The ATP Binding Cassette Multidrug Transporter LmrA and Lipid Transporter MsbA Have Overlapping Substrate Specificities*

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LmrA is an ATP binding cassette (ABC) multidrug transporter in Lactococcus lactis that is a structural and functional homologue of the human multidrug resistance P-glycoprotein MDR1 (ABCB1). LmrA is also homologous to MsbA, an essential ABC transporter in Escherichia coli involved in the trafficking of lipids, including Lipid A. We have compared the substrate specificities of LmrA and MsbA in detail. Surprisingly, LmrA was able to functionally substitute for a temperature-sensitive mutant MsbA in E. coli WD2 at non-permissive temperatures, suggesting that LmrA could transport lipid A. LmrA also exhibited a Lipid A-stimulated, vanadate-sensitive ATPase activity. Reciprocally, the expression of MsbA conferred multidrug resistance on E. coli. Similar to LmrA, MsbA interacted with photoactivatable substrate [³²H]azidopine, displayed a daunomycin, vinblastine, and Hoechst 33342-stimulated vanadate-sensitive ATPase activity, and mediated the transport of ethidium from cell and Hoechst 33342 in proteoliposomes containing purified and functionally reconstituted protein. Taken together, these data demonstrate that MsbA and LmrA have overlapping substrate specificities. Our observations imply the presence of structural elements in the recently published crystal structure of MsbA in E. coli and Vibrio cholera (Chang, G., and Roth, C. B. (2001) Science 293, 1793–1800; Chang, G. (2003) J. Mol. Biol. 330, 419–430) that support drug-protein interactions and suggest a possible role for LmrA in lipid trafficking in L. lactis.

Multidrug transporters interfere with the drug-based control of cancer and infectious diseases by mediating the extrusion of cytotoxic drugs from the cell (1, 2). Whereas ion-coupled transporters have a dominant role in efflux-based multidrug resistance in prokaryotic organisms, ABC binding cassette (ABC) transporters have such a role in eukaryotic organisms (3). Certain human ABC transporters are well conserved in bacteria. For example, LmrA in the Gram-positive bacterium Lactococcus lactis is homologous to the human multidrug resistance P-glycoprotein MDR1 (ABCB1) (4, 5), overexpression of which confers resistance on human cancer cells to chemotherapy (6). LmrA and P-glycoprotein MDR1 have very similar specificities for chemotherapy drugs and modulators, and surprisingly, LmrA can functionally substitute for P-glycoprotein MDR1 in human lung fibroblast cells (7).

One of the controversial issues regarding multidrug transporters is their role in cell physiology. Multidrug transporters may have a purely protective function, but they may also be involved in the transport of substrates (e.g. lipids and lipid soluble metabolites) that share physico-chemical properties with drugs (8, 9). Interestingly, LmrA and P-glycoprotein MDR1 share a significant sequence similarity with MsbA, an essential ABC transporter in Gram-negative Escherichia coli that is involved in the biogenesis of the outer membrane (10). The outer membrane is an asymmetric bilayer composed of glycerophospholipids on its inner leaflet and Lipid A, the hydrophobic anchor of lipopolysaccharides, on the outer leaflet. The Lipid A moiety is a hexa-acylated disaccharide of glucosamine and is a potent activator of innate immunity in mammals via the Toll-like receptor 4 (11, 12). The enzymes that synthesize phospholipids and Lipid A are well characterized and are present in the cytoplasm and cytoplasmic membrane (13). Although the precise mechanisms by which phospholipids and Lipid A are targeted to the outer membrane are unknown, recent observations demonstrate that MsbA is required for the export of all newly made lipopolysaccharides and phospholipids to this compartment of the cell envelope (10, 14).

MsbA was the first ABC transporter to be crystallized and analyzed by x-ray crystallography, allowing the determination of its structure at 4.5 Å resolution for MsbA in E. coli (15) and at 3.8 Å resolution for MsbA in Vibrio cholera (16). The implications of the MsbA structure for the structure and function of LmrA and P-glycoprotein MDR1 make a comparison of the substrate specificities of these transporters highly relevant. Here, we report that the lmrA gene can complement the msbA gene in E. coli and that MsbA and LmrA have overlapping specificities for Lipid A and chemotherapeutic and cytotoxic drugs.

