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Membrane-Spanning Peptides Induce Phospholipid Flop: A Model for Phospholipid Translocation across the Inner Membrane of *E. coli*

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ABSTRACT: The mechanism by which phospholipids translocate (flop) across the *E. coli* inner membrane remains to be elucidated. We tested the hypothesis that the membrane-spanning domains of proteins catalyze phospholipid flop by their mere presence in the membrane. As a model, peptides mimicking the transmembrane stretches of proteins, with the amino acid sequence GXXL$(AL)_n$XXA (with X = K, H, or W and n = 8 or 12), were incorporated in large unilamellar vesicles composed of *E. coli* phospholipids. Phospholipid flop was measured by assaying the increase in accessibility to dithionite of a 2,6-(7-nitro-2,1,3-benzoxadiazol-4-yl)aminocaproyl (C_6NBD)-labeled phospholipid analogue, initially exclusively present in the inner leaflet of the vesicle membrane. Fast flop of C_6NBD-phosphatidylglycerol (C_6NBD-PG) was observed in vesicles in which GKKL$(AL)_{12}$KKA was incorporated, with the apparent first-order flop rate constant (K_{flop}) linearly increasing with peptide:phospholipid molar ratios, reaching a translocation half-time of ∼10 min at a 1:250 peptide:phospholipid molar ratio at 25 °C. The peptides of the series GXXL$(AL)_8$XXA also induced flop of C_6NBD-PG, supporting the hypothesis that transmembrane parts of proteins mediate phospholipid translocation. In this series, K_{flop} decreased in the order X = K > H > W, indicating that peptide−lipid interactions in the interfacial region of the membrane modulate the efficiency of a peptide to cause flop. For the peptides tested, flop of C_6NBD-phosphatidylethanolamine (C_6NBD-PE) was substantially slower than that of C_6NBD-PG. In vesicles without peptide, flop was negligible both for C_6NBD-PG and for C_6NBD-PE. A model for peptide-induced flop is proposed, which takes into account the observed peptide and lipid specificity.

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In the growing bacterial cell, newly synthesized phospholipids have to be translocated from their site of synthesis, the cytoplasmic leaflet of the plasma membrane (*1*), to the periplasmic leaflet. In Gram-negative bacteria, additionally transport from the plasma membrane to the inner leaflet of the outer membrane has to occur. Both processes have been studied in vivo and in vitro [for a recent review, see (*2*)], but the molecular mechanisms of these transport processes remain to be elucidated.

In vivo pulse labeling studies using the Gram-positive bacterium *B. megaterium* have shown that its major phospholipid, phosphatidylethanolamine (PE)[1] (*3*), is translocated rapidly from the inner to the outer leaflet with a half-time of translocation ($t_{1/2}$) of ∼3 min at 37 °C (*4*). This translocation process was demonstrated to be independent of sources of metabolic energy (*5*). A study on transport of phospholipids from the inner membrane to the outer membrane in the Gram-negative bacterium *E. coli* (*6*) showed that also this process is fast ($t_{1/2}$ = 2.8 min for PE), and independent of ATP and ongoing lipid and protein synthesis. Depletion of the pmf inhibited transport to the outer membrane. Recently the ATP-binding protein MsbA has been implicated in transport of phospholipids to the outer membrane of *E. coli* (*7*).

To dissect the mechanism of phospholipid transmembrane transport in bacteria, several in vitro approaches using isolated membranes have been followed. The translocation of newly synthesized PE across membrane vesicles of the *E. coli* plasma membrane was shown to be fast ($t_{1/2}$ <1 min), independent of ATP and proton motive force, and not affected by treatment with the sulfhydryl reagent *N*-ethylmaleimide (NEM) or protease (*8*). Similar results were obtained using short-chain NBD-labeled phospholipids (*9*). In vitro studies using membrane vesicles of *B. megaterium* showed that also in this system, short-chain NBD-labeled phospholipid analogues are rapidly translocated with half-times on the order of tens of seconds. This process is aspecific for the phospholipid headgroup and not affected by treating the vesicle preparations with NEM (*10*). In contrast to the results obtained in *E. coli* mentioned above, the process could be slowed by protease treatment of the vesicles.

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[1] Abbreviations: ATP, adenosine triphosphate; C_6NBD-PE, 1-palmitoyl-2,6-(7-nitro-2,1,3-benzoxadiazol-4-yl)aminocaproyl-*sn*-glycero-3-phosphoethanolamine; C_6NBD-PG, 1-palmitoyl-2,6-(7-nitro-2,1,3-benzoxadiazol-4-yl)aminocaproyl-*sn*-glycero-3-phospho-1-glycerol; CL, cardiolipin; NEM, *N*-ethylmaleimide; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL, phospholipid(s); pmf, proton motive force; TEA, triethanolamine; TFE, trifluoroethanol; TLC, thin-layer chromatography.

