Phospholipid flipping in biogenic membranes is a key feature of membrane bilayer assembly. Flipping is facilitated by proteinaceous transporters (flippases) that do not need metabolic energy to function. No flippase has yet been identified. The architecture of the E. coli protein translocon suggests that it could account for the flippase activity in the bacterial inner membrane. To test this possibility, we used E. coli cells depleted of SecYE or YidC to assay flipping in proteoliposomes reconstituted from detergent extracts of their inner membranes. We conclude that the protein translocon contributes minimally, if at all, to phospholipid flippase activity in the inner membrane.

Key words: Flippase / Phospholipid flip-flop / Reconstitution / Translocon / YidC.

The translocation of phospholipids across a membrane bilayer is central to the process of membrane assembly (Menon, 1995; Huijbregts et al., 2000; Sprong et al., 2001). Phospholipid biosynthesis in biogenic (self-synthesizing) membranes, such as the endoplasmic reticulum (ER) of eukaryotes and the bacterial cytoplasmic membrane, occurs in the cytoplasmic leaflet of the membrane. For bilayer growth, some of these lipids have to be transferred (flipped) to the exoplasmic (lumenal or periplasmic) leaflet. Transbilayer movement of phospholipids has been characterized in the ER (Bishop and Bell, 1985; Backer and Dawidowicz, 1987; Herrmann et al., 1990; Buton et al., 1996; Marx et al., 2000; Nicolson and Mayinger, 2000) and the bacterial cytoplasmic membrane (Rothman and Kennedy, 1977; Langley and Kennedy, 1979; Huijbregts et al., 1996, 1998; Hrafnsdóttir et al., 1997; Hrafnsdóttir and Menon, 2000; Kubelt et al., 2002) and demonstrated to occur via a rapid, metabolic energy-independent, bi-directional, facilitated diffusion process that is unspecific with respect to phospholipid headgroup and requires the participation of membrane proteins (Backer and Dawidowicz, 1987; Menon et al., 2000; Hrafnsdóttir and Menon, 2000). The membrane proteins involved are presumed to act as transport facilitators, providing a low-energy pathway for phospholipids to translocate between the two leaflets of the membrane bilayer. These facilitators, termed ‘flippases’, have yet to be identified.

A number of pore-forming peptides act as flippases (Fattal et al., 1994; Matsuzaki et al., 1996; Matsuzaki, 1998; Moll et al., 1998; Huang, 2000), suggesting that oligomeric, membrane-embedded pores can provide a transverse diffusion conduit for phospholipids. It can be envisaged that during transbilayer transit, the polar headgroups of phospholipids transit the aqueous lumen of the pore while the acyl chains ‘grease’ the packing space between peptide monomers in the pore complex. The most notable endogenous pore in the E. coli inner membrane is the protein-conducting channel, or translocon, which mediates the transmembrane translocation of secretory proteins, as well as the insertion of transmembrane proteins (Dalbey and Robinson, 1999; Johnson and van Waes, 1999; Driessen et al., 2001). The E. coli translocon is composed of the oligomeric SecYE translocase and several accessory factors (Dalbey and Robinson, 1999; Driessen et al., 2001). One of these accessory proteins, YidC, is required for the membrane integration of proteins that use the SecYEG translocon, as well as those such as the M13 coat protein and the N-terminal region of leader peptidase, that are inserted independently of SecYEG (Samuelson et al., 2000). YidC is a homolog of mitochondrial Oxa1p (Scotti et al., 2000) and chloroplast Albino3 (Moore et al., 2000) proteins, which are both implicated in membrane protein translocation in their respective organelles.

