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On the energy-dependence of Hoechst 33342 transport by the ABC transporter LmrA

Henrietta Venter, Saroj Velamakanni, Lekshmy Balakrishnan [1], *Hendrik W. van Veen* *

Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1PD, UK

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ABSTRACT

LmrA is an ATP-binding cassette (ABC) multidrug transporter from *Lactococcus lactis*, and is a structural homologue of the human multidrug resistance P-glycoprotein (ABCB1), the overexpression of which is associated with multidrug resistance in tumours. We recently observed that a truncated version of LmrA lacking the nucleotide-binding domain mediates a proton motive force-dependent ethidium transport reaction by catalyzing proton-ethidium symport. This finding raised the question whether proton motive force-dependent transport can also be observed for other drugs, and whether this reaction is also relevant for full-length LmrA. Furthermore, the observations on LmrA-MD raised the question whether ATP-dependent transport by LmrA in intact cells could be due to the activity of independent ABC transporters that might become upregulated in the lactococcal cells due to the overexpression of LmrA; the recently identified ABC multidrug transporter LmrCD was put forward as a possible candidate. Here, we investigated the energy coupling to the transport of the amphiphilic dye Hoechst 33342 in proteoliposomes containing purified LmrA. For this purpose, LmrA was obtained from lactococcal cells lacking the genomic *lmrA* and *lmrCD* genes, in which LmrA was expressed from a plasmid. To separate ATP-dependence from proton motive force-dependence, we also used mutant LmrA proteins, which were affected in their ability to hydrolyse ATP. Our studies in proteoliposomes demonstrate that LmrA can catalyze Hoechst 33342 transport independent of auxiliary proteins, in an ATP-dependent fashion and a transmembrane chemical proton gradient (interior acidic)-dependent fashion.

1. Introduction

The ATP-binding cassette (ABC) multidrug transporters are pharmacologically important proteins in humans as they can confer drug resistance on cancer cells, and play a role in the distribution and elimination of drugs in our body [1]. To date, three major ABC multidrug efflux systems have been identified: the multidrug resistance P-glycoprotein (also termed ABCB1), the multidrug resistance-associated protein 1 (ABCC1), and the breast cancer resistance protein (ABCG2), of which ABCB1 has been studied most extensively [2,3].

Homologs of ABCB1 are also found in prokaryotic organisms [4]. Among these, LmrA from *Lactococcus lactis* represents a useful model for ABCB1. LmrA is a half-transporter composed of an amino-terminal membrane domain (MD), consisting of six transmembrane segments, followed by a

* *Corresponding author*. Tel.: +44 1223 765295; fax: +44 1223 334100.
E-mail address: hwv20@cam.ac.uk (H.W. van Veen).

[1] Present address: Trends in Pharmacological Sciences, Elsevier Ltd., 84 Theobald's Road, London WC1X 8RR, UK.

Abbreviations: ABC, ATP binding cassette; ΔpH, transmembrane chemical proton gradient; Δp, proton motive force; MD, membrane domain; NBD, nucleotide-binding domain; TNP-ATP, 2′-(or-3′)-O-(trinitrophenyl)-adenosine 5′-triphosphate; Wt, wildtype.

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hydrophilic nucleotide-binding domain (NBD) [5]. The protein dimerises to form the minimal functional unit with two MDs and two NBDs [6]. In contrast, the two half-transporters are fused into a single polypeptide in ABCB1 [3]. The MDs form the pathways for drugs across the membrane, whereas the NBDs couple drug transport to ATP binding/hydrolysis. LmrA and each half of ABCB1 share 34% identical residues with an additional 16% conservative substitutions [5]. The sequence identity between LmrA and the N- and C-terminal halves of ABCB1 is observed throughout their lengths. This structural similarity translates into a functional similarity, as LmrA exhibits a similar drug and modulator specificity as the human protein [7]. In addition, LmrA can functionally substitute for ABCB1 in lung fibroblast cells [7]. Interestingly, LmrA can mediate reversible transport [8]. This observation raises the possibility of a pharmacological intervention of multidrug resistance in which modulators might enable reverse transport-associated drug delivery in cells overexpressing LmrA-like ABC transporters.

