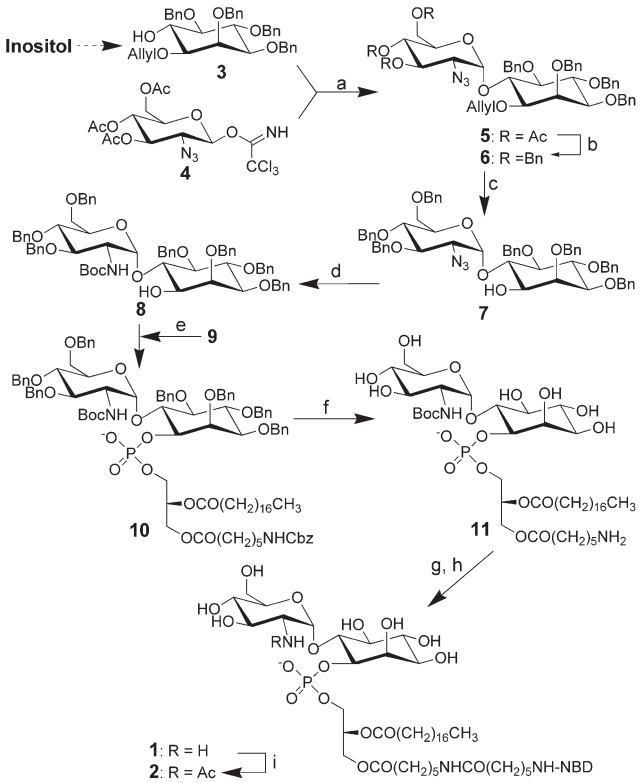

Scheme 1 Synthesis of NBD-GlcNAc-PI and NBD-GlcN-PI. *Reagents and conditions*: (a) TMSOTf, CH_2Cl_2, 0 °C, 0.5 h, 90%, (b) NaOMe, CH_2Cl_2–MeOH, rt, 24 h; then NaH, BnBr, DMF, 80%, (c) $PdCl_2$, NaOAc, AcOH–H_2O, rt, 48 h, 64%, (d) propanedithiol, Py–H_2O, Et_3N, rt, 24 h; then Boc_2O, rt, 12 h, 66%, (e) lipid-H-phosphonate **9**, Py, Piv–Cl, rt, 0.5 h; then I_2 in Py–H_2O, rt, 0.5 h, 57%, (f) $Pd(OH)_2$, MeOH–CH_2Cl_2–H_2O, H_2, 12 h, 85%, (g) NBD-X,SE, DMF, Et_3N, 2 h, rt, (h) TFA–CH_2Cl_2–CH_3CN, 4 : 4 : 2, rt, 2 h, 55%, (i) Ac_2O, $NaHCO_3$–MeOH, rt, 0.5 h, quant.

Our synthetic design involved three chiral building blocks: (a) 1-allyl-2,3,4,5-tetra-*O*-benzyl-D-myo-inositol **3** made in 7 steps from bis-cyclohexylidene-D-inositol, (b) the 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-β-D-glucosyl donor **4** prepared from tri-*O*-acetyl-D-glucal by azidonitration and (c) the phosphatidyl donor **9** with a protected terminal amine in the *sn*-1 acyl chain prepared from 1,2-isopropylidene-*sn*-glycerol. Glycosylation of **3** with **4** gave the α-glucosaminyl(1 → 6)inositol intermediate **5** followed by deprotection of the allyl group and replacement of the azido group with NHBoc to enable the eventual selective coupling of the NBD probe. Phospholipidation of **8** with **9** using H-phosphonate chemistry gave the protected GPI intermediate **10**. The next three steps, removal of benzyls, coupling of the NBD probe, and deprotection of the NHBoc group, provided NBD-GlcN-PI (**1**), which on *N*-acetylation gave NBD-GlcNAc-PI (**2**).