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In marked contrast with the fast flip-flop in bacterial membranes, lipid translocation is orders of magnitude slower in model membranes consisting of only phospholipids (*11*), also when these model membranes are prepared from phospholipids found in the bacterial inner membrane (*9*). This makes it highly likely that proteins are involved in translocation of newly synthesized phospholipids.

Consequently, the presence of a putative phospholipid transporter has been hypothesized, which would enable phospholipids to flop, i.e., to translocate from the cytoplasmic to the periplasmic leaflet of the plasma membrane. Despite many efforts, the existence of such a transporter has not (yet) been demonstrated in bacterial membranes, consistent with the finding that interfering with protein function by addition of NEM does not block the process (*8*, *10*). Alternatively, the mere *presence* in membranes of proteins, *without* a dedicated phospholipid transport function, may alter the properties of the bacterial plasma membrane sufficiently to enable phospholipids to flop. We set out to investigate this hypothesis.

All transmembrane proteins in the *E. coli* inner membrane discovered so far have membrane-spanning domains that are in an α-helical conformation (*12*). As a simple model for these proteins, we incorporated peptides that are composed of a hydrophobic core of alternating alanine-leucine residues flanked on both sides by two interfacial anchoring residues X (so-called XALP), into large unilamellar vesicles prepared from an *E. coli* phospholipid extract. This type of peptides has been extensively studied and was shown to adopt an α-helical transmembrane orientation in phospholipid bilayers (*13*, *14*, *17*).

The in vitro dithionite assay described by McIntyre and Sleight (*15*) was used to monitor the flop of NBD-phospholipids across the bilayer of liposomes in which the model peptides were incorporated. All peptides tested induced flop of C_6NBD-PG, with rates depending on the type of peptide. Peptide-induced flop of the C_6NBD-PE probe was generally slower than flop of C_6NBD-PG. The data presented in this study support the hypothesis that the presence of membrane-spanning domains of proteins is sufficient for inducing phospholipid translocation across the bacterial plasma membrane.

EXPERIMENTAL PROCEDURES

Materials. C_6NBD-PG and C_6NBD-PE as well as their long-chain (C_{12}) analogues were obtained from Avanti Polar Lipids (Alabaster, AL), and used without further purification. They were dissolved in chloroform to a ~1 mM stock solution which was periodically checked by thin-layer chromatography (TLC) as described (*16*). Stock solutions in chloroform were stored at −20 °C under nitrogen. The peptides were synthesized as described (*13*). Stock solutions in TFE (0.48 mM) were prepared on the basis of weight and stored at −20 °C. The sequence of the peptides used is shown in Table 1. The tripeptide KWK was obtained from Sigma (St. Louis, MO).

E. coli phospholipids were obtained from the wild-type strain W3899 (*18*), grown in LB medium at 37 °C to the late log phase (OD_{660} ~ 0.8). Cells were harvested by centrifugation, washed with 0.9% (w/v) NaCl, and resuspended. Phospholipids were extracted from the cells according to Bligh and Dyer (*19*), and subsequently purified on a silica gel column by elution with chloroform/methanol [1:1 (v/v)] as described (*20*). The phospholipids were stored under nitrogen as a stock solution of approximately 10 mM in chloroform at −20 °C. The phospholipid extract was analyzed by TLC and contained 84% PE, 13% PG, and 3% CL (mol %), typical for wild-type *E. coli* (*21*). Phospholipid compositions and concentrations are presented based on lipid phosphorus.

Table 1: Amino Acid Sequences of the Model Peptides[a]

name	amino acid sequence
KALP31	Ac-GKKL $(AL)_{12}$ KKA-amide
KALP23	Ac-GKKL $(AL)_8$ KKA-amide
WALP23	Ac-GWWL $(AL)_8$ WWA-amide
HALP23	Ac-GHHL $(AL)_8$ HHA-amide
DALP23	Ac-GDDL $(AL)_8$ DDA-amide

[a] Ac denotes an acetyl group attached to the *N*-terminus.

Preparation of Vesicles. E. coli phospholipids, an NBD-phospholipid analogue (0.2% of total PL), and peptide at the appropriate molar ratio (1:2000 to 1:250 peptide:PL), all in organic solvent, were mixed and dried, first by rotatory evaporation and subsequently under vacuum overnight. The lipid film was hydrated by adding buffer Z (10 mM KCl, 50 mM TEA, 1 mM EDTA, pH 7.5), supplemented with 20 mM $K_3Fe(CN)_6$, to a final concentration of 5 mM PL. $K_3Fe(CN)_6$ was included to scavenge the minor amounts of dithionite permeating into the vesicle lumen. Where indicated, a 0.6 mM aliquot of the water-soluble tripeptide KWK was present in the buffer. After freeze−thawing the suspension 5 times, unilamellar vesicles were formed by extruding the suspension 6 times through 200 nm membrane filters (Anotop 10, Whatman, Maidstone, U.K.) using a 1 mL syringe, essentially as described by Hope et al. (*22*). $K_3Fe(CN)_6$ located outside the vesicles was removed by gel filtration on a Sephadex G 50 spin column in buffer Z as described (*23*).