To further analyze drug transport in the absence of NBD activity, we previously studied the functional properties of a truncated form of LmrA lacking the NBD (termed LmrA-MD) [9]. Remarkably, LmrA-MD sensitized *L. lactis* to drugs and toxic compounds, including ethidium and Hoechst 33342, by mediating their uptake into the cell. The subsequent binding of ethidium and Hoechst 33342 to chromosomal DNA causes local unwinding, and hence, inhibition of DNA replication and transcription. Detailed studies on the mechanism of LmrA-MD-mediated ethidium transport indicated that this uptake reaction is coupled to the proton motive force (Δp) via ethidium-proton symport [9,10], and suggested a link between the mechanisms of LmrA and secondary-active (ion-coupled) transporters [9,11].

Here, we investigated the relevance of these observations on LmrA-MD for full-length LmrA using Hoechst 33342 as an alternative transport substrate, instead of ethidium. In addition, we tested the Δp-dependence and ATP-dependence of the transport reaction in experiments in which wildtype (Wt) protein was compared with NBD mutants that are affected in their ability to hydrolyse the nucleotide. Recently, a heterodimeric ABC multidrug transporter LmrCD was discovered in *L. lactis* with a drug specificity that includes ethidium and Hoechst 33342 [12]. This observation has led us to purify exogenously expressed LmrA proteins from a lactococcal strain in which the genomic *lmrA* and *lmrCD* genes were deleted, to exclude a potential interference in our measurements by activities of endogenous LmrA or LmrCD.

2. Materials and methods

2.1. Construction of *L. lactis* ΔlmrA ΔlmrCD

The deletion of *lmrCD* was introduced in the genome of *L. lactis* NZ9000 Δ*lmrA* by a gene replacement method as previously described [12–14], with modifications. Genomic DNA was extracted from *L. lactis* MG1363 using a DNeasy kit (Qiagen) according to the manufacturers instructions. The contiguous *lmrC lmrD* genes were PCR amplified from the genomic DNA with primer LMRA1 (5′-CGC CCA TGG GGA AGC ATA AAT GGG TTG CCT TAT T-3′) to introduce an Nco1 restriction site at the 5′ end and primer LMRA2 (5′-GCG TCT AGA TTC AAA AAC GAA TTG ATT ATG-3′) to introduce an Xba1 restriction site at the 3′ end. The resulting 3.7 kb fragment was ligated into the *L. lactis* expression vector pNZ8048 [15] using restriction sites NcoI and XbaI to yield pNZLmrCD. The construct was digested overnight at 37 °C in the presence of ScaI. Subsequently, Mva1/BstN1 was added after which the incubation was continued for a further 90 min at 60 °C. The double digestion with Sca1 and Mva1/BstN1 removed a 2.1 kb internal fragment from *lmrCD* (Fig. 1). The truncated 5 kb plasmid was treated with Klenow enzyme to create blunt ends, ligated to yield pNZΔ*lmrCD*, and subsequently transformed into electrocompetent *L. lactis* MG1363 cells. The DNA fragment containing Δ*lmrCD* was subcloned into pORI280 as an NcoI–XbaI fragment, yielding pORIΔ*lmrCD*. As neither the pORI plasmid nor *L. lactis* contain *repA* for plasmid replication, the plasmid was maintained in *E. coli* strain EC1000 (*repA*$^+$) in the presence of 100 μg/ml erythromycin. pORIΔ*lmrCD* was transformed into electrocompetent cells of *L. lactis* NZ9000 Δ*lmrA* [16]. Cells were allowed to recover for 1.5 h in recovery medium (M17 containing 0.5 M sucrose, 0.5% glucose, 2 mM $MgSO_4$, and 0.2 mM $CaCl_2$) in the presence of 50 ng/ml erythromycin. The erythromycin concentration was increased to 5 μg/ml and cells were allowed to recover for another 1.5 h before they were plated on M17 agar plates containing 0.5 M sucrose, 0.5% glucose, 5 μg/ml erythromycin, and 120 μg/ml X-gal. As pORI Δ*lmrCD* cannot replicate in *L. lactis*, selection of erythromycin resistance yielded cells where the plasmid had integrated into the genome. These colonies were all blue due to the expression of β-galactosidase from the pORI vector. After 48 h the colonies were picked and grown overnight in M17 containing 0.5% glucose. Cells were spun down and resuspended in 50 mM KPi buffer (pH 7.0) to an OD_{660} of 0.5 and subsequently incubated at 30 °C for 24 and 48 h before serial dilutions of the cells were plated on M17 agar plates containing 0.5 M sucrose, 0.5% glucose, 5 μg/ml erythromycin, and 120 μg/ml X-gal. About 20% of the colonies were white, indicating that the second recombination event took place resulting in the loss of pORI DNA. White colonies were picked and screened for the loss of pORIΔ*lmrCD* by their inability to grow in the presence of erythromycin. To verify the substitution of Wt *lmrCD* by Δ*lmrCD*, PCR was performed on genomic DNA using primers LMRA1 and LMRA2 (Fig. 1B). The appropriate strain was termed *L. lactis* NZ9000 Δ*lmrA* Δ*lmrCD*.