We made unilamellar proteoliposomes from TE, egg phosphatidylcholine (egg PC) and trace amounts of NBD-GPI (**1** or **2**). Protein-free liposomes were prepared by omitting TE. Using ES-MS we found no evidence of degradation of the NBD-GPI's during reconstitution. Since the NBD-GPI's are presumed to be symmetrically distributed in the vesicle membrane,[4] addition of dithionite to liposomes should cause ~50% fluorescence loss; treatment of flippase-active proteoliposomes should yield ~100%

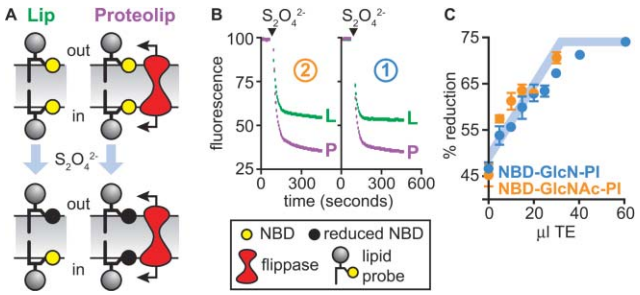

Fig. 3 Flipping of NBD-GPI's. (A) Schematic showing the predicted 50% fluorescence loss on adding dithionite to symmetrically labeled liposomes (or inactive proteoliposomes) (left; labeled 'Lip'), compared with 100% loss in flippase-active proteoliposomes ('Proteolip') due to flipping of NBD-GPIs.[4] The looping back of the NBD-bearing acyl-chain positions the polar NBD group at the membrane–water interface where it can react with dithionite.[6,7] (B) Fluorescence traces of assays with liposomes (L) and proteoliposomes (P) containing NBD-GPI's (**1** or **2**). (C) Protein dependence of the extent of dithionite reduction of NBD-GPI's. The blue line is a fit of the data based on a model described here (see ESI† for details) and elsewhere.[4]

loss, since NBD-lipids in the inner leaflet will flip out and become accessible to dithionite (Fig. 3A).[4]

Fluorescence dropped rapidly by ~45% when dithionite was added to NBD-GPI-labeled liposomes (Fig. 3B, green traces), and was eliminated when the vesicles were detergent-permeabilized indicating that dithionite was sufficient to reduce all NBD present and that the NBD-GPI probes were roughly symmetrically distributed. For proteoliposomes, dithionite caused a similarly rapid, yet greater fluorescence loss (Fig. 3B, purple traces) that (a) was reduced by protease treatment, (b) did not require ATP and (c) depended on the amount of TE used for reconstitution (Fig. 3C). Although fluorescence loss on dithionite addition is predicted to range from ~50–100% depending on the number of flippase-equipped vesicles in the sample (which in turn depends on the amount of TE used for reconstitution)[4] (Fig. 3A), the measured range was narrower (~45–75%; Fig. 3C), as reported previously for other phospholipid probes.[4] The reason for this is not fully understood.

The protein-dependence profile was the same for both GPI probes (Fig. 3C). As detailed and validated elsewhere,[4] at the inflection point of the profile, *i.e.*, the point at which the ~75% reduction plateau is first reached, each vesicle has one flippase. This occurs at TE ~ 32 µl where the protein : phospholipid ratio of the vesicles is ~21 µg µmol^{-1}. Using this we estimate that the flippase(s) responsible for transporting the GPI probes represents ~0.6% by weight of ER proteins in the TE (see below).[4]

Interestingly, the protein-dependence profile obtained in assays of NBD-PC flipping was similar to that obtained for the GPI probes (Fig. 3C and ESI†). It is possible, therefore, that the GPI probes are transported by the 'membrane building' ER glycerophospholipid flippase(s)[4] responsible for flipping NBD-PC. Alternatively, the two NBD-GPIs and NBD-PC may be flipped by different flippases that are nevertheless similarly abundant in the TE. Although our assay does not provide data on flipping kinetics, it is clear that both GPI probes are transported rapidly, on a time scale of ~1 min (Fig. 3A), similar to that measured for glycerophospholipid flipping in the ER.[7] Thus, in our reconstituted

system, the flippase(s) appears unable to distinguish between GlcNAc-PI, an intermediate that is not consumed in the ER lumen, and GlcN-PI, a substrate for lumenal mannosyl transferases (Fig. 1).

In summary, we have described the first generation of functional fluorescent GPI probes and used them to demonstrate that the ER possesses a transporter(s) capable of flipping GPIs in an ATP-independent manner. We are now in a position to track down this elusive transport protein(s) and to investigate the mechanism underlying this basic biological process.

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