These symmetrically labeled vesicles were incubated for 5 min with sodium dithionite ($Na_2S_2O_4$), added from a 1 M stock solution in 2 M Tris, pH 11, to a final concentration of 25 mM and pH 8.2, after which excess $S_2O_4^{2-}$ was removed by gel filtration as above. This procedure resulted in asymmetrically labeled vesicles that were immediately used in the flop assay. For the control experiments, vesicles without peptide were prepared following the same procedure. Sample preparation and measurements were performed at 25 °C.

Flop Assay. The asymmetrically labeled vesicles were incubated at a concentration of ~3 mM in buffer Z. At different time points, 50 μL samples were taken, and the amount of NBD-phospholipid susceptible to reduction by sodium dithionite was determined as the percentile fluorescence decrease after 180 s of incubation with 8 mM dithionite. These conditions were shown to effectively reduce the NBD-phospholipid analogues located in the outer leaflet of the vesicle without significant influx of dithionite into the vesicles (*9*). All fluorescence measurements were performed in buffer Z, in a 1 mL quartz cuvette at 25 °C under continuous stirring, using an SLM Aminco SPF 500C fluorometer. The excitation wavelength was set at 460 nm, and the emission was recorded at 534 nm, with slit widths

of 5 nm. From these measurements, the percentage of NBD-phospholipid flopped to the outer leaflet at each time point was calculated according to

$$\text{flop (\%)} = [1 - (F_{180}/F_0)] \times 100 \quad (1)$$

with F_0 and F_{180} the fluorescence intensities after 0 and 180 s of reaction with dithionite, respectively. From the time dependence of the percentage of flop [flop(t)], the apparent relative first-order flop rate constants (K_{flop}) were calculated by fitting the data (least-squares fit) to eq 2 (see below), which describes the process of flip-flop in a vesicle assuming that the inward and outward rate constants are equal (*24*):

$$\text{flop}(t) = C_{offset} + 0.5(1 - e^{-K_{flop} \cdot t}) \quad (2)$$

with the factor 0.5 representing the theoretical maximum value (50% redistribution of the probe), C_{offset} the average percentage of flop of vesicles without peptide, and t the time of incubation (h).

Incorporation of the Peptides in Vesicles. Due to interference of the phospholipids, conventional protein assays could not be used. Instead circular dichroism was applied to determine the efficiency of peptide incorporation. Vesicles (3 μmol of PL) with peptides incorporated at a 1:250 molar ratio were prepared, pelleted by ultracentrifugation at 435000g_{max} in a TLA120.2 rotor (Beckman Coulter, USA), and resuspended in a small volume of buffer. Subsequently, 9 volumes of TFE were added in order to solubilize the membranes and to induce an α-helical conformation in the peptides. Twenty spectra were recorded on a Jasco J600 spectropolarimeter using a 10 mm path length cell, 1 nm bandwidth, 0.2 nm resolution, 1 s response time, and a scan speed of 20 nm/min, and subsequently averaged. The ellipticity at 222 nm was read and calibrated using the ellipticity of the corresponding peptide dissolved at a 3 μM concentration in the same mixture of phospholipids, buffer, and TFE. The ellipticity at 222 nm is linear with the concentration of peptide (data not shown).

To get further insight into the orientation of the peptides within the bilayer, oriented CD measurements were carried out as described (*13*) for WALP23 and KALP23. Measurements were performed on a Jasco 810 spectropolarimeter, operated at a 2 nm bandwith, 0.2 nm resolution, and a scanning speed of 50 nm·min^{-1}, and confirmed the transmembrane incorporation of HALP23, KALP31, and KALP23 (not shown), as was previously shown for WALP23 and KALP23 (*13*).

Miscellaneous. The protein content of *E. coli* inner membranes was determined before and after carbonate extraction of membrane-associated proteins (*25*). The Micro BCA protein assay (Pierce) was used according to the manufacturers' instructions. The inner membrane phospholipids were extracted (*19*) and quantified according to Rouser et al. (*26*). Vesicle size was determined by dynamic light scattering in a Zetasizer (Malvern Instruments Ltd., U.K.), operated at a wavelength of 633 nm.

The average number of transmembrane helices per protein was calculated for a subset of membrane proteins from *E. coli* ($n = 182$) obtained from TMbase (http://www.isrec.isb-sib.ch/ftp-server/tmbase/). The average molecular weight of the membrane proteins was derived from the SwissProt database (http://www.expasy.ch/sprot/sprot-top.html).