2.2. Nucleotide-binding domain mutants of LmrA

The Walker A ΔK388 mutation was introduced in the *lmrA* gene in the *E. coli* vector pGHLmrA [10] by PCR using KODHotstart DNA polymerase (Novagen) and the forward primer 5′-GGT GGT GGT TCA ACC ATC TTC TCA CTT TTA G-3′ and reverse primer 5′-AGA TGG TTG AAC CAC CAC CAG AAG GAC CAG C-3′. The mutant *lmrA* gene was then subcloned into pNZ8048 as an NcoI-XbaI fragment downstream of the *nisA* promoter, yielding pNHLmrA ΔK388. The construction of the E512Q mutant of LmrA was described previously [8]. Mutated *lmrA*

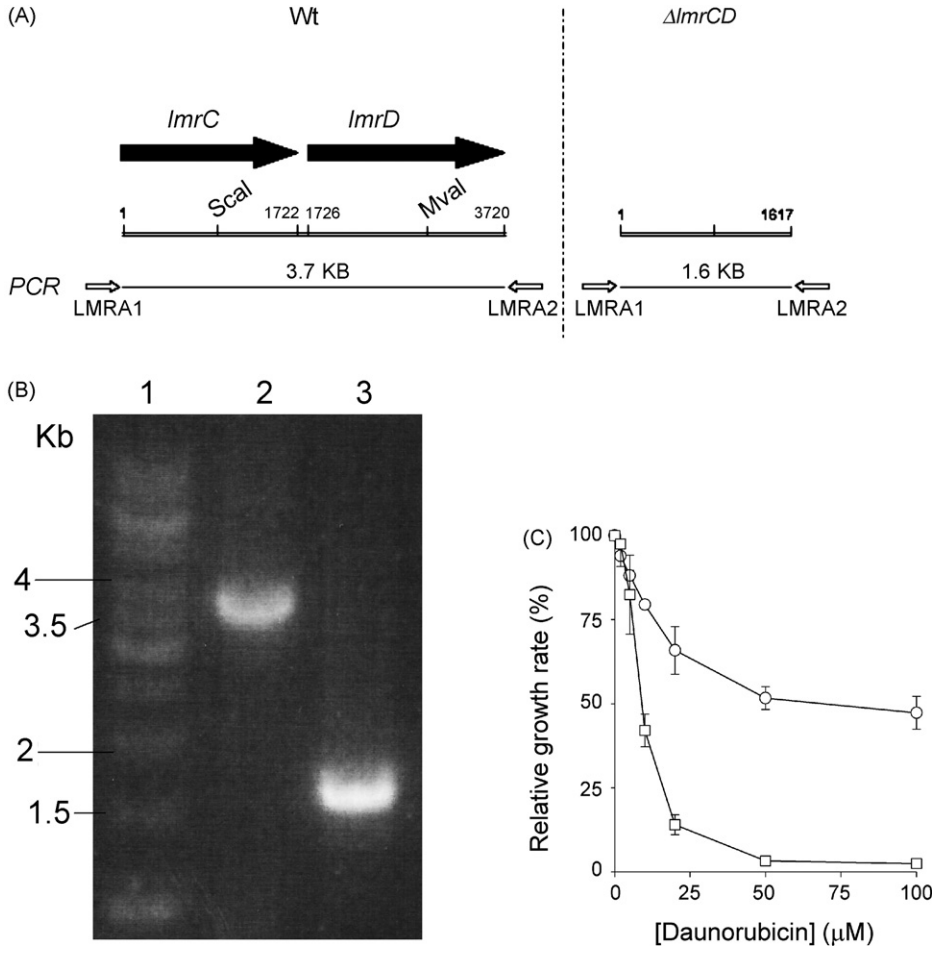

Fig. 1 – Analysis of the *lmrCD* locus in *L. lactis* strains. (A) Cartoon showing the localization of the *lmrC* and *lmrD* genes in the genome of the lactococcal cells. Two different primers LMRA1 and LMRA2 were used in a PCR reaction on genomic DNA to distinguish between *L. lactis* NZ9000 Δ*lmrA* (Wt *lmrCD*) and *L. lactis* NZ9000 Δ*lmrA* Δ*lmrCD*. (B) The PCR products were separated on a 1% agarose gel. Lane 1, migration of DNA ladder; Lane 2, genomic DNA from the Wt strain. Lane 3, genomic DNA from the deletion strain. (C) Sensitivity of *L. lactis* strains to daunorubicin. The relative growth rate of *L. lactis* NZ9000 Δ*lmrA* Wt *lmrCD* (○) or *L. lactis* NZ9000 Δ*lmrA* Δ*lmrCD* (□) is plotted as a function of the daunorubicin concentration in the culture medium. The maximum specific growth rate (μ_m) was determined at each daunorubicin concentration and is presented as a percentage of μ_m in the absence of daunorubicin. Without the drug, the $\mu_m$values for the Wt and Δ*lmrA* Δ*lmrCD* cells were 0.522 ± 0.030 h^{-1} and 0.384 ± 0.021 h^{-1}, respectively.

genes were sequenced to ensure that only the intended changes were introduced.

2.3. *Preparation of inside-out membrane vesicles*

Preparation of inside-out membrane vesicles from *L. lactis* NZ9000 Δ*lmrA* Δ*lmrCD* cells without or with expression of LmrA proteins was performed as described for *L. lactis* NZ9000 [6,17,18].

2.4. *Hoechst 33342 transport in membrane vesicles*

Hoechst 33342 transport in inside-out membrane vesicles (0.5 mg protein/ml) was measured by fluorescence spectroscopy [6,17,18]. In the experiments, the inside-out membrane vesicles were present in 100 mM KPi buffer (pH 7.4) containing 2 mM $MgSO_4$, 5 mM phosphocreatine, 0.1 mg/ml creatine kinase, and 2.5 mM Na-ATP.

2.5. *Purification of LmrA proteins*