**EXPERIMENTAL PROCEDURES**

Bacterial Strains, Plasmids, and Growth Conditions—E. coli WD2 was maintained aerobically at 30 °C in Luria Broth (LB) medium supplemented with 20 mM glucose and 12 µg/ml tetracycline (14). For growth of E. coli WD2 strains harboring plasmid pACYC184 (vector), pZZ34 (pACYC184 containing msbA), pGK13 (vector), or pGKLmrA (pGK13 containing lmrA), LB medium was also supplemented with 12 µg/ml chloramphenicol (1, 10, 14). E. coli Novablue DE3 cells (Novagen, WI) harboring pET28b (vector) or pWTD1 (pET28b containing msbA) with a coding region for an amino-terminal hexa-histidine tag were grown at 30 °C in LB medium with 20 mM glucose and 30 µg/ml kanamycin (17). L. lactis NZ9000 containing pNZ8048 (vector) or...
pNZ8048 (pNZS048 containing lmrA with a coding region for an amino-terminal hexa-histidine tag) was grown to an A_{600} of 0.3 and incubated for 2 h at 30 °C in the presence of 40 g/ml nisin. Protein expression in the E. coli DE3 cells was induced at an A_{600} of 0.5 through the addition of 0.4 mM isopropyl-1-thio-β-D-galactopyranoside (Melford Laboratories Ltd., Suffolk, UK) followed by incubation for 2 h at 30 °C. Cells were harvested by centrifugation at 13,000 × g for 15 min. The cell pellet was washed at 4 °C in 50 mM potassium HEPES (pH 7.4), resuspended in 150 mM MgSO_{4}, and then resuspended in 1 ml of potassium phosphate (pH 7.0) and frozen and thawed. Subsequently, 4 mg/ml lysosome, 10 mM MgSO_{4}, and Complete protease inhibitor mixture (Roche Applied Science), and incubated for 30 min at 30 °C. Cells were broken by passage twice through a Basic Z 0.75-kilowatt Benchtop Cell Disruptor (Constant Systems, Northants, UK) at 20,000 p.s.i. Potassium EDTA (pH 7.4) was then added to a final concentration of 50 mM potassium phosphate buffer (pH 7.4) containing 5 mM β-mercaptoethanol and stored in 150-μl aliquots in liquid N2.

Preparation of Inside-out Membrane Vesicles—For protein expression in L. lactis, cells were grown to an A_{600} of 0.3 and incubated for 2 h at 30 °C in M17 medium (Difco) supplemented with 20 mM glucose and 5 μg/ml chloramphenicol (18).

Liposomes—Lipids were extracted from 10 liters of L. lactis culture as described (18), with modifications. L. lactis NZ9000 cells harboring pNZS048 (vector) were grown to an A_{600} of about 1 and harvested by centrifugation at 13,000 × g for 15 min at 4 °C. Inside-out membrane vesicles were harvested by centrifugation at 125,000 × g for 30 min at 4 °C. The membrane vesicles were resuspended to a protein concentration of 30 mg of membrane protein/ml in 20 mM potassium HEPES (pH 7.4) containing 5 mM β-mercaptoethanol and stored in 150-μl aliquots in liquid N2.

