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The reconstituted *Escherichia coli* MsbA protein displays lipid flippase activity

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The MsbA protein is an essential ABC (ATP-binding-cassette) superfamily member in Gram-negative bacteria. This 65 kDa membrane protein is thought to function as a homodimeric ATP-dependent lipid translocase or flippase that transports lipid A from the inner to the outer leaflet of the cytoplasmic membrane. We have previously shown that purified MsbA from *Escherichia coli* displays high ATPase activity, and binds to lipids and lipid-like molecules, including lipid A, with affinity in the low micromolar range. Bacterial membrane vesicles isolated from *E. coli* overexpressing His_6-tagged MsbA displayed ATP-dependent translocation of several fluorescently NBD (7-nitrobenz-2-oxa-1,3-diazole)-labelled phospholipid species. Purified MsbA was reconstituted into proteoliposomes of *E. coli* lipid and its ability to translocate NBD-labelled lipid derivatives was characterized. In this system, the protein displayed maximal lipid flippase activity of 7.7 nmol of lipid translocated per mg of protein over a 20 min period for an acyl chain-labelled PE (phosphatidylethanolamine) derivative. The protein showed the highest rates of flippase activity when reconstituted into an *E. coli* lipid mixture. Substantial flippase activity was also observed for a variety of other NBD-labelled phospholipids and glycolipids, including molecules labelled on either the headgroup or the acyl chain. Lipid flippase activity required ATP hydrolysis, and was dependent on the concentration of ATP and NBD–lipid. Translocation of NBD–PE was inhibited by the presence of the putative physiological substrate lipid A. The present paper represents the first report of a direct measurement of the lipid flippase activity of purified MsbA in a reconstituted system.

Key words: ATP-binding-cassette (ABC) superfamily, lipid A, lipid flippase, MsbA, phosphatidylethanolamine, reconstitution.

INTRODUCTION

Gram-negative bacteria such as *Escherichia coli* possess both an inner (cytoplasmic) membrane and an outer membrane, separated by a layer of peptidoglycan and the periplasmic space. The cytoplasmic membrane is primarily composed of integral membrane proteins and glycerol-phospholipids such as PG (phosphatidylglycerol), PE (phosphatidylethanolamine) and cardiolipin. The cytoplasmic membrane lipids are distributed asymmetrically, with the inner leaflet of Gram-positive bacteria being enriched in PE and the outer leaflet enriched in PG ([1]; bacterial lipid topology is reviewed in [2]). The distribution of phospholipids between the inner and outer leaflets of the cytoplasmic membrane of Gram-negative bacteria is not known, since experimental measurements are complicated by the presence of the outer membrane [2]. The outer membrane lipid distribution is also asymmetric. The inner leaflet consists primarily of glycerophospholipids; however, the outer leaflet is composed mainly of lipid A, the hexa-acylated hydrophobic core lipid of LPS (lipopolysaccharide) [3].

Our understanding of how these lipids traffic to the outer membrane, and how lipid asymmetry is maintained, is becoming clearer. The LptDE (Imp–RlpB) complex is required for assembly of LPS at the outer membrane [4], and the essential inner membrane proteins YjgP (LptF) and YjgQ (LptG), along with the cytoplasmic ATPase LptB, are proposed to actively extract LPS from the cytoplasmic membrane outer leaflet [5]. Lipid A, like phospholipids, is initially synthesized by the biosynthetic machinery on the cytoplasmic membrane inner leaflet, and must be translocated to the outer leaflet in the first step of its movement to the outer membrane. This function may be mediated by MsbA, an essential protein of the cytoplasmic membrane and a member of the ABC (ATP-binding cassette) superfamily [6,7]. MsbA is a 'half-transporter', and is presumed to function as a homodimer of 64.5 kDa polypeptides. X-ray crystal structures of MsbA from three closely related bacterial species have been solved [8].

Evidence in the literature supports a role for MsbA in translocation of lipid A, and perhaps also phospholipids, between the inner and outer leaflets of the cytoplasmic membrane. MsbA is an essential gene in *E. coli*, and mutations result in cytoplasmic membrane accumulation of phospholipids, lipid A and its derivatives [9]. In cells expressing missense MsbA mutants, PE and lipid A are accessible from the cytoplasmic face of the membrane rather than the periplasmic face [10], consistent with a functional role for MsbA in the translocation of these lipids between the cytoplasmic membrane leaflets. However, phospholipid translocation by MsbA remains controversial, and in *Neisseria meningitides*, it was concluded that MsbA plays a role in lipid A translocation, but is not strictly required for the translocation of glycerophospholipids [11]. Kol et al. [12,13] have studied phospholipid flip–flop mediated by a variety of bacterial peptides and proteins. In their reconstituted system, transmembrane peptides were able to mediate flip–flop of fluorescently labelled PG and PE; however, MsbA-mediated phospholipid flipping could not be demonstrated at a ratio of *E. coli* lipid/MsbA of ∼10:1 (w/w), either in the absence or

Abbreviations used: ABC, ATP-binding-cassette; AMP-PNP, adenosine 5′-[β,γ-imido]triphosphate; AlF_x, aluminium fluoride; BeF_x, beryllium fluoride; DLS, dynamic light scattering; DM, *n*-dodecyl-β-D-maltoside; DTE, dithioerythritol; LPS, lipopolysaccharide; NB, nucleotide-binding; NBD, 7-nitrobenz-2-oxa-1,3-diazole; NBD–GlcCer, NBD–C_6-glucosylceramide; NBD–LacCer, NBD–C_6-lactosylceramide; Ni-NTA, Ni^{2+}-nitrilotriacetate; OG, octyl-β-D-glucopyranoside; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; Pgp, P-glycoprotein; PS, phosphatidylserine; RaLPS, LPS from the Ra mutant of *Escherichia coli*; ReLPS, deep rough chemotype LPS; SM, sphingomyelin; TM, transmembrane; V_i, sodium orthovanadate.

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presence of ATP. Although low levels of ATPase activity were associated with the proteoliposomes, it is possible that either MsbA was not reconstituted in a functional manner in this system or some other factor required for phospholipid translocation was lacking.

In previous work [14], we have shown that the ATPase activity of purified MsbA is modulated by a variety of lipids and lipid-based molecules, including lipid A, the LPS precursors RaLPS (LPS from the Ra mutant of *E. coli*) and ReLPS (deep rough chemotype LPS), and phospholipid-based amphipathic drugs. It was demonstrated for the first time that MsbA binds directly to these molecules, as assessed by quenching of the protein intrinsic tryptophan fluorescence, with affinities in the low micromolar range. These observations lend support to a role for MsbA in the translocation of LPS and phospholipids.

In the present paper, we report for the first time that bacterial membranes isolated from *E. coli* overexpressing His_6-tagged wild-type MsbA display ATP-dependent translocation of a variety of fluorescently NBD (7-nitrobenz-2-oxa-1,3-diazole)-labelled lipid species. Purified and functionally active MsbA reconstituted into proteoliposomes of *E. coli* lipid possessed flippase activity for both headgroup- and acyl chain-labelled derivatives of the phospholipids PE and PS (phosphatidylserine), as well as chain-labelled PG, PC (phosphatidylcholine) and SM (sphingomyelin). The protein showed the highest rates of flippase activity when reconstituted into an *E. coli* lipid mixture. The present study demonstrates for the first time that MsbA has the functional ability to carry out ATP-dependent translocation of phospholipids *in vitro* in a reconstituted system.