The solubilization of His_6-tagged LmrA proteins from inside-out membrane vesicles with *n*-dodecyl-D-maltoside and the purification of the solubilized proteins with nickel–nitrilotriacetic acid resin were performed as previously described [9,18,19].

2.6. *TNP-nucleotide-binding assay*

Purified LmrA proteins were diluted to 25 μg/ml in 100 mM (K)HEPES buffer (pH 7.4). Increasing volumes of a 0.5 mM solution of 2′-(or-3′)-O-(trinitrophenyl)-adenosine 5′-triphosphate trisodium salt (TNP-ATP) (Molecular Probes) were added to 2 ml of the protein solution and fluorescence of bound TNP-nucleotide was measured in a LS 55B luminescence spectrometer (Perkin-Elmer Life Sciences) using excitation and emis-

sion wavelengths of 408 and 550 nm, respectively, and slit widths of 10 and 15 nm, respectively. Fluorescence was corrected for non-specific binding (less than 40% of total binding) using equimolar amounts of purified LmrA-MD.

2.7. ATPase assay

The ATPase activity of purified LmrA was measured from the release of Pi from ATP in a colorimetric malachite green assay. Purified protein (2.5 μg) in 100 mM (K)HEPES buffer (pH 7.4) containing 2.5 mM $MgSO_4$ and Na-ATP at concentrations as indicated in Fig. 2B, was incubated at 30 °C in a 96-well plate in a total reaction volume of 30 μl/well. Following incubation for 3 min, the ATPase reaction in each well was terminated by addition of 150 μl of freshly activated malachite green solution. This solution was prepared by mixing a solution of malachite green (17 mg/3.75 ml of ultrapure H_2O) and ammonium heptamolybdate (0.525 g/12.5 ml 4N HCl), followed by the addition of ultrapure H_2O to a final volume to 50 ml. The solution was left on ice for 4 h before it was used. The malachite green solution was filtered over a 0.45 μM-pore size acrodisc filter (Millipore) prior to use and supplemented with a 1:100 dilution of Triton-X100 from a 10% stock solution. After the addition of the malachite green solution, the 96-well plate was removed from the ice and incubated 5 min at 20 °C. Finally, 75 μl of 34% (w/v) citric acid was added to each well, and the plate was further incubated for 30 min at 30 °C in the dark to allow color development. The OD_{600} of the samples was measured in a Spectramax microplate reader (Molecular Devices), and compared with the OD_{600} of standards containing 0.5–5 nmol Pi. Readings were corrected for Pi contamination originating from the ATP, protein, and buffer.