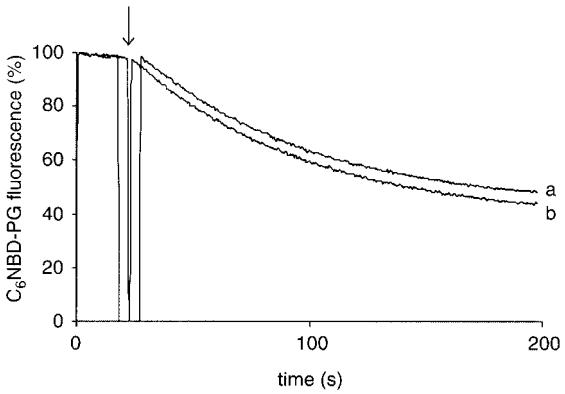

FIGURE 1: On-line fluorescence measurements of the dithionite reduction of C_6NBD-PG (0.2% of total PL) present in both leaflets of vesicles, composed of *E. coli* phospholipids, without (a) and with (b) the model peptide KALP31 incorporated at a 1:2000 peptide:PL molar ratio. At the time point indicated by the arrow, sodium dithionite was added to a final concentration of 8 mM to the vesicles which were present at a concentration of ~150 μM (PL). The fluorescence is presented as the percentage of the initial fluorescence.

RESULTS

Validation of the Method. To be able to monitor peptide-induced transmembrane movement of phospholipids in model membranes with an assay, based on the irreversible quenching of the fluorescence of NBD-phospholipids by dithionite, unilamellar peptide-containing vesicles are required which contain a NBD-phospholipid in the inner leaflet only, and which are impermeable to this reagent. To test the feasibility of this approach, KALP31 (Table 1) was selected as a model peptide and C_6NBD-PG as a model lipid. KALP31 has a hydrophobic length of 25 amino acids and thus is well capable of spanning the *E. coli* phospholipid bilayer. C_6-NBD-PG exhibits fast flip-flop in *E. coli* inner membranes (*9*). Figure 1 shows the reduction by $S_2O_4^{2-}$ of the fluorescence of C_6NBD-PG, initially present in both leaflets of the vesicle. When vesicles without peptide are used (trace a), the fluorescence intensity decreases until it levels off at ~48% of the initial value after a 3 min incubation with 8 mM sodium dithionite. Vesicles prepared from a mixed film containing the model peptide KALP31 in a 1:2000 peptide:PL molar ratio show a very similar time-dependent reduction of fluorescence upon incubation with dithionite, resulting in about the same protected pool of C_6NBD-PG (44%, trace b). The diameter of vesicles with and without peptide was between 180 and 200 nm. These results show that the vesicles are unilamellar and essentially impermeable to dithionite under these conditions. Similar results were obtained for the C_6NBD-PE probe, and for the other peptides used in this study (not shown).

We next tested whether we could use the asymmetrically labeled vesicles for monitoring flop of C_6NBD-PG. Vesicles with KALP31 incorporated at a 1:2000 molar ratio were incubated at room temperature, and the accessibility of the NBD probe to dithionite was assayed at different time points as shown in Figure 2, traces c−e. The amount of probe available for dithionite reduction increases with the time of

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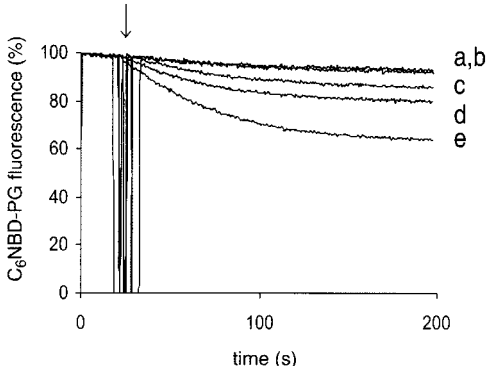

FIGURE 2: Fluorometric determination of the amount of C_6NBD-PG accessible to $S_2O_4^{2-}$ in asymmetrically labeled vesicles (probe initially only in the inner leaflet) without (a, b) and with (c−e) KALP31 incorporated at a 1:2000 peptide:PL molar ratio. The vesicles were incubated at RT for 27 min (a), 23 min (c), 41 min (d), 165 min (e), and 169 min (b), after which the amount of probe that had flopped was determined by addition of 8 mM sodium dithionite (arrow) to ~150 μM (PL) vesicles in the cuvette. Fluorescence intensities are presented as a percentage of the initial fluorescence.