EXPERIMENTAL

Materials

ATP, AMP-PNP (adenosine 5′-[β,γ-imido]triphosphate), DTE (dithioerythritol), lipid A, NBD–GlcCer (NBD–C_6-glucosylceramide), NBD–LacCer (NBD–C_6-lactosylceramide), OG (octyl-β-D-glucopyranoside), RaLPS, ReLPS, sodium dithionite, V_i (sodium orthovanadate), Triton X-100, $BeSO_4 4H_2O$ (beryllium sulfate tetrahydrate) and $AlCl_3$ were purchased from Sigma–Aldrich. *E. coli* lipid, egg PC and all other NBD-labelled lipids were purchased from Avanti Polar Lipids. The suppliers provide detailed information on the structures of the various NBD–lipids. The designation (16:0, 6:0), for example, indicates that the NBD–lipid contains a 16-carbon acyl chain and a 6-carbon acyl chain, which carries the NBD group. Creatine kinase and phosphocreatine were obtained from Roche Diagnostics. DM (*n*-dodecyl-β-D-maltoside) was obtained through Alexis Biochemicals, and Ni-NTA (Ni^{2+}-nitrilotriacetate) agarose resin was purchased from Qiagen. Lipid A, the rough chemotype ReLPS and the smooth chemotype RaLPS, were prepared as stock solutions in reconstitution buffer [50 mM Hepes, 100 mM KCl, 5 mM $MgCl_2$ and 2 mM DTE (pH 7.5)]. All other lipid stocks were prepared in 4:1 (v/v) chloroform/methanol and stored at −20 °C.

MsbA expression and purification

A plasmid containing wild-type N-terminally His_6-tagged *E. coli* MsbA was provided by Dr Candice Klug (Medical College of Wisconsin, Milwaukee, WI, U.S.A.) [15]. The protein was overexpressed in *E. coli* as described previously [14]. Inside–out membrane vesicles were prepared by cell lysis using a French Press at a pressure of 10000 psi (1 psi = 6.9 kPa), followed by low- and high-speed centrifugation of the cell lysis mixture [15], and were used directly for flippase measurements. For further purification of MsbA, vesicles were lysed with 1 % DM and MsbA was bound to a Ni-NTA column equilibrated with buffer containing 0.1 % OG and 10 mM imidazole. After washing with buffer containing 0.1 % OG and 10 mM, followed by 50 mM, imidazole, the protein was eluted with 200 mM imidazole in buffer containing 0.1 % OG. MsbA was isolated to over 90 % purity [14]. Protein was assayed by the method of Peterson [16] using a BSA standard.

Reconstitution of MsbA into proteoliposomes

MsbA was reconstituted into proteoliposomes of 5 mg of *E. coli* lipid, egg PC, 1:1 (w/w) egg PC/*E. coli* lipid, or 7:3 (w/w) PE/PG, each containing 0.3 % of the chosen NBD-labelled lipid. MsbA was initially reconstituted into *E. coli* lipid proteoliposomes containing NBD–PE using DM and Bio-Beads for detergent removal as described previously [14]; however, stable baseline fluorescence intensities following dithionite quenching were difficult to achieve. This suggested that not all of the detergent was removed during reconstitution, resulting in some membrane leakiness. We modified the purification procedure to exchange DM for OG (see above) while the MsbA was bound to the Ni-NTA column, and the reconstitution procedure then used removal of OG by gel-filtration chromatography as follows. The dried lipid sample was dissolved in 250 μl of 1 % (w/w) OG and 1 ml of the desired concentration of purified MsbA in OG was added [the lipid/protein ratio was typically 10:1 (w/w)]. The sample was incubated on ice for 30 min with occasional mixing, and then applied to a 15 cm Sephadex G50 gel-filtration column equilibrated with reconstitution buffer [50 mM Hepes, 100 mM KCl, 5 mM $MgCl_2$ and 2 mM DTE (pH 7.5)]. Reconstituted proteoliposomes eluted in the void volume of the column. The desired fractions (∼0.5 ml each) were pooled and diluted to a final volume of 3 ml. Liposomes of *E. coli* lipid containing NBD-labelled phospholipid, but with no incorporated MsbA, were also prepared in the same way. For time-course experiments up to 90 min, two samples of proteoliposomes were prepared in parallel from the same batch of MsbA, and the appropriate fractions from each column were pooled to give a total volume of 6 ml. Baselines for MsbA proteoliposomes prepared using OG/gel filtration were relatively stable in flippase experiments.

Proteoliposome size measurements

E. coli lipid proteoliposomes containing reconstituted MsbA were prepared using the OG gel-filtration method as described above, and diluted 50-fold in reconstitution buffer. Size-distribution measurements based on intensity were carried out by DLS (dynamic light scattering) using a Zetasizer Nano-S instrument (Malvern Instruments) at 22 °C, with a count rate of 260 per s and a measurement duration of 80 s.

Lipid flippase assay using NBD-labelled lipids

The ability of MsbA reconstituted into proteoliposomes to flip a variety of NBD-labelled lipids was determined essentially as described previously [17], with the following modifications. Flippase experiments were carried out in MsbA reconstitution buffer, 5 mM ATP was used with an ATP-regenerating system [18] (30 μg/ml creatine kinase and 3.5 mM phosphocreatine), and 4 mM sodium dithionite was employed to quench the NBD fluorescence. Duplicate samples were assayed in independent experiments, and representative data-sets are shown.

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For experiments involving *E. coli* membrane vesicles, NBD–lipid (15 μg) was added in 100 μl of ethanol solution to the exterior of 100 μl of vesicle suspension (600 μg of protein) in a total volume of 1 ml of reconstitution buffer. Vesicles were incubated on ice for 5 min and then centrifuged at 37 000 ***g*** for 20 min to sediment the membrane vesicles. The pellet was resuspended in 3 ml of reconstitution buffer and lipid flippase activity was determined as described above for proteoliposome samples. Control membranes, where used, were from *E. coli* transformed with empty vector, and a sample of identical protein content (600 μg) was used.

Dependence of flippase activity on NBD–lipid concentration

The dependence of MsbA-mediated flippase activity on the concentration of NBD–PE (16:0, 6:0) was determined as described above, except that MsbA was reconstituted into *E. coli* lipid containing 0.1–0.6 % (w/w) NBD–PE (16:0, 6:0), using OG and gel-filtration chromatography.

Nucleotide-dependence of flippase activity

Nucleotide-dependence experiments were carried out as described above, with the following changes. Final ATP concentrations were in the range 0–20 mM, with the usual concentrations of ATP-regenerating system reagents. For experiments involving AMP and GTP, the nucleotide was added at a concentration of 5 mM, and for AMP-PNP a 1 mM concentration was used, in the absence of ATP, but in the presence of the regenerating system. For experiments involving V_i dependence, 5 mM ATP and the regenerating system were added, along with 100 or 200 μM V_i, followed by incubation at 37 °C as usual. For experiments involving BeF_x (beryllium fluoride) and AlF_x (aluminium fluoride), the samples were prepared essentially as described previously for Pgp (P-glycoprotein)-trapped complexes [19].