Lipoproteins—Lipoproteins were extracted from 10 liters of L. lactis culture as described (18), with modifications. L. lactis NZ9000 cells harboring pNZS048 (vector) were grown to an A_{600} of about 1 and harvested by centrifugation at 13,000 × g for 15 min at 4 °C. Inside-out membrane vesicles were harvested by centrifugation at 125,000 × g for 30 min at 4 °C. All subsequent steps took place in glass containers in an N2 atmosphere in the dark. Cells were stirred overnight in 200 ml of chloroform and 400 ml of methanol at 4 °C. Undissolved particles were removed by centrifugation at 1,000 × g for 10 min at 4 °C. Water (100 ml) was added, and the mixture was stirred for 3 h at 20 °C. Phases were separated overnight in a separation funnel, and the lower layer containing the lipids was collected. Lipids were dried in a rotary evaporator and dissolved in chloroform to a final lipid concentration of 0.1 g/ml. One ml of the mixture was dripped into 10 ml of ice-cold acetone containing 5 mM β-mercaptoethanol, stirred overnight at 4 °C, and centrifuged for 15 min at 1,000 × g at 20 °C to remove insoluble particles. The lipid solution was dried under N2 gas and dissolved in 15 ml of diethyl ether containing 5 mM β-mercaptoethanol. Subsequently, the lipid mixture was stirred for 1 h at 20 °C and centrifuged at 10,000 × g at 20 °C. The lipid solution was dried in a rotary evaporator, rehydrated in 20 ml potassium phosphate (pH 7.4) containing 5 mM β-mercaptoethanol by vortexing for 30 min, and resuspended by sonication in the same buffer to 20 mg/ml lipid/mg. Liposomes were stored in liquid N2.

Reconstitution of Purified MsbA in Proteoliposomes—The purification of amino-terminal His6-tagged MsbA was performed according to previously described methods (17, 18) with modifications. Inside-out membrane vesicles were solubilized on ice for 1 h in Buffer A (50 mM potassium HEPES (pH 7.4), 10% (v/v) glycerol, 0.5 mM NaCl, 5 mM β-mercaptoethanol, 5 mM MgSO_{4} containing 2% (v/v) lauryl dimethylamine N-oxide (Fluka Biochemika, Buchs, Switzerland). The mixture was centrifuged at 125,000 × g for 30 min, after which the solubilized protein in the supernatant was mixed with Ni^{2+}-nitrilotriacetic acid resin (Qiagen, West Sussex, UK) for 30 min and washed 3 times with Buffer A. After incubation of inside-out vesicles and [3H]azidopine at 30 °C for 10 min, the mixture was transferred to fresh Buffer A and washed 3 times and resuspended in phosphate buffer to an A_{600} of 0.5. The uptake of 2 µM ethidium in cells was measured at 42 °C in a 96-well plate using a Spectrmax Gemini XPS microplate reader (Molecular Devices). The excitation and emission wavelengths were 500 and 580 nm, respectively.

E. coli DE3 Transport—E. coli WD2 cells were harvested in mid-exponential phase, washed three times in 50 mM potassium phosphate buffer (pH 7.4) containing 5 mM MgSO_{4} and deenergized in the presence of 0.5 mM nigericin and 5 mM 2,5-diphenyloxazole (Sigma). Cells were thawed three times and resuspended in phosphate buffer to an A_{600} of 0.5. The uptake of 2 µM ethidium in cells was measured at 42 °C in a 96-well plate using a Spectrmax Gemini XPS microplate reader (Molecular Devices). The excitation and emission wavelengths were 500 and 580 nm, respectively.
significantly higher than those of non-expressing cells. These results suggested that LmrA could functionally replace the temperature-sensitive A270T mutant MsbA protein in *E. coli* WD2 at the non-permissive temperature.

**Resistance to Ethidium Bromide**—LmrA is known to confer ethidium resistance on *E. coli* by mediating the active extrusion of ethidium from the cells (4). In view of the functional complementation of *E. coli* msbA by lactococcal *lmrA*, it was interesting to determine whether MsbA expression conferred ethidium resistance on *E. coli* WD2 cells. The growth rate of *E. coli* WD2 cells harboring pZZ34 (*msbA*) or pACYC184 (vector) was measured in liquid cultures containing increasing concentrations of ethidium bromide. At 30 °C, no significant differences were observed between the concentrations of ethidium necessary to reduce the growth rates of MsbA-expressing cells and non-expressing control cells by 50% (IC₅₀) (Fig. 2). However, at 42 °C MsbA-expressing cells showed a significantly higher IC₅₀ value compared with control cells. Hence, the expression of MsbA in *E. coli* conferred ethidium resistance on the cells under these conditions.