incubation, indicating that in the presence of KALP31 flop of C_6NBD-PG occurs. The percentage of flop, defined as the percentage of the initial fluorescence that disappeared due to reaction with dithionite after 180 s, increases from 15% after 23 min to 37% after 165 min of incubation (Figure 2). It should be noted that the maximum percentage of flop attainable is ~50%, assuming that the probe is distributed evenly between the two leaflets at equilibrium. In marked contrast to the peptide-containing vesicles, the C_6NBD-PG in vesicles without peptide remains virtually inaccessible to dithionite (Figure 2, traces a and b). This lack of phospholipid flop in model membranes from *E. coli* phospholipid extract without protein or peptide is in agreement with earlier observations (*9*). These results show that the dithionite method can be used to study transmembrane movement of NBD-phospholipids in peptide-containing vesicles and demonstrate that incorporation of a transmembrane model peptide at a low peptide:PL molar ratio induces flop of C_6NBD-PG.

Peptide-Induced Flop Is Concentration-Dependent. We next investigated whether increasing the peptide concentration would increase the flop rate. From fluorescence traces as depicted in Figure 2, the percentage of flop in vesicles with varying KALP31:PL molar ratios was determined and plotted against the time of incubation (Figure 3). Curve a shows flop induced by KALP31 present at a 1:2000 peptide:PL molar ratio. Increasing the peptide:PL molar ratio to 1:1000 (b) or 1:250 (c) results in a strong increase of the flop rate such that at the highest concentration tested complete redistribution of the probe occurs within ~1.5 h at 25 °C. The slightly higher amount of accessible probe at equilibrium, compared to the expected value of ~50%, might be due to the method, as also in the peptide-free vesicles ~5% of the fluorescence is quenched by dithionite. However, this is clearly independent of the time of incubation and therefore does not reflect flop. The apparent first-order flop rate constant (K_{flop}), determined as described under Experimental Procedures, is linearly dependent on the peptide concentration in the concentration range tested (Figure 3B). The half-time of flop of C_6NBD-PG induced by KALP31 at a 1:250 molar ratio, calculated from the apparent first-order

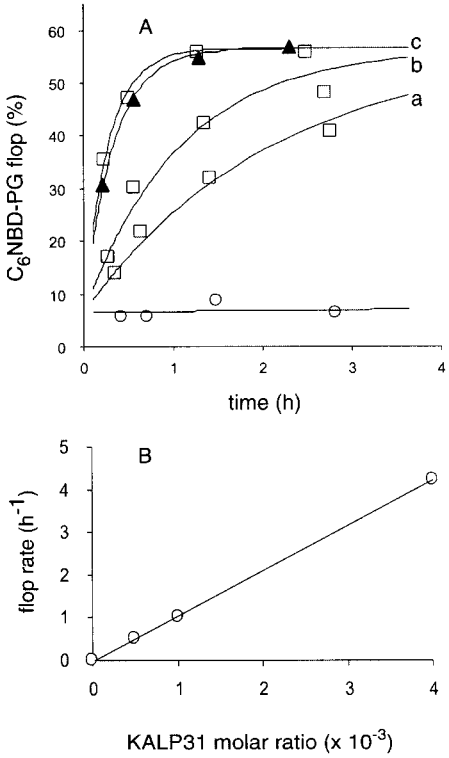

FIGURE 3: Time-dependent flop of C_6NBD-PG in vesicles with the peptide KALP31 (squares) incorporated at a 1:2000 (a), 1:1000 (b), and 1:250 (c) peptide:PL molar ratio, and in vesicles with the peptide KALP23 incorporated (1:250, solid triangles). The data points are mean values from two independent experiments; variation between the data points never exceeded 11% of the average value. For comparison, the flop in vesicles without peptide (circles) is also shown. Panel B shows the averaged ($n = 2$) calculated K_{flop} plotted against the peptide:lipid molar ratio. Least-squares analysis of the unaveraged data yielded $r^2 = 0.72$. For experimental details, see the legend of Figure 2 and Experimental Procedures.

flop rate constant, is 10 min, which is at least 2 orders of magnitude faster than flop in peptide-free vesicles.

Flop Rates Depend on the Type of Peptide. Since the standard deviation of the average length of transmembrane helices of proteins in the inner membrane of *E. coli* is considerable (*14*), KALP23, which has a hydrophobic length eight amino acids shorter than KALP31 (see Table 1), was also tested in the flop assay. We observed that KALP23 induces flop with a similar efficiency as KALP31 (Figure 3, black triangles vs trace c), suggesting that the transmembrane length, at least of these peptides, is not an important determinant for phospholipid flop.

To investigate whether the lysine-flanked peptides (KALP31 and KALP23) are unique in their ability to induce flop of C_6NBD-PG, peptides with other flanking residues were tested. Figure 4 shows that also the uncharged, tryptophan-flanked peptide WALP23 and the partially charged, histidine-flanked HALP23 induce flop of C_6NBD-PG, supporting our hypothesis that the presence of transmembrane helices in the *E. coli* inner membrane is sufficient to allow for phospholipids to flop. Interestingly, flop induced by WALP23 is relatively slow compared to KALP23 (Figure 4).