Inhibition of MsbA-mediated lipid flipping by bacterial lipids

Fluorescence traces were recorded as described above with the following modifications. A 20 μl aliquot of lipid A, RaLPS or ReLPS in reconstitution buffer was added 5 min prior to initiation of NBD–PE flipping by addition of ATP and the regenerating system. Control samples were incubated either without added lipid but with ATP (positive control, 100 % flippase activity) or with the highest concentration of exogenous lipid used but without the addition of ATP and the regenerating system (negative control, 0 % flippase activity).

RESULTS

Reconstitution of MsbA into proteoliposomes

The purification of N-terminally His_6-tagged *E. coli* MsbA has been described previously in detail [14]. Membrane vesicles were isolated from BL21 DE3 *E. coli* overexpressing the protein and contained approx. 5–10 % MsbA, as assessed by SDS/PAGE. Further purification of MsbA by Ni-NTA chromatography using the detergent DM resulted in $\sim$90 % pure MsbA [14]. In the present study we obtained MsbA of similar purity following exchange of DM for OG (see the Experimental section). MsbA was reconstituted into various lipids using removal of OG by gel-filtration chromatography. When purified MsbA was reconstituted into *E. coli* lipid using this method, DLS measurements indicated a bimodal distribution of vesicles (Supplementary Figure S1 at http://www.BiochemJ.org/bj/429/bj4290195add.htm). Native

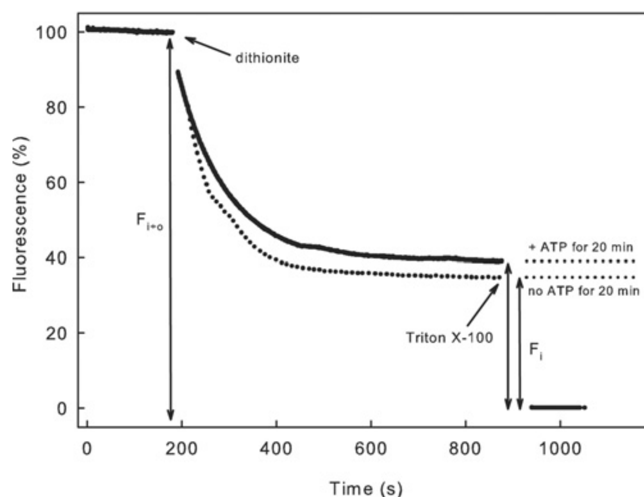

Figure 1 ATP-dependent translocation of NBD–PE by reconstituted MsbA

Proteoliposomes of *E. coli* lipid containing MsbA and 0.3 % NBD–PE (16:0, 6:0) were incubated at 37 °C in the presence or absence of ATP and a regenerating system for 20 min. After termination of the reaction, NBD–PE fluorescence emission (excitation at 464 nm, emission at 536 nm) was monitored at 20 °C until a stable baseline was established. After 3 min, 4 mM dithionite was added (indicated by an interruption in the trace), and after a relatively stable baseline was again reached, 1 % (w/v) Triton X-100 was added to establish the background fluorescence. Traces consist of raw fluorescence data (1 point per s) presented as a scatter plot, and were normalized to the fluorescence intensity recorded prior to dithionite addition, which was taken as 100 %. The vertical arrows represent the total fluorescence of NBD–PE in both leaflets (F_{i+o}), and the inner leaflet (F_i). The Figure shows a representative fluorescence trace. Experiments with reconstituted proteoliposomes of the same lipid composition gave highly reproducible traces.

E. coli membrane vesicles, purified MsbA in detergent, and reconstituted protein in proteoliposomes displayed a high level of ATPase activity that was stimulated and inhibited by the presence of substrates [14], suggesting that the protein was functional. We investigated whether MsbA can function as a lipid translocase or flippase in native membrane vesicles and reconstituted proteoliposomes. We studied translocation of primarily NBD–PE, since the work of Doerrler et al. [10] suggested that this lipid may be flipped by MsbA, and PE is the predominant phospholipid in *E. coli*, making up 70–80 % of the total phospholipid pool [2].

Fluorescence assay for MsbA-mediated lipid translocation

We modified a previously described lipid flippase assay [17,20,21] to assess whether MsbA can mediate the transbilayer movement of fluorescent phospholipid derivatives in both inside–out native membrane vesicles and reconstituted proteoliposomes. In the case of reconstituted proteoliposomes, NBD–lipid was mixed with the host lipids before rehydration and was thus incorporated into both leaflets of the bilayer. After incubation of reconstituted proteoliposomes at 37 °C in the absence or presence of ATP, the total fluorescence intensity of NBD–lipid in both leaflets of the membrane was monitored at 20 °C at an emission wavelength of 536 nm (F_{i+o}; Figure 1). NBD fluorescence was chemically quenched by dithionite, which is membrane-impermeant under the conditions used in the assay. After quenching the outer leaflet fluorescence, a new fluorescence baseline was obtained, arising from the NBD–lipids in the inner leaflet, which are inaccessible to dithionite (F_i; Figure 1). Addition of Triton X-100 to permeabilize the vesicles allowed dithionite to quench the inner leaflet NBD–lipid fluorescence. MsbA molecules reconstituted into proteoliposomes with their NB (nucleotide-binding) domains on the outer surface have access

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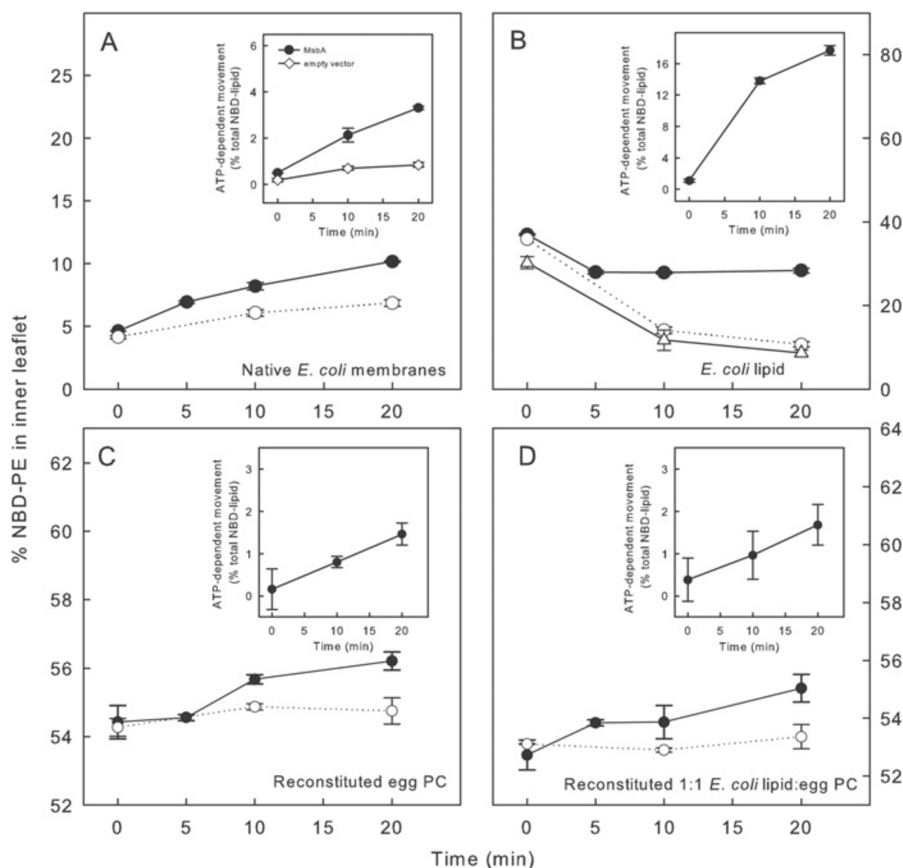