To test whether ethidium efflux from the cell was the underlying mechanism of drug resistance in *E. coli* WD2 expressing MsbA at 42 °C, fluorimetric ethidium transport assays were performed (Fig. 3). Suspensions of deenergized *E. coli* WD2 cells containing pZZ34 (*msbA*) or pACYC184 (vector) were first allowed to accumulate ethidium. When metabolic energy was then generated in the cells through the addition of glucose, ethidium extrusion was observed in cells expressing MsbA but not in control cells (Fig. 3A). Upon the addition of glucose, a significant ethidium efflux was also observed for *E. coli* WD2 cells expressing LmrA compared with control cells (Fig. 3B).

**MsbA Interacts with Multiple Drugs**—To further investigate drug-protein interactions in MsbA, the photoaffinity compound [³H]azidopine was used. In previous work, this 1,4-dihydropyridine derivative was shown to be photo-incorporated into LmrA² and the human multidrug resistance P-glycoprotein MDR1 (20). Inside-out membrane vesicles were prepared from *E. coli* DE3 cells harboring pET28b (vector) or plasmid pWTD1, which allowed the expression of His₆-tagged MsbA under control of an isopropyl-1-thio-β-D-galactopyranoside-inducible T7 promoter (17). After photo-cross-linking of the membrane vesicles in the presence of [³H]azidopine, a ~65-kDa band was detected on the autoradiogram in *E. coli* membrane vesicles containing MsbA and in lactococcal membrane vesicles containing LmrA but not in *E. coli* control membrane vesicles (Fig. 4).

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² L. Balakrishnan and H. W. van Veen, unpublished data.
The membrane vesicles were incubated in the presence of 0.5 μM [3H]azidopine, after which the probe was photocross-linked to interacting proteins by irradiation at 312 nm. Total membrane proteins were then separated by 10% SDS-PAGE and analyzed by autoradiography. The migration of molecular mass markers is indicated.

The specificity of His6-tagged MsbA for drugs was also assessed through measurements of the MsbA-associated ATPase activity in inside-out membrane vesicles. In contrast to control membrane vesicles, MsbA-containing membrane vesicles displayed a significant amount of vanadate-sensitive ATPase activity that was stimulated up to 3-fold in the presence of daunomycin (Fig. 5A), a chemotherapeutic drug transported by LmrA and human P-glycoprotein MDR1. The concentration of daunomycin required for half-maximal stimulation (SC50) of the MsbA-associated ATPase activity was about 25 μM. Vinblastine and Hoechst 33342, which are substrates for LmrA and P-glycoprotein MDR1, stimulated the MsbA ATPase activity 4- and 6-fold, respectively, with SC50 values of 28 and 49 μM, respectively. Interestingly, these drugs stimulated the LmrA ATPase activity to a similar extent as observed for the MsbA ATPase activity but at significantly lower SC50 values of 5 μM for daunomycin, 6 μM for vinblastine, and 1.7 μM for Hoechst 33342.

In addition to the observation that the MsbA ATPase activity is stimulated by Hoechst 33342, evidence for the interaction between MsbA and Hoechst 33342 was obtained in Hoechst 33342 transport measurements in proteoliposomes prepared from lactococcal lipids containing Ni2+-nitrilotriacetic acid affinity-purified and functionally reconstituted His6-tagged MsbA. The protein was greater than 97% pure as judged by silver-stained SDS-PAGE (data not shown). Hoechst 33342 is only fluorescent when it is present in the phospholipid environment of the membrane and essentially non-fluorescent in the aqueous phase (18, 21, 22). The addition of Hoechst 33342 to the proteoliposomes resulted in a rapid increase in fluorescence up to a steady state level due to the partitioning of the dye in the membrane. The subsequent addition of Mg-ATP resulted in a rapid quenching of the Hoechst 33342 fluorescence in proteoliposomes containing MsbA (Fig. 5B). The quenching of Hoechst 33342 was not observed in MsbA-containing proteoliposomes in the presence of the non-hydrolyzable ATP analogue AMP-PNP or in empty liposomes in the presence of Mg-ATP or AMP-PNP. These observations point to the MsbA-dependent transport of Hoechst 33342 from the phospholipid bilayer into the aqueous lumen of the membrane vesicles, similar to previous observations for LmrA and P-glycoprotein MDR1 (5, 21, 22). Taken together these data demonstrate the interaction between MsbA and cytotoxic drugs.