As the flop rates depend on the peptide to phospholipid molar ratio (see Figure 3), the different flop rates for WALP23 and KALP23 might be due to different extents of membrane incorporation of the peptides. To exclude this

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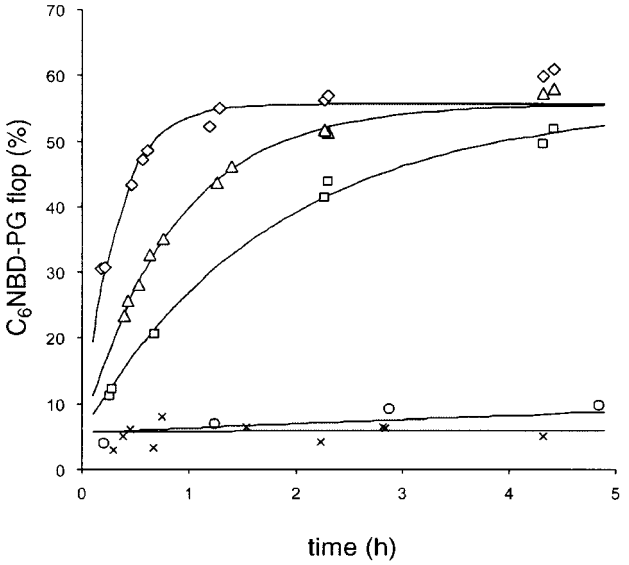

FIGURE 4: Time course of flop of C_6NBD-PG in vesicles with KALP23 (diamonds), HALP23 (triangles), and WALP23 (squares), all incorporated at a 1:250 peptide:PL molar ratio, and without peptide (crosses). Data points of at least two independent experiments are depicted. The curves represent the best fits (least-squares analysis) of the data to eq 2 which yielded K_{flop} values of 3.2 h^{-1} (KALP23), 1.2 h^{-1} (HALP23), 0.6 h^{-1} (WALP23), and 0.0 h^{-1} (no peptide). Also shown is the time-dependent flop induced by the tripeptide KWK (circles), present inside and outside the vesicles at a concentration of 0.6 mM. KWK was included inside the vesicles by adding it to the buffer in which the mixed film was hydrated (inside), and added to the vesicle suspension (outside) at $t = 0$ of the flop assay (data of a single experiment are shown).

possibility, the incorporation efficiency of these peptides was quantified using circular dichroism (see Experimental Procedures), taking advantage of the property of the peptides to adopt an α-helical conformation in TFE. As a percentage of the total amount of material in the mixed film, the recoveries of phospholipid were 70% for vesicles with KALP23 and 73% for vesicles with WALP23. Peptide recoveries were 59% for KALP23 and 61% for WALP23, yielding efficiencies of incorporation of 84%, both for KALP23 and for WALP23. This result rules out the possibility that the observed differences in flop rate are due to differences in the extent of incorporation of the peptides.

The difference in efficiency of flop induced by KALP23 and WALP23 indicates that the presence of charged amino acid residues in the headgroup region of the membrane enhances C_6NBD-PG flop rates. We therefore tested also the negatively charged DALP23, but found that this peptide did not associate with the vesicles (data not shown).

To investigate whether the presence of lysines in the interfacial region of the bilayer is sufficient for inducing flop, we tested the ability of the water-soluble, positively charged tripeptide KWK to induce C_6NBD-PG flop in the same assay. This peptide is known to partition into the membrane−water interface (*27, 28)*. Figure 4 shows that the presence of 0.6 mM KWK (a peptide:PL bulk molar ratio of 1:5) at both sides of the membrane does not induce any flop. Given the propensity of this peptide to associate with negatively charged lipids in membranes (*27*), the surface concentration of lysines should be comparable to that of the vesicles containing KALP23, indicating that the presence of the transmembrane segment is necessary for flop of C_6NBD-PG to occur.