Figure 2 MsbA-mediated NBD–PE flippase activity in *E. coli* membrane vesicles and reconstituted proteoliposomes

(**A**) NBD–PE (16:0, 6:0) was incorporated into membrane vesicles isolated from *E. coli* overexpressing MsbA, which were incubated at 37 °C for 0–20 min in the presence (●) and absence (○) of 5 mM ATP and a regenerating system (added at time zero). The distribution of NBD–lipid between the two bilayer leaflets was determined using dithionite quenching. Experiments were also carried out using membrane vesicles isolated from cells transformed with empty vector (inset). Similar experiments were carried out (**B**) using *E. coli* lipid proteoliposomes containing NBD–PE (16:0, 6:0) and MsbA. Data are also shown for liposomes of *E. coli* lipid and NBD–PE (16:0, 6:0) alone with no incorporated MsbA (△) in the absence of ATP. (**C**) Egg PC proteoliposomes containing NBD–PE and MsbA. (**D**) *E. coli* lipid/egg PC 1:1 (w/w) proteoliposomes containing NBD–PE and MsbA. Inset graphs show ATP-dependent movement of NBD–lipids, calculated by normalizing the curves in the presence of ATP (●) to those in the absence of ATP (○). Data points are the means of duplicate determinations; error bars represent the range and, where not visible, they fall within the symbols.

to ATP, therefore unidirectional translocation of NBD–lipids from the outer to the inner leaflet is expected. The difference in inner leaflet fluorescence intensity between vesicles incubated in the absence and presence of ATP represents the net NBD–lipid translocated during the incubation period (Figure 1).

MsbA mediates transbilayer movement of NBD–lipids in *E. coli* membrane vesicles

For inside–out membrane vesicles isolated from cells overexpressing MsbA, NBD–PE (16:0, 6:0) was inserted asymmetrically into only the outer leaflet by addition from ethanol solution. In these vesicles, the NB domains of all MsbA molecules are exposed on the outer surface with access to ATP. There was an increase in the inner leaflet fluorescence upon addition of ATP, attributable to ATP-dependent translocation of the NBD–PE from the outer to the inner leaflet (Figure 2A). Only ∼25 % of this level of translocation was observed for membrane vesicles prepared from cells transformed with empty vector (Figure 2A, inset), confirming that the bulk of the ATP-dependent movement of NBD–PE was due to the presence of overexpressed MsbA. We examined the ATP-dependent change in inner leaflet fluorescence (Figure 1) for a variety of NBD-labelled lipids, including PE, PS, PG and PC derivatives, where the NBD fluorophore was linked to one of the acyl chains, SM with NBD on one of the acyl chains, and PE and PS with the NBD label on the headgroup (Table 1). In each case, we observed an ATP-dependent increase in the NBD fluorescence in the inner leaflet after incubation for 20 min at 37 °C, relative to an identical sample incubated in the absence of ATP (Table 1). We observed a greater percentage of NBD–lipids transported into the inner leaflet for PE labelled in the acyl chain relative to its headgroup-labelled counterpart. Curiously, a very large amount of NBD–SM (>13 %) was translocated to the inner leaflet compared with the other NBD–lipids examined (1–5 %). Vesicles from cells transformed with empty vector displayed a low level of NBD–SM translocation, similar to that of empty vector experiments with NBD–PE (results not shown). SM is not found in the *E. coli* cytoplasmic membrane and it is not clear why there is such a large ATP-dependent movement of this lipid, which was the only sphingolipid examined in bacterial membranes.

MsbA mediates transbilayer movement of NBD–lipids in reconstituted lipid systems

Purified MsbA in OG was reconstituted into a mixture of *E. coli* lipid containing 0.3 % acyl chain-labelled NBD–PE (16:0, 6:0), and the amount of NBD–lipid in the two membrane leaflets was measured. NBD–PE displays an asymmetric distribution between

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Table 1 Lipid flippase activity of MsbA for NBD-labelled lipids

The change in the inner leaflet level of NBD–lipid was determined in membrane vesicles isolated from *E. coli* overexpressing MsbA, and in reconstituted proteoliposomes containing purified MsbA. For membrane vesicles, values are the difference between the percentage of total NBD–lipid in the inner leaflet with and without ATP after 20 min incubation at 37°C. For *E. coli* lipid proteoliposomes containing MsbA and 0.3% NBD-derivative, the extent of translocation was determined as the difference between samples with and without ATP after 20 min incubation at 37°C, per mg of reconstituted protein. The data represent means ± range for duplicate samples. nd, not determined.

	Membrane vesicles	Reconstituted proteoliposomes
Lipid	Change in NBD–lipid in inner leaflet (%)	NBD–lipid translocated (nmol/mg of protein)
NBD–PE (16:0, 6:0)	2.86 ± 1.24‡	7.71 ± 1.07†
NBD–PE (18:1)*	1.23 ± 0.75‡	2.51 ± 0.23†
NBD–PS (16:0, 6:0)	3.28 ± 0.98†	4.05 ± 0.78†
NBD–PS (18:1)*	3.16 ± 0.29†	1.62 ± 0.12†
NBD–PG (16:0, 6:0)	5.18 ± 0.45†	4.69 ± 1.11‡
NBD–PC (16:0, 6:0)	2.30 ± 0.46‡	3.59 ± 0.55†
NBD–C_{12}-SM	13.3 ± 1.7†	2.57 ± 0.30†
NBD–GlcCer	nd	4.27 ± 0.24†
NBD–LacCer	nd	2.91 ± 0.51†

*Headgroup-labelled NBD–lipid.
†Inner leaflet distributions of NBD–lipid were significantly different for samples incubated in the absence and presence of ATP (P<0.05).
‡Inner leaflet distributions of NBD–lipid were significantly different for samples incubated in the absence and presence of ATP (P<0.1).

the two bilayer leaflets in both the absence and presence of MsbA, with higher amounts seen in the outer leaflet. A preference for either the inner or outer bilayer leaflet was previously reported for several different NBD–lipids in reconstituted egg PC vesicles [21]. In liposomes of *E. coli* lipid alone, the apparent inner leaflet concentrations of NBD–PE decreased over time during incubation at 37 °C in the absence of ATP (Figure 2B). Vesicles composed of egg PC or a 1:1 (w/w) mixture of *E. coli* lipid/egg PC did not exhibit this decrease, which appears to be due to the specific properties of the *E. coli* lipid mixture. In the absence of ATP, *E. coli* lipid proteoliposomes reconstituted with MsbA also showed a decrease in apparent inner leaflet NBD–PE, similar to that seen for liposomes of *E. coli* lipid alone (Figure 2B). While quenching of the outer leaflet fluorescence in MsbA proteoliposomes of *E. coli* lipid is slower than in egg PC, relatively stable baselines are achieved (Figure 1), indicating that the vesicles are not permeable to dithionite. *E. coli* lipid membranes may have higher fluidity, which would more readily allow the spontaneous flip–flop of NBD–lipids in the absence of ATP. Also, *E. coli* lipid often contains hydrophobic peptides, and these may mediate the spontaneous flip–flop of some NBD–lipids, since a variety of TM (transmembrane) domains and hydrophobic peptides have been shown to mediate translocation of NBD–PG between membrane leaflets [12,13,22,23]. This ATP-independent movement of NBD–lipids is not expected to interfere with ATP-dependent movement mediated by MsbA. However, increased flip–flop of NBD–lipid from the inner to the outer leaflet would counteract the action of MsbA, and therefore result in an underestimate of its activity.