We previously hypothesized that the protein translocon could act as a phospholipid flippase (Menon et al., 2000). The translocon is evolutionarily conserved from mycoplasma to man and located in precisely the membranes that are endowed with flippase activity (Johnson and van Waes, 1999; Driessen et al., 2001). The translocon has a gated transverse aqueous channel that can open laterally toward the lipid bilayer to allow membrane insertion of hydrophobic protein transmembrane segments (Figure 1). It is likely that phospholipids line the lateral channel of the translocon, as shown in Figure 1, since photocrosslinking experiments show that translocating peptides can contact both the translocon and phospholipid (Martoglio et al., 1995; Mothes et al., 1997; Johnson and van Waes, 1999; Heinrich et al., 2000). Thus
the amphipathic architecture of the protein translocon could allow constitutive bi-directional phospholipid translocation between membrane leaflets in a fashion similar to that proposed for pore-forming peptides, with no requirement for metabolic energy.

YidC was chosen as a candidate because it is a conserved protein that was recently shown to have a function in the insertion of transmembrane sequences (Samuelson et al., 2000). YidC is associated with the translocon pore (Scotti et al., 2000; Beck et al., 2001) and appears to handle nascent transmembrane segments in a way that facilitates their transition from the pore into the lipid milieu (Beck et al., 2001; van der Laan et al., 2001; Samuelson et al., 2001; Urbanus et al., 2001). This suggests that YidC might be involved in the lateral opening of the SecYEG pore, a key feature of the hypothesis that the translocon machinery might facilitate phospholipid flip-flop (Figure 1).

To test the involvement of SecYE and YidC in phospholipid flip-flop, we used a reconstitution procedure that was described previously (Menon et al., 2000; Hrafnbsdöttir and Menon, 2000) in conjunction with a flippase assay using [3H]dibutyryl phosphatidylcholine (dilC4PC), a water-soluble radiolabeled phospholipid analog of phosphatidylcholine, as the transport substrate (Bishop and Bell, 1985; Menon et al., 2000; Hrafnsdottir and Menon, 2000). Proteoliposomes derived from detergent extracts of Bacillus membranes as well as rat liver ER were previously shown to be competent to transport dilC4PC as well as a long-chain, membrane-integrated phospholipid, dipalmitoyl-PC (Hrafnsdöttir and Menon, 2000; Gummadi and Menon, 2002). Rather than relying on kinetic information to measure phospholipid translocation, the dilC4PC transport assay in reconstituted vesicles provides a direct measure of the abundance of flippase units as a proportion of other Triton X-100-soluble membrane proteins (Menon et al., 2000; Hrafnsdöttir and Menon, 2000).

Although choline is not a naturally-occurring E. coli lipid head group, phosphatidylcholine was shown to be translocated in Bacillus megaterium and E. coli membranes as efficiently as the naturally occurring ethanolamine phospholipid (Hrafnsdóttir et al., 1997; Kubelt et al., 2002). Furthermore, it was shown (i) that transport depends on an intact membrane, (ii) that net transport stops when the intravesicular concentration of dilC4PC is equal to the concentration of dilC4PC in the extravesicular space, and (iii) that the intravesicular volume revealed by dilC4PC is consistent with that deduced from morphometric analyses and light scattering, as well as that deduced from measuring trapped markers such as [3H]inulin. These earlier experiments also showed that the transport amplitude does not increase once the protein concentration in proteoliposomes exceeds ~100 µg/µmol phospholipid. This last observation implies that dilC4PC is transported into the vesicles, and is not simply binding to vesicle protein (Menon et al., 2000; Hrafnbsdöttir and Menon, 2000).

To test the role of the protein translocon in phospholipid translocation we used bacterial strains in which the only copy of secE or yidC is located on a plasmid under the control of the AraBAD promoter. The depletion strains – CM124 (Traxler and Murphy, 1996) for SecE and JS7131 (Samuelson et al., 2000) for YidC—were grown in Luria Broth (LB) supplemented with 0.2% arabinose (LBA), centrifuged, washed in LB and diluted into LBA or LB + 0.2% glucose (LBG). The cultures were grown for 3–4 hours to deplete the glucose-grown CM124 cultures of SecE as reported (Traxler and Murphy, 1996), and the JS7131 cultures of YidC (Figure 2B). Loss of SecE resulted in the concomitant depletion of other translocon components as shown by the loss of SecY in LBG cultures (Figure 2A). Cultures were checked for reversion by plating on LBG and LBA agar plates. Strain JS7131 exhibited a rate of reversion of about 10^{-4} in an overnight arabinose culture, while no CM124 revertants were observed. Inner membranes were separated on sucrose density gradients according to Zhou et al. (Zhou et al., 1998) (Figure 3).