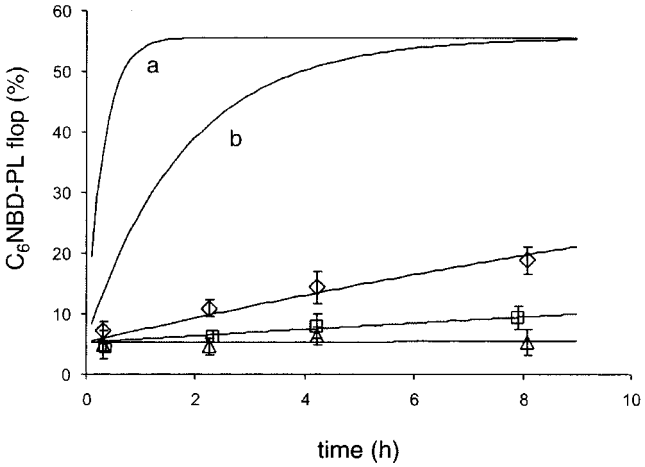

FIGURE 5: Time-dependent flop of C_6NBD-PE in vesicles with KALP23 (diamonds) and WALP23 (squares) incorporated at a 1:250 peptide:PL molar ratio. The data points from at least three independent experiments are shown. For comparison, flop of C_6NBD-PG induced by KALP23 (a) and WALP23 (b) from Figure 4 is also shown. Vesicles without peptide were tested as a control (triangles).

Phospholipid Headgroup Dependence of Flop. The results reported so far show that C_6NBD-PG is flopped from the inner to the outer leaflet of a vesicle, when a positively charged (KALP23) or a neutral peptide (WALP23) is incorporated in the membrane. Since a clear difference in flop efficiency of these two peptides was observed, they were selected to examine peptide-induced flop of a zwitterionic probe, C_6NBD-PE, an analogue of the major phospholipid of *E. coli*. Also for this probe, KALP23 (1:250 peptide:PL molar ratio) caused a gradual redistribution (Figure 5, diamonds), with a translocation half-time of ∼16 h, significantly faster than flop of C_6NBD-PE in peptide-free vesicles which was essentially negligible (triangles). However, compared to KALP23-induced flop of C_6NBD-PG (curve a), flop of C_6NBD-PE is reduced. WALP23-induced flop of C_6NBD-PE was hardly detectable during the time-course of the experiment (Figure 5, squares). The longer chain analogues C_{12}NBD-PG and C_{12}NBD-PE displayed a similar phospholipid headgroup dependence for KALP23-induced flop with flop rates comparable to their short-chain counterparts (not shown).

DISCUSSION

To test the hypothesis that the presence of transmembrane proteins in the cytoplasmic membrane of *E. coli* is sufficient to allow phospholipids to translocate across the bilayer, transmembrane peptides were incorporated in vesicles prepared from an *E. coli* phospholipid extract. Phospholipid flop was measured by assaying the dithionite accessibility of an NBD-phospholipid analogue. The peptides used in this study have been well characterized and used as a model for the α-helical transmembrane stretches of proteins (*14*).

The results show that the incorporation of transmembrane peptides in vesicles, composed of *E. coli* phospholipids, enable NBD-phospholipids to translocate across the bilayer and thus support our hypothesis. In the absence of peptides,

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flop is negligible for both types of phospholipids, in agreement with earlier studies (*9*). The half-times for translocation depend on the type of peptide and the type of phospholipid used.

The incorporation in vesicles of the lysine-flanked peptide KALP31 at increasing peptide:PL molar ratios results in increasing flop rates (Figure 3B). Mechanistically important is the linear relationship between K_{flop} and the peptide:PL molar ratio, indicating that flop is mediated by peptide monomers. This is in agreement with results obtained from electron spin resonance experiments which suggested that no significant aggregation of WALP and KALP peptides occurs (*13*). The shorter peptide KALP23 induces flop of C_6NBD-PG with about the same efficiency as KALP31, suggesting that at least under the conditions used here, the *length* of the membrane-spanning peptide is not an important parameter for flop. In contrast, the tripeptide KWK, which partitions into the membrane−water interface, does not induce flop, leading to the conclusion that the hydrophobic core of the peptide is essential for inducing phospholipid flop.

Membrane-spanning peptides with different flanking residues (WALP23 and HALP23) also mediate flop of C_6NBD-PG, again showing that the decisive factor for the occurrence of flop is the presence of a transmembrane α-helix. Interestingly, the peptides WALP23 and KALP23 induce flop at different rates. The tryptophan residues in WALP23 are thought to be preferentially located near the carbonyl groups of the acyl chains, deeper in the membrane than the lysines in KALP23 that prefer a more hydrophilic location (*13*). As a consequence, the tryptophans have less interaction with the polar headgroups, which could account for the slower rate of flop induced by WALP23 as compared to KALP23. Moreover, lysines have a less defined localization with respect to the phospholipids (*13*), which may give the peptide more motional freedom. This could increase local perturbation of the membrane, resulting in faster phospholipid flop.

Additionally, electrostatic interactions between the peptide and the phospholipid may influence the efficiency of flop induced by a peptide. Possibly, the positively charged KALPs attract negatively charged phospholipids, including the C_6-NBD-PG, which would lead to their relative enrichment at the site where flop occurs. This is in agreement with the order of efficiency of inducing flop: KALP23 > HALP23 > WALP23. Moreover, it provides a possible explanation for the observed differences in flop rate between the zwitterionic C_6NBD-PE and the anionic C_6NBD-PG.