LmrA Interacts with Lipid A—In view of the genetic evidence that LmrA can substitute for MsbA in E. coli WD2 and, hence, appears able to transport Lipid A in E. coli, it was of interest to study the interaction between Lipid A and LmrA through measurements of the LmrA-associated ATPase activity in inside-out membrane vesicles prepared from L. lactis. As shown in Fig. 6, Lipid A stimulated the vanadate-sensitive ATPase activity in LmrA-containing inside-out membrane vesicles 2.1-fold with a SC50 of 3 μM. No effect of Lipid A was observed on the vanadate-sensitive ATPase activity in control inside-out membrane vesicles without LmrA. Similar to the experiments on LmrA, Lipid A also stimulated the MsbA-associated ATPase activity in E. coli DE3 membrane vesicles 2-fold with an SC50 of about 7 μM.

**DISCUSSION**

An increasing number of transporters are appreciated for their ability to transport a variety of lipids in addition to their ability to transport multiple drugs. ABC transporters such as the human multidrug resistance P-glycoprotein MDR1 and multidrug resistance-associated protein MRP1 (ABCC1) are...
LmrA and MsbA Have Overlapping Substrate Specificities

Our insight into the detailed mechanisms by which phospho-
lipid transporters, as observed in eukaryotic cells (34, 35), which
would shuttle Lipid A from the outer leaflet of the inner mem-
brane to the inner leaflet of the outer membrane by diffusion or
in a transporter-dependent fashion (e.g. analogous to LolCDE/
Lola (30)). The transport of Lipid A from the cytoplasmic mem-
brane to the outer membrane in E. coli could also involve endo-
genous lipid flippase, catalyzing the transbilayer move-
ment of lipids from the outer leaflet of the plasma membrane
rather than the outer leaflet (21, 22, 31). The functional substitu-
tion of MsbA by LmrA in E. coli WD2 at non-permissive tempera-
tures implies that this mode of transport is also relevant for MsbA and,
therefore, that MsbA catalyzes the transbilayer movement of Lipid A in the cytoplasmic
membrane.

However, pumping from the inner leaflet of the cytoplasmic
membrane does not explain how MsbA would mediate the transloca-
tion of its lipid substrates to the outer membrane. In this
context, the hemolysin A secretion system HlyBD-TolC in E. coli (32, 33) represents an interesting example of how mem-
brane transporters, including the MsbA homologue HlyB, and a
periplasmic accessory protein act together in an export reaction
across the cell envelope. Although MsbA and LmrA are hom-
ologous proteins, the subtle differences between their primary
structures make precise compulsory protein-protein interac-
tions between the MsbA or LmrA and additional components of
the Lipid A transport machinery in E. coli less likely. In-
stead, the transport of Lipid A in E. coli could involve lipid
transfer proteins, as observed in eukaryotic cells (34, 35), which
would shuttle Lipid A from the outer leaflet of the inner
membrane to the inner leaflet of the outer membrane by diffusion or
in a transporter-dependent fashion (e.g. analogous to LolCDE/
Lola (30)). The transport of Lipid A from the cytoplasmic mem-
brane to the outer membrane in E. coli could also involve endo-
genous multidrug transporters, such as AcrAB-TolC (28,
29), which can transport lipophilic substrates from the outer
leaflet of the cytoplasmic membrane. Alternatively, MsbA-me-
diated Lipid A transport from the cytoplasmic membrane to the outer
membrane may occur at contact sites between these membranes,
known as Bayer junctions (36).

Our insight into the detailed mechanisms by which phospho-
lipids are transported across and between membranes in mi-
croorganisms and mammalian cells is limited (37). This aspect of the biogenesis of biological membranes has lagged far behind the tremendous advances in other areas, such as understanding solute transport and membrane protein traffic and sorting, made over the last two decades. We conclude that LmrA and MsbA have overlapping substrate specificities, providing an example of the close link that exists between lipid transporters and multidrug transporters. Our conclusion implies the presence of structural elements in MsbA supporting drug-protein interactions and may also point to a physiological role of LmrA in lipid transport in _L. lactis_.

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