In the presence of ATP, MsbA translocated NBD–PE (16:0, 6:0) to the inner leaflet, resulting in net movement of the lipid to the inner leaflet during the 20 min incubation at 37 °C (Figures 2B–2D). In proteoliposomes of *E. coli* lipid, the difference between the inner leaflet concentrations of NBD–PE in the presence and absence of ATP indicated that MsbA mediated the translocation of ~7.7 nmol of this lipid per mg of protein over 20 min under

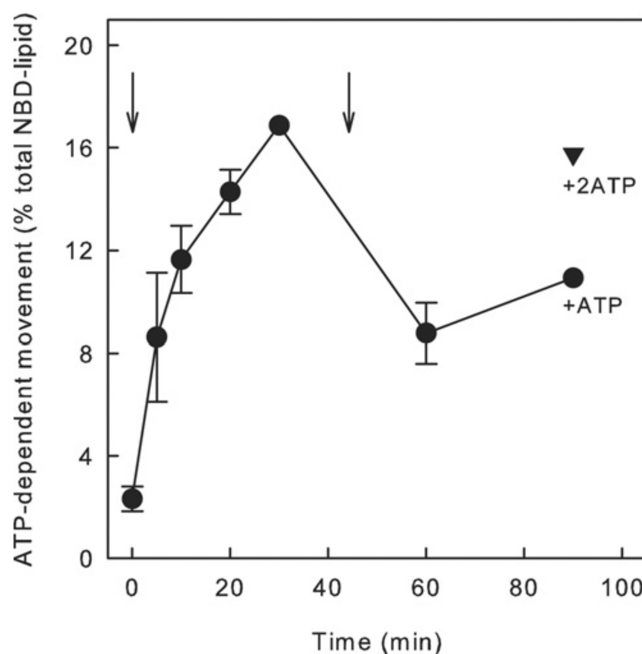

Figure 3 Time course of translocation of NBD–PE by MsbA

Proteoliposomes of *E. coli* lipid containing 0.3 % NBD–PE (16:0, 6:0) and reconstituted MsbA were incubated for 0–90 min in the presence or absence of 5 mM ATP and a regenerating system, or with 5 mM ATP and a regenerating system (arrow at time zero) for the first 45 min, after which an additional aliquot of ATP/regenerating system was added (arrow at 45 min) and the sample was incubated for a further 45 min (▼, +2ATP). Data points (●) represent the difference in the means of duplicate determinations in the presence and absence of ATP. Error bars represent the range and, where not visible, they fall within the symbols.

the assay conditions. Reconstituted MsbA also mediated flipping of NBD–PE that was asymmetrically incorporated into the outer leaflet of preformed proteoliposomes (results not shown).

In proteoliposomes of egg PC, the level of inner leaflet NBD–PE remained unchanged in the absence of ATP, and NBD–lipid was translocated into the inner leaflet in the presence of ATP (Figure 2C). However, the flippase activity was considerably lower than that observed for proteoliposomes of *E. coli* lipid, possibly due to inhibition of MsbA activity by PC, which is not a major component of *E. coli* membranes. Alternatively, MsbA may require one of the components of the *E. coli* lipid mixture for activity. Since a 1:1 (w/w) mixture of egg PC and *E. coli* lipid also exhibits low lipid flippase activity (Figure 2D), inhibition of activity by PC seems a more likely explanation. These results confirm that, in proteoliposomes of *E. coli* lipid, egg PC or a mixture of lipids, MsbA mediates ATP-dependent translocation of NBD-labelled PE.

Characterization of MsbA-mediated flippase activity

MsbA-mediated translocation of NBD–PE was further characterized in terms of time-dependence, ATP-dependence and NBD–lipid-dependence. Reconstituted proteoliposomes of MsbA in *E. coli* lipid were incubated for 0–90 min in the absence or presence of ATP and a regenerating system (Figure 3). There was a rapid increase in the net movement of NBD–PE within the first 30 min after adding ATP. Beyond 30 min, the amount of NBD–PE translocated was significantly reduced; however, the distribution was maintained by the addition of a second aliquot of ATP at 45 min. The ATP-regenerating system employed in the present study was previously used successfully to maintain

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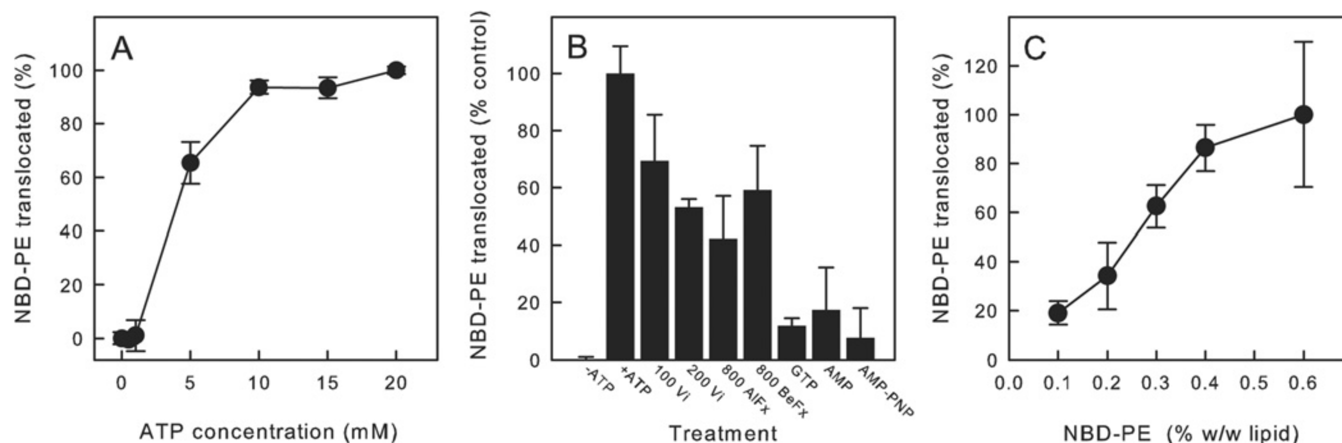

Figure 4 Characterization of MsbA-mediated translocation of NBD–PE

(**A**) ATP dependence of NBD–PE (16:0, 6:0) translocation. *E. coli* lipid proteoliposomes were incubated at 37 °C for 20 min in the presence of 0–20 mM ATP and a regenerating system. The transbilayer distribution of NBD–PE was determined, and the data were normalized to the value with 20 mM ATP, which was taken as 100 %. Data points represent means ± range for duplicate determinations. (**B**) Effect of nucleotides, V_i, AlF_x and BeF_x on translocation of NBD–PE. *E. coli* lipid proteoliposomes were incubated at 37 °C for 20 min in the absence of ATP, or in the presence of the regenerating system+5 mM ATP, AMP, GTP or AMP-PNP, 5 mM ATP+100 or 200 μM V_i, or 5 mM ATP+800 μM AlF_x or 800 μM BeF_x. The transbilayer distribution of NBD–PE was determined, and the data were normalized to the value with 5 mM ATP, which was taken as 100 %. Data points represent means ± range for duplicate determinations. (**C**) Dependence of translocation on the concentration of NBD–PE in the bilayer. Reconstituted proteoliposomes of *E. coli* lipid containing increasing amounts of NBD–PE were incubated for 20 min at 37 °C, and the extent of translocation was determined. Data were normalized to the extent of translocation with 0.6 % NBD–PE, which was taken as 100 %, and are represented as the means ± range for duplicate determinations.