The different flop rates of PE and PG may also arise from the intrinsic properties of these molecules. One possibility is that PG travels across the membrane as an uncharged species, i.e., with a protonated phosphate group. It has been shown that PG can be distributed asymmetrically between the two leaflets of a peptide-free model membrane in response to a transmembrane pH gradient. At the side of low pH, PG is protonated and subsequently translocated to the other side, where it is trapped by deprotonation due to a basic pH (*29*). It has to be noted, however, that in our study the bulk pH of 7.5 at which the experiments are performed is well above the pK_a of PG, which is ∼3 (*29*). This implies that only a small portion of the probe molecules is uncharged.

In summary, our studies lead to the following model. We propose that peptide-mediated flop is primarily the result of local perturbances of the bilayer structure in the vicinity of the peptide, and that the transmembrane α-helix contributes most to this perturbance. The flanking residues of the peptides may determine the dynamics of the interaction between the peptides and the phospholipids and thereby the rate of phospholipid flop.

Peptide-induced flop has also been demonstrated for membrane-permeabilizing peptide antibiotics (e.g., magainin). These form oligomeric pores in the membrane and are thought to induce flop by the formation of a curved lipid surface with the polar headgroups toward the water-filled pore connecting the inner leaflet with the outer leaflet of the membrane (*30*). This would allow phospholipids to move from one leaflet to the other without their polar headgroup actually having to cross the hydrophobic core of the membrane. By a similar mechanism referred to as pore-mediated flop, the synthetic peptide GALA induces rapid flop in model membranes (*24*). Unlike the pore-forming peptides, the peptides used in the present study cannot form amphipatic α-helices with a hydrophilic and a hydrophobic surface. This, and the notion that KALP31 as a monomer induces flop (Figure 3B), strongly suggests that the peptides used in this study induce flop by a mechanism distinctly different from that of these antibiotics.

Interestingly, in earlier studies on model membranes containing the single membrane-spanning protein glycophorin, phospholipid flop was observed, demonstrating that this phenomenon is not restricted to peptides (*31*). More recently, a study published by Hrafnsdottir et al. (*32*) showed that phospholipid translocation activity could be reconstituted in proteoliposomes from *B. subtilis* membrane proteins and DOPC. Proteinase K treatment of these vesicles inhibited phospholipid translocation. This is indicative for the presence of proteins with specific phospholipid transport activity. Alternatively, in the context of the present data, this might also be explained by the loss of residues interacting with the phosholipid headgroups, resulting in reduced flip-flop per se, or in a different orientation and/or aggregational state of the remaining helices. In the same study, a *B. subtilis* membrane protein extract was fractionated using glycerol density centrifugation. The different fractions showed varying translocation activity after reconstitution, with the fraction banding at ∼4 S exhibiting the highest activity. It would be interesting to see what the general characteristics of the proteins in this fraction are. Instead of (a) dedicated flippase-(s), it might contain proteins that have a high number of transmembrane helices per total mass. Although transport activity was found in several fractions, also a membrane protein fraction with negligible activity was isolated, indicating that not all membrane proteins are capable of inducing phospholipid flop.

The flop rates in our model system are generally lower than transport rates observed in in vivo studies (*4*, *6*) or in isolated bacterial cytoplasmic membranes (*8*, *9*). The highest translocation half-time obtained here for C_6NBD-PG induced by KALP31, in a 1:250 molar ratio, is ∼10 min at 25 °C. To compare this molar ratio to the in vivo situation, we made a rough estimate of the concentration of transmembrane helices in the inner membrane of *E. coli*. The amount of transmembrane protein after carbonate extraction was determined to be ∼0.8 μg/nmol of PL. The subset of 182 *E. coli* membrane proteins obtained from TMbase has an

average mass of 43 kDa, and an average of 6 transmembrane helices per protein. From these data, we estimate the ratio of transmembrane helices to phospholipid to be around 1:9 in the *E. coli* inner membrane. Since the peptides and lipids we used do not form stable vesicles at a 1:9 molar ratio (*33*), a model system resembling the in vivo situation cannot be tested. If the data from Figure 3B are linearly extrapolated to a 1:9 molar ratio, the translocation half-time of C_6NBD-PG would be ~22 s, which is of the same order of magnitude found for translocation half-times in the in vivo and in vitro studies cited above.

The observation that membrane-spanning peptides can induce flop in a model membrane of *E. coli* phospholipids supports our hypothesis that the transmembrane regions of integral membrane proteins in the *E. coli* inner membrane are sufficient to allow phospholipids to flop. Interestingly, preliminary experiments indicate that also *E. coli* leader peptidase, a protein with two membrane-spanning α-helices (*34*), induces flop in vesicles of *E. coli* phospholipids.

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