We reconstituted proteoliposomes from a Triton X-100 extract of inner membranes from LBA- or LBG-grown CM124 or JS7131 cells and assayed for dilC4PC trans-

![Fig. 1 Model (Top View and Perspective View) Showing How the SecYE Protein Translocon Could Facilitate Transbilayer Phospholipid Diffusion. The translocon is depicted as a trimeric structure with a single pore and three lateral openings (other oligomeric arrangements would work equally well for the model). The hydrophobic lateral opening and transverse aqueous pore of the translocon combine to form a diffusion conduit for phospholipids, connecting the opposite leaflets of the bilayer. YidC is thought to be important for the membrane insertion of hydrophobic sequences, and thus could have a potential role in providing a favorable environment for the phospholipid acyl chains.](image-url)
port. Experiments with proteoliposomes containing proteins from a Triton X-100 extract of wild-type cells showed that diC$_4$PC was transported into the vesicles, whereas protein-free liposomes were incapable of transport (Figure 4A), and that the extent of transport after 12 minutes incubation with diC$_4$PC depended on the proportion of transport-competent vesicles within the total vesicle population (Figure 4B). A convenient expression of specific activity of transport is to normalize the maximum extent of diC$_4$PC transport to the total intravesicular volume of the sample (determined by a soluble content marker, [³H]inulin) and to the protein/phospholipid ratio of the preparation (Menon et al., 2000; Hrafnisdóttir and Menon, 2000). Depletion of SecY or YidC in reconstituted vesicles relative to bulk membrane protein was verified by Western blot with equal amounts of vesicle protein. Depleted proteoliposomes contained less than 5% of the SecY or YidC in control proteoliposomes. The extent of transport after 12 minutes incubation with diC$_4$PC varied linearly with the protein/phospholipid ratio for all samples. The slopes of the dose-response lines (that is, the specific activities) were not significantly different between samples depleted of SecYE or YidC and the corresponding replete samples (Figure 4C, D), indicating that depletion of SecYE and YidC does not reduce the activity of phospholipid flippase in the inner membrane. These results demonstrate that the protein translocation apparatus is not necessary for the efficient transbilayer diffusion of phospholipids observed in the inner membrane.

Whereas our approach does not address whether the translocon has some capacity for inefficient facilitation of phospholipid movement on its own, it does eliminate it as a primary source of transbilayer movement. The diC$_4$PC transport assay in reconstituted vesicles allows us to consider the possibility of redundancy, i.e. whether the translocon or YidC can function as one of several flippases in the inner membrane. Reconstitution of proteoliposomes in the protein/phospholipid range where some in-
The travesicular space is inaccessible to diC4PC (Figure 4B) creates a system that is sensitive to the number of transporters and provides a means for testing redundancy. Consider the example of two molecularly distinct flippases present in equal amounts in the *E. coli* inner membrane. If these flippases account for all flippase units in the inner membrane, and if the expression of one of them is reduced by ~20-fold as in our experiments with SecYE, then one would observe a ~48% decrease in the slope of the dose-response line depicted in Figure 4B. The data (Figure 4) indicate that if SecYE translocase or YidC contributed at all to the flippase activity in the inner membrane, they would represent a minute fraction (<5%) of the flippase units in the inner membrane.

The experiments presented here demonstrate reconstitution of flippase activity from *E. coli* inner membranes. They also provide a general approach to determine whether a candidate protein is necessary for efficient bidirectional phospholipid transbilayer movement, and for gauging the extent of its contribution to the population of flippase units in the inner membrane. It is expected that this approach will become increasingly useful as biochemical purification and genetic analysis reveal other candidates for this essential activity.

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