ATP concentrations for both transport and flippase assays using Pgp [17,21,24–28]. When measuring Pgp-mediated NBD–PC flipping in similar experiments, ATP did not become limiting up to 90 min [17], which suggests that the substantially higher flippase activity of MsbA may be responsible for the observed ATP depletion over time periods longer than 30 min. Horio et al. [18] observed a drop-off in drug uptake into plasma membrane vesicles containing Pgp at 90 min relative to 30 min, and suggested that this was due to ATP depletion, and Doige and Sharom [29] made a similar observation. On the basis of the ATP-concentration-dependence of MsbA ATP hydrolysis [14] and lipid translocation (see below; Figure 4A), if the ATP concentration in the assay fell below 5 mM, there would be a significant decrease in flippase activity.

We next examined the ATP-dependence of the translocation process. Proteoliposomes were incubated in the presence of 0–20 mM ATP and the flippase activity was determined over 20 min (Figure 4A). At concentrations below 1 mM ATP, a low level of ATP-dependent flippase activity was observed. With 5 mM ATP, the concentration chosen for ATPase experiments and the initial flippase characterization, there was substantial translocation of NBD–PE by MsbA, and maximal translocation occurred at 10 mM ATP. We chose to maintain the ATP concentration in the assay at 5 mM to allow comparison between different experiments, and because this concentration provided a substantial level of flippase activity. Flippase activity was essentially supported only by ATP, with GTP, AMP and the non-hydrolysable ATP analogue AMP-PNP yielding low levels of translocation over 20 min (Figure 4B). The pentavalent orthophosphate analogue V_i is known to be trapped in the active site of some ABC proteins, blocking ATPase activity after a single catalytic turnover. The presence of 100–200 μM V_i inhibited the measured flippase activity of MsbA by approx. 50 % (Figure 4B). We previously found that V_i was not able to completely eliminate the ATPase activity of the protein [14]. Trapping of MsbA with 800 μM BeF_x and AlF_x also resulted in incomplete inhibition of activity (Figure 4B).

The NBD–PE (16:0, 6:0) content of the proteoliposomes was varied to determine the dependence of MsbA-mediated flipping on the concentration of this lipid. Activity increased up to a concentration of 0.6 % (Figure 4C). The typical assay concentration of 0.3 % NBD–PE gave a good activity of ~60 % of the maximum value observed, and again we chose to use this concentration in further experiments.

MsbA-mediated flippase activity was determined for several different NBD-labelled phospholipids in reconstituted *E. coli* lipid proteoliposomes (Figure 5). In the presence of ATP there was substantial net movement to the inner leaflet for all NBD–lipids tested (Table 1). We observed that the maximal flippase activity of 7.7 nmol per mg of protein over 20 min occurred for NBD–PE (16:0, 6:0), which has the NBD fluorophore in the acyl chain. As was observed for native bacterial membrane vesicles, acyl chain-labelled lipids gave higher flippase activities than the corresponding headgroup-labelled lipids. Flippase activity was highest for derivatives of the common bacterial membrane phospholipids PE, PS and PG, with lower activity observed for PC. The general trends observed for NBD–lipids in reconstituted proteoliposomes were similar to those seen in membrane vesicles, with the exception of SM, which gave the lowest observed flippase activity of the chain-labelled lipids. The origin of the high level of SM translocation observed in bacterial membrane vesicles, which appears to arise from either a direct or indirect effect of MsbA overexpression, is unknown.

MsbA is proposed to function as a flippase for lipid A, which possesses a large sugar headgroup. Since NBD-labelled derivatives of lipid A are not commercially available, we tested NBD–glycosphingolipids instead. We found that an NBD–lipid with a single sugar headgroup (NBD–GlcCer) was translocated at a rate comparable with that of NBD–phospholipids (Table 1). Although addition of another sugar to the headgroup (NBD–LacCer) lowered the extent of translocation of this molecule by MsbA, it was still higher than the headgroup-labelled phospholipids (Table 1). This suggests that MsbA can accommodate lipids with bulky carbohydrate headgroups.

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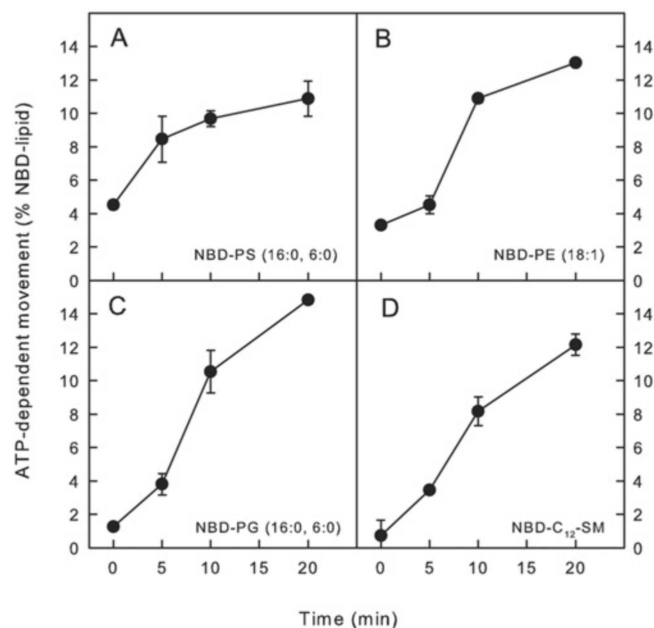

Figure 5 Time course of the MsbA-mediated translocation of four NBD-labelled lipids

Proteoliposomes of *E. coli* lipid containing reconstituted MsbA and 0.3 % (**A**) NBD–PS (16:0, 6:0), (**B**) NBD–PE (18:1) (**C**) NBD–PG (16:0, 6:0) and (**D**) NBD–C_{12}-SM were incubated for 0–20 min in the presence or absence of 5 mM ATP and a regenerating system. Data points represent the ATP-dependent movement of NBD–lipid, calculated from the difference in the means of duplicate determinations in the presence and absence of ATP. Error bars represent the range and, where not visible, they fall within the symbols.