2.8. Reconstitution of LmrA proteins

LmrA proteins were reconstituted into proteoliposomes in an inside-out fashion at a protein:lipid ratio of 1:100 (wt:wt) [9,18]. Dried total *E. coli* lipids/egg phosphatidylcholine mixtures were rehydrated in 100 mM KPi buffer (pH 6.0) containing 2 mM $MgSO_4$. After extrusion through a 400 nm polycarbonate filter the liposomes were destabilised by the addition of 2 mM TritonX-100 instead of the *n*-dodecyl-β-D-maltoside as described in the original method [19].

2.9. Hoechst 33342 transport in proteoliposomes

To impose a ΔpH (interior acidic) in proteoliposomes by pH jump, proteoliposomes in 100 mM KPi buffer (pH 6.0) were diluted 100-fold in 100 mM KPi buffer (pH 7.0) containing 2 mM $MgSO_4$. ATP-dependent Hoechst 33342 transport was measured by diluting the liposomes 100-fold in KPi buffer (pH 6.0) containing 2 mM $MgSO_4$, 5 mM phosphocreatine and 0.1 mg/ml creatine kinase. To impose the ΔpH in the presence of ATP, proteoliposomes were diluted 100-fold in 100 mM KPi buffer (pH 7.0) containing 2 mM $MgSO_4$ and the ATP regenerating system. The fluorescence recording was started and after 30 s, Hoechst 33342 (0.25 μM) was added. Where required, 2.5 mM ATP was added at time points indicated in Fig. 4, and Hoechst 33342 fluorescence was measured as a function of time in an LS 55B luminescence spectrometer (Perkin-Elmer Life Sciences) with excitation and emission wavelengths of 355 and 457 nm, respectively, and slit widths of 2.5 nm each.

2.10. Data analysis

All statistical analyses were performed with the Student's *t*-test with a 95% confidence interval for the sample mean. The data represent at least four independent observations with different batches of membrane vesicles and (proteo)liposomes.

3. Results

3.1. Construction of *L. lactis* Δ*lmrA* Δ*lmrCD*

To investigate LmrA activity in a background lacking genomic *lmrA* and *lmrCD*, we deleted/replaced the contiguous *lmrC* and *lmrD* genes in *L. lactis* NZ9000 Δ*lmrA* by a truncated Δ*lmrCD* region (Fig. 1A). For this purpose a DNA fragment containing the Δ*lmrCD* region was cloned into pORI280, which lacks *repA* and cannot replicate in *L. lactis*. The gene deletion method relies crucially on the temporary integration of pORI280 into the target genome due to a single crossover between homologous regions in Δ*lmrCD* and *lmrC* or *lmrD*. Due to the erythromycin-resistance marker on pORI280, this co-integrate is stable in the presence of erythromycin, but its resolution by a second crossover event between homologous regions in Δ*lmrCD* and *lmrC* or *lmrD* can be readily detected by screening for the loss of the erythromycin resistance phenotype or β-galactosidase activity associated with pORI280 when the erythromycin-selective pressure is removed. Depending on where the second crossover event occurs, either the lactococcal plasmid will revert to its original composition or the Δ*lmrCD* region will be incorporated in the genome, resulting in the deletion/replacement of *lmrC* and *lmrD* by Δ*lmrCD*. A PCR reaction, with primers LMRA1 and LMRA2, was used to discriminate between reversion and deletion. As expected, PCR of genomic DNA of the Δ*lmrCD* strain yielded a 1.6 kb fragment, whereas a 3.7 kb fragment was obtained for the organism containing Wt *lmrCD* (Fig. 1B).

The drug resistance phenotype of the Δ*lmrCD* strain was tested in cell cytotoxicity assays. In these assays, the growth rate of cells was measured in liquid cultures containing increasing concentrations of the anthracycline daunorubicin. The concentration of drug necessary to reduce the growth rate of cells by 50% (IC_{50}) was significantly lower for the Δ*lmrCD* strain compared to the Wt strain (IC_{50} of 7 μM for *L. lactis* NZ9000 Δ*lmrA* Δ*lmrCD* versus >100 μM for *L. lactis* NZ9000 Δ*lmrA* Wt *lmrCD*) (Fig. 1C). Hence, consistent with previous observations [12] the *lmrCD* deletion renders the cells hypersensitive to daunorubicin. In subsequent experiments described in this paper, LmrA proteins were expressed in the Δ*lmrA* Δ*lmrCD* background, excluding a cross-contamination of the activity of recombinant LmrA proteins by the activity of endogenously expressed LmrA or LmrCD.

3.2. Expression and functionality of NBD mutants of LmrA

To facilitate studies on the energetics of LmrA-mediated transport, we included NBD mutants of LmrA, which were

Table 1 – ATP binding and ATPase activities of purified LmrA proteins

Protein	TNP-ATP binding		ATPase	
	K_d (μM)	B_{max} (a.u.)	K_m (mM)	V_{max} (nmol/min/mg)
Wt LmrA	0.8 ± 0.1	38.2 ± 1.0	0.5 ± 0.1	125.5 ± 7.5
E512Q LmrA	0.2 ± 0.0	34.4 ± 0.6	3.0 ± 0.9	121.4 ± 5.3
ΔK388 LmrA	0.8 ± 0.1	36.5 ± 0.8	[a]	[a]

[a]ATPase activity was undetectable.

affected in their ability to hydrolyse ATP. In E512Q LmrA the highly conserved glutamate which is located immediately downstream of the Walker B asparate (D511 in LmrA), was replaced by glutamine [8]. In addition, we generated the ΔK388 LmrA mutant, which lacked the catalytic lysine residue in the Walker A motif. Coomassie Brilliant Blue-stained SDS-PAGE gels demonstrated that the NBD mutants were expressed in the plasma membrane of *L. lactis* at a similar level as Wt LmrA (data not shown).

The ability of purified NBD mutants to bind ATP was investigated in a binding assay with a fluorescent ATP analogue TNP-ATP (Fig. 2A). The E512Q and ΔK388 mutants bound TNP-ATP with a similar maximal binding (B_{max}) as Wt LmrA (34.4 ± 0.6 a.u. for E512Q LmrA, 36.5 ± 0.8 a.u. for ΔK388 LmrA, and 38.2 ± 1.0 a.u. for Wt LmrA), while the dissociation constant (K_d) was decreased by fourfold for E512Q LmrA and remained unaltered for ΔK388 LmrA compared to Wt LmrA (K_d = 0.2 ± 0.0 μM for E512Q LmrA versus 0.8 ± 0.1 μM for ΔK388 LmrA and 0.8 ± 0.1 μM for Wt LmrA) (Table 1).

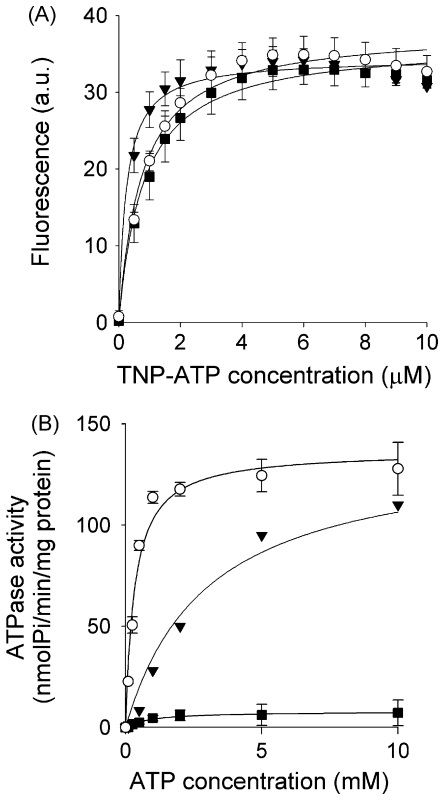

Fig. 2 – Characterisation of NBD mutants of LmrA. (A) Binding of TNP-ATP to purified Wt and mutant LmrA proteins (15 μg/ml) in 100 mM (K)HEPES buffer (pH 7.4) was measured by fluorimetry. (B) ATPase activity of purified LmrA proteins (83 μg/ml) was determined from the liberation of Pi from ATP. Wt LmrA (○), E512Q LmrA (▼), and ΔK388 LmrA (■).

We also assessed the ATPase activity of purified Wt and mutant LmrA proteins over a range of ATP concentrations (Fig. 2B). Analysis of the data showed that: (i) the ATPase activities of Wt LmrA and the E512Q mutant were saturable with a maximum activity (V_{max}) of 125.5 ± 7.5 nmol/min/mg and 121.4 ± 5.3 nmol/min/mg, respectively, (ii) E512Q LmrA had a significantly reduced apparent affinity for ATP in the hydrolysis reaction compared to Wt LmrA (K_m = 3.0 ± 0.9 mM for E512Q LmrA versus 0.5 ± 0.1 mM for Wt LmrA), and (iii) the ΔK388 LmrA mutant was a null mutant, which could not hydrolyse ATP at a significant level in our assay (Table 1).

3.3. *Hoechst 33342 transport in membrane vesicles*

One limitation inherent in studying the energetic requirements of LmrA-mediated transport in intact cells is the difficulty to control the nucleotide, drug and ion concentrations in the cytosol. We therefore investigated the transport activity of the NBD mutants of LmrA in inside-out membrane vesicles and proteoliposomes, where the composition of the environment on both sides of the membrane can be manipulated in detail. The transport of Hoechst 33342 in both systems can be monitored from the changes in the fluorescence of this environment-sensitive dye. After the addition of Hoechst 33342 to inside-out membrane vesicles containing LmrA, a rapid increase in fluorescence was observed as Hoechst 33342 bound to the phospholipid bilayer (Fig. 3). Upon addition of ATP, Hoechst 33342 fluorescence was quenched at a rate of 0.74 ± 0.10 a.u./s in LmrA-containing membrane vesicles which, at least in part, reflects the movement of Hoechst 33342 from the membrane into the aqueous environment [20]. The rate of fluorescence quenching was much slower for the control vesicles without LmrA (0.13 ± 0.05 a.u./s) (Fig. 3, traces 1 and 2), pointing to the involvement of LmrA in the transport reaction. Surprisingly, we found a rate of 0.24 ± 0.06 a.u./s for E512Q LmrA, which was significantly reduced compared to Wt, but which remained significantly above control (Fig. 3, trace 3). Furthermore, the rate of fluorescence quenching obtained for ΔK388 LmrA of 0.70 ± 0.07 a.u./s was similar to Wt (Fig. 3, trace 4). It was interesting to note that quenching of Hoechst 33342 fluorescence was also obtained for LmrA-MD, which lacks the NBD, and which is expressed at a twofold lower level than LmrA [9] (Fig. 3, trace 5). The ATP added to the membrane vesicles might directly energize LmrA activity through ATP binding/hydrolysis by the NBD. However, ATP is also used by the F_0F_1 H^+-

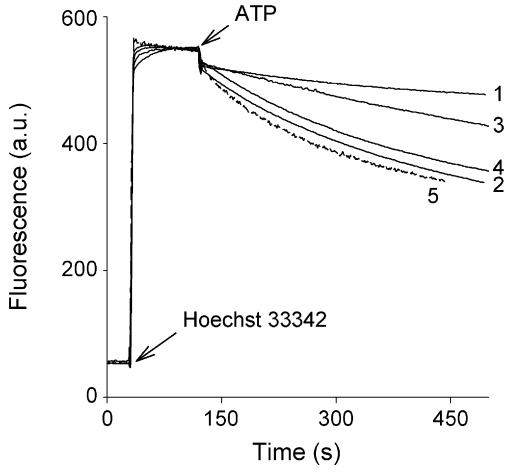

Fig. 3 – Hoechst 33342 transport in inside-out membrane vesicles. Membrane vesicles were diluted to a protein concentration of 0.5 mg/ml in 100 mM KPi (pH 7.0) containing 2 mM $MgSO_4$ and an ATP-regenerating system. Hoechst 33342 (0.25 μM) was added where indicated and the increase in the fluorescence of the dye was followed in time until a steady state was reached. After 2 min, active transport of Hoechst 33342 was initiated by the addition of 2.5 mM ATP. Trace 1, non-expressing control; trace 2, Wt LmrA; trace 3, E512Q LmrA; trace 4, ΔK388 LmrA; trace 5, Hoechst 33342 transport in inside-out membrane vesicles containing truncated LmrA without the NBD (LmrA-MD) [9].

ATPase to pump protons into the lumen of the membrane vesicles, thereby generating a Δp (interior positive and acidic) across the membrane. In view of our previous finding that LmrA-MD mediates proton-ethidium symport [9,10], and the current observation that LmrA-MD can mediate Hoechst 33342 transport in inside-out membrane vesicles in the absence of the NBD (Fig. 3, trace 5), ATP might also energize LmrA activity in membrane vesicles by an indirect mechanism based on Δp-dependent, secondary-active transport.

3.4. *Hoechst 33342 transport in proteoliposomes*

To separate ATP-dependence of Hoechst 33342 transport by LmrA from Δp-dependence, experiments were performed in proteoliposomes containing purified Wt LmrA, E512Q LmrA or ΔK388 LmrA in an inside-out fashion, similar to the orientation of these proteins in inside-out membrane vesicles. In contrast to membrane vesicles, proteoliposomes lack the F_0F_1 H^+-ATPase and other primary-active and secondary-active transporters, allowing a study of the ATP-dependence of LmrA-mediated transport in the absence of potentially interfering transport processes. Analysis of proteoliposomes on Coomassie Brilliant Blue-stained SDS-PAGE gels demonstrated the equal incorporation of Wt LmrA and NBD mutants in the liposomal membrane (data not shown). The proteoliposomes were used in Hoechst 33342 transport assays in the absence or presence of 2.5 mM ATP and/or a ΔpH (interior acidic), which was imposed by a pH jump (pH_{in} 6.0/pH_{out} 7.0). The magnitude of the ΔpH and the concentration of ATP in these experiments are both realistic and physiologically relevant. In the presence of ATP only, Hoechst 33342 transport activity in proteoliposomes was observed for Wt LmrA, for E512Q LmrA at a reduced level, but not for ΔK388 LmrA (Fig. 4A). Strikingly, the mutant proteins were equally active as Wt LmrA in the presence of the ΔpH (interior acidic) only (Fig. 4B). In the simultaneous presence of the ΔpH and ATP, the relative fluorescence changes of Hoechst 33342 in Wt LmrA or E512Q LmrA-containing proteoliposomes versus empty control liposomes displayed similar trends as observed in inside-out membrane vesicles (Figs. 4C and 3, respectively). In the proteoliposomes, the E512Q and ΔK388 LmrA mutants were both transport-active compared to the empty control liposomes, which in the case of ΔK388 LmrA must be due to the dependency on the ΔpH. No differences in Hoechst 33342 transport were observed between empty control liposomes and Wt or mutant LmrA-containing proteoliposomes in the absence of ATP and the ΔpH, pointing to a direct role of LmrA proteins in the transport activities observed (Fig. 4D). Taken together, these experiments show that LmrA-mediated Hoechst 33342 transport can be driven independently by the ΔpH and by ATP binding/hydrolysis.

4. Discussion

The experiments in this paper strongly support the concept that Hoechst 33342 transport by LmrA exhibits a dual mode of energy coupling. Most of our knowledge about the ATP dependence of drug transport by LmrA originates from ATPase measurements in membrane vesicles and transport studies in intact cells [5,6]. Our experiments in proteoliposomes containing purified and functionally reconstituted LmrA complement these earlier studies and demonstrate that this protein can mediate Hoechst 33342 transport in an ATP-dependent fashion in the absence of a possible contribution by LmrCD. Consistent with the lack of ATPase activity of ΔK388 LmrA (Fig. 2B), this mutant protein was not transport-active in proteoliposomes in the presence of ATP only (Fig. 4A). We detected residual Hoechst 33342 transport activity for the E512Q LmrA (Fig. 4A), in agreement with the reduced rate of ATP hydrolysis by this mutant protein compared to Wt at the 2.5 mM concentration of ATP used in the transport assay (Fig. 2B). These results indicate that, as expected for an ABC transporter, the ATP-dependent transport of Hoechst 33342 by LmrA is related to its ATPase activity.

Compared to membrane vesicles where levels of LmrA upon overexpression can be as high as 30% of total membrane protein [19], the amount of protein in the proteoliposomes is strongly reduced. Using an average M_w of 750 g/mol for a phospholipid molecule and average surface area of 39 $Å^2$/phospholipid molecule in a lipid bilayer [21], it can be calculated that about 40 LmrA homodimers were incorporated per proteoliposome in the reconstitution procedure. Hence, the Hoechst 33342 fluorescence changes obtained in the proteoliposomes (Fig. 4) are associated with a relatively small number of transporters, and are therefore highly significant. These calculations are also relevant for previous observations on ATP-dependent transport of C6-nitro-2,1,3-benzoxadiazol-