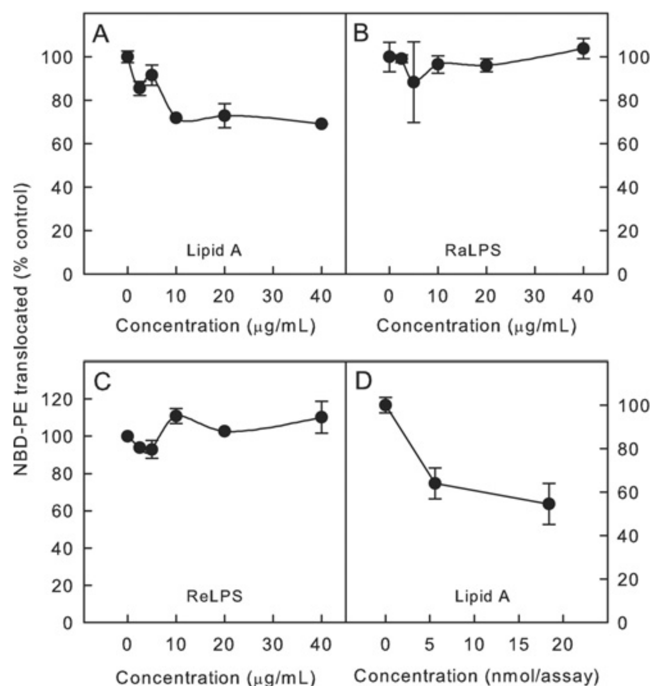

Figure 6 Inhibition of ATP-dependent MsbA-mediated NBD–PE translocation by bacterial lipids

Net translocation of NBD–PE (16:0, 6:0) in proteoliposomes of *E. coli* lipid and MsbA was assessed in the presence of various concentrations of (**A**) lipid A, (**B**) RaLPS and (**C**) ReLPS, relative to a control without added lipid (taken as 100 %), and a control with added lipid but without ATP (taken as 0 %), or (**D**) incorporation of various concentrations of lipid A during preparation of proteoliposomes. The transbilayer distribution of NBD–PE was determined after incubation with 5 mM ATP and a regenerating system for 20 min at 37 °C. Data points represent means ± range for duplicate determinations; where not visible, error bars fall within the symbols.

Inhibition of MsbA-mediated lipid flipping by bacterial lipids

We monitored the MsbA-mediated flipping of NBD–PE after addition to the exterior of proteoliposomes of increasing concentrations of lipid A, RaLPS or ReLPS (Figures 6A–6C). Lipid A inhibited translocation by approx. 30 % at 10–40 μg/ml, a concentration at which it stimulates the ATPase activity of MsbA [14]. The inhibition may be due to competition for flipping of NBD–PE. Neither RaLPS nor ReLPS affected MsbA flippase activity significantly at the concentrations used (Figures 6B and 6C), although the ATPase activity was modestly affected in this concentration range [14]. Since we do not know how much lipid A incorporates into preformed vesicles, MsbA proteoliposomes of *E. coli* lipid were prepared with various amounts of lipid A incorporated during their formation. Under these conditions, lipid A inhibited NBD–PE flipping by ~45 % at ~18 nmol per assay (Figure 6D). Thus translocation of NBD–PE was inhibited by the presence of a putative physiological substrate, lipid A.

DISCUSSION

We have shown previously that MsbA binds directly to lipids and lipid-like molecules, including RaLPS, ReLPS and lipid A, its proposed physiological substrate [14]. In the present paper we report for the first time the measurement of lipid flippase activity mediated by purified MsbA protein. The present study is the first direct demonstration of lipid translocation mediated by MsbA, and avoids the ambiguity of whole-cell or membrane systems, where multiple proteins are present, by using purified reconstituted MsbA. We show that, in proteoliposomes, MsbA mediates the ATP-dependent flipping of a variety of NBD-labelled phospholipid derivatives. Among the chain-labelled derivatives tested, the flippase activity was highest with NBD–PE. The inner leaflet of the outer membrane of *E. coli* has a high proportion of PE [2], and the fact that MsbA translocates this phospholipid to the greatest extent suggests that it may indeed be involved in the pathway for phospholipid trafficking to the outer membrane.

We previously used a similar NBD–lipid dithionite-quenching assay to show that Pgp functions as an ATP-dependent flippase [17,21]. MsbA apparently mediated the translocation of ~7.7 nmol of NBD–PE per mg of protein over 20 min, which is substantially greater than the flippase activity observed for Pgp for a variety of NBD–lipids, which was in the range 0.36–1.83 nmol per mg of protein over 20 min [17,21]. Thus MsbA is a relatively high-activity flippase for NBD-labelled lipids. It seems highly unlikely that a minor contaminant in the preparation could be responsible for this activity. The MsbA is of relatively high purity, and has ATP-dependent flippase activity that is inhibited by vanadate (characteristic of ABC proteins) and lipid A. Flippase activity is likely to be a dynamic situation, with lipids continuously moving from the inner to the outer leaflet down a concentration gradient, and MsbA continuously moving them back, as occurs for Pgp-mediated drug transport [24,25,29]. Therefore it seems likely that the true flippase activity of the protein is in fact much higher. In addition, MsbA is reconstituted in both inward- and outward-facing orientations [14], and since only the inward-facing protein has access to ATP, the flippase activity measured here is further underestimated.

PC appeared to have an inhibitory effect on MsbA flippase activity. MsbA reconstituted into PC bilayers also displayed lower ATPase activity than in other lipid environments (P. D. W. Eckford and F. J. Sharom, unpublished work). Indeed reconstitution of

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MsbA into a mixture of 7:3 (w/w) PE/PG to better mimic native *E. coli* membranes resulted in greater ATP-dependent NBD–PE translocation than membranes composed of PC (results not shown). MsbA in PE/PG proteoliposomes had somewhat lower flippase activity than the same preparation reconstituted into *E. coli* lipid. MsbA was also functional in a 1:1 (w/w) mixture of *E. coli* lipid and egg PG or dioleoylPG (results not shown), although again, its activity was lower than in *E. coli* lipid alone. Thus we conclude that MsbA can function as an NBD–PE flippase in a variety of reconstituted systems.

The NBD fluorophore exploited to determine lipid distribution between the two bilayer leaflets is small, and expected to be minimally perturbing. The NBD group linked to the small molecule DTE does not stimulate the ATPase activity of either MsbA (P. D. W. Eckford and F. J. Sharom, unpublished work) or Pgp [21] in the concentration range used in the assay, and is thus not expected to interact with either protein. The possibility remains that an interaction of NBD-labelled lipid might take place that does not occur with the corresponding unlabelled lipid, and addition of a relatively hydrophilic NBD moiety to the lipid, particularly in the acyl chain region, could result in altered protein binding affinity compared with an unlabelled lipid. Translocation of natural unmodified membrane lipids may be measured by techniques involving enzymatic degradation of lipids in the outer leaflet; however, this is highly perturbing to the vesicle system and is also technically challenging [30]. Overall, the NBD reduction assay remains the most reliable method for determination of lipid flippase activity.

Transmembrane movement of NBD–lipids in *E. coli* membrane vesicles has been measured by other researchers, using either back-extraction with BSA [31] or by a dithionite-quenching technique [32], similar to the method used in the present study. Non-specific flipping of NBD–lipids across the inner leaflet membrane in a protein-dependent process was reported in the absence of ATP, with $t_{\frac{1}{2}}$ (half-life) ranging from 1 to 3 min for NBD–PE (14:0, 6:0) [31] to 7 min for NBD–PE (16:0, 6:0) [32]. The bacterial species, strain, phospholipid type and assay method can all affect the results obtained. We used the *E. coli* strain BL21 DE3 overexpressing large amounts of MsbA. Clearly, we observed different results from those of Huijbregts et al. [32], who used similar NBD-labelled phospholipids in vesicles from the *E. coli* strain W3899. First, we were able to use the dithionite assay at room temperature (22 °C), since we obtained a stable fluorescence baseline, while they did not. Secondly, although we did see ATP-independent inward movement of NBD–PE in *E. coli* membrane vesicles over time at 37 °C, the $t_{\frac{1}{2}}$ was longer than 7 min, and we saw a much larger ATP-dependent change attributable to MsbA.

Membrane-spanning domains of proteins [12] and TM peptides [13,23] can mediate the flipping of NBD–PG (and PE at a lower rate) simply by their presence in the membrane. The rate of transbilayer movement of NBD–PC and unlabelled PC is also increased by certain TM and pore-forming peptides [33,34]. In the case of MsbA, translocation in proteoliposomes is dependent on ATP and NBD–lipid concentration, and is inhibited by V_i and the substrate lipid A, suggesting that lipid transport is integral to the function of the protein.

Kol et al. [12] were unable to demonstrate MsbA-mediated flipping of NBD–PG in reconstituted proteoliposomes, but they used 2 mM ATP and incubation times of up to 3 h. In addition, only low levels of ATPase activity were associated with their proteoliposomes, ∼10 nmol/min per mg of protein, whereas our MsbA preparation displayed ∼100-fold higher activity [14]. We measured translocation of 4.69 nmol of NBD–PG per mg of MsbA over a 20 min incubation time with 5 mM ATP (Table 1). However, at an ATP concentration of 2 mM, MsbA-mediated flipping of NBD–PE was substantially lower. At longer incubation times (over 30 min), it was apparent that the ATP concentration became limiting even in the presence of an ATP-regenerating system, and the ability of MsbA to maintain a higher proportion of NBD–PE on the inner leaflet was reduced. These two factors may explain the failure of previous attempts to detect the flippase activity of MsbA.

Our demonstration that *E. coli* MsbA can flip phospholipids is in agreement with the work of Doerrler et al. [10], who showed that PE and lipid A accessibility in MsbA mutants was consistent with MsbA-mediated translocation of these lipids. However, Tefsen et al. [11] showed that, in *N. meningitidis*, MsbA is implicated in LPS translocation, but is not strictly required for phospholipid translocation because this process was not altered in MsbA mutants. This does not rule out the possibility that MsbA can flip phospholipids, however, but rather indicates that there is at least one other pathway for phospholipid movement to the outer membrane in this species. The present study examines *in vitro* flippase activity, and it still remains to be demonstrated that MsbA plays a direct role in phospholipid transport *in vivo*.

It is unlikely that MsbA functions simply as a general transporter of amphipathic compounds carrying the NBD group, rather than as a specific phospholipid flippase. We previously showed that lipid A, lipid-like compounds and amphipathic drugs are able to modulate the MsbA ATPase activity [14]. Like Pgp, MsbA binds a number of these species with a broad range of affinities varying from 0.25 to 50 μM [14], again implying specificity, and supporting the idea that they are translocated. The fact that MsbA flips different lipid species with characteristic rates implies the existence of specificity, and suggests that the headgroup and acyl chains are important for transport, not simply the presence of the NBD label. This is in contrast with the results of Huijbregts et al. [32], who found identical rates of translocation of NBD–PC, NBD–PE and NBD–PS in *E. coli* membrane vesicles in the absence of ATP, implying that there was no specificity. The headgroup may be an important recognition point for binding of lipids to MsbA. MsbA showed relatively high activity with NBD-labelled LacCer (Table 1), whereas Pgp flippase activity is very low with this lipid [17]. This suggests that MsbA can accommodate lipids with bulky carbohydrate headgroups, which may be involved in substrate recognition.

There is evidence in the literature that MsbA can confer drug resistance and is a drug efflux pump [35,36]. Recently, we reported that purified MsbA binds various amphipathic drugs at a location distinct from the site where lipid A binds [37]. Pgp also binds drugs and a variety of lipid-like molecules with high affinity [27] and can translocate fluorescently labelled lipids in reconstituted proteoliposomes [17,21]. MsbA may function in a similar manner, as a dual-lipid flippase and amphipathic drug pump. It is not yet known whether MsbA acts as a drug efflux pump to protect *E. coli* cells from harmful substances, although its expression in some Gram-positive bacteria has thus far not been otherwise explained.

Lipid A, which is known to bind to MsbA with an affinity of 6.4 μM, inhibited the flippase activity of the protein in the concentration range tested, despite its stimulation of the ATPase activity at similar concentrations [14]. This inhibition could represent competition between NBD–PE and lipid A for translocation by MsbA, suggesting that translocation of NBD–phospholipids and LPS precursors may occur via the same pathway. The mechanism whereby lipid molecules are translocated between bilayer leaflets is not known for any lipid flippase. For MsbA, one can imagine a model whereby the carbohydrate portion of lipid A is sequestered within a large flexible substrate-binding pocket, the existence of which was shown recently in the X-ray crystal structure of Pgp [38]. The lipid

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molecule may be translocated to the other membrane leaflet either by dragging the acyl chains through the hydrophobic interior of the lipid bilayer, or by binding of the entire molecule within the substrate-binding pocket. Lipid flippases play a very important role in both prokaryotic and eukaryotic membranes, but up to now it has not been possible to study their structure, function or mechanism in detail, since no lipid flippase has been purified in sufficient quantities to allow biochemical characterization. The reconstituted MsbA system should prove invaluable in studies to elucidate the mechanism of ATP-driven lipid translocation by ABC proteins.

AUTHOR CONTRIBUTION

Paul Eckford conducted the experiments, analysed the data and co-wrote the paper. Frances Sharom supervised the study, was involved in experimental design, and co-wrote the paper.

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online data

SUPPLEMENTARY ONLINE DATA

The reconstituted *Escherichia coli* MsbA protein displays lipid flippase activity

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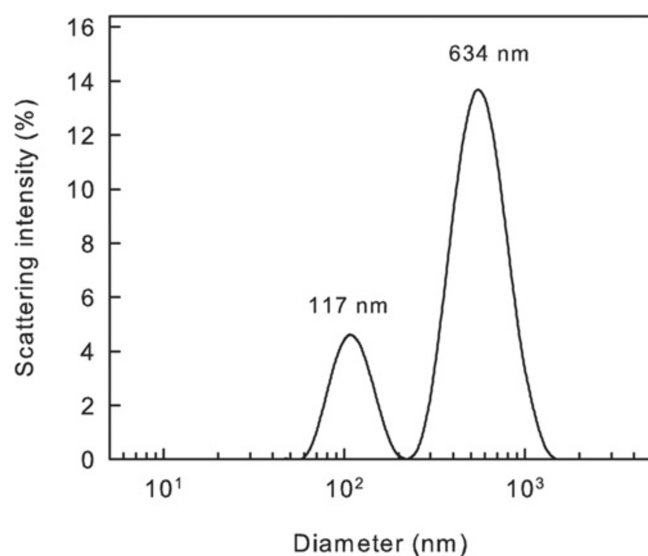

Figure S1 Size distribution of MsbA proteoliposomes

DLS size-distribution intensity measurements were carried out for *E. coli* lipid–MsbA proteoliposomes prepared by the OG gel-filtration method. Data represent the mean ± S.D. ($n = 3$). A smaller population of vesicles was centred at approx. 117 nm diameter and a larger group at approx. 634 nm in diameter. In the absence of MsbA, liposomes prepared by the same method consisted of two somewhat smaller-sized populations (79 nm and 524 nm; results not shown). Romsicki and Sharom [1] found that egg PC and reconstituted Pgp–egg PC proteoliposomes prepared by gel filtration also displayed a multimodal size distribution.

REFERENCE

1 Romsicki, Y. and Sharom, F. J. (2001) Phospholipid flippase activity of the reconstituted P-glycoprotein multidrug transporter. Biochemistry **40**, 6937–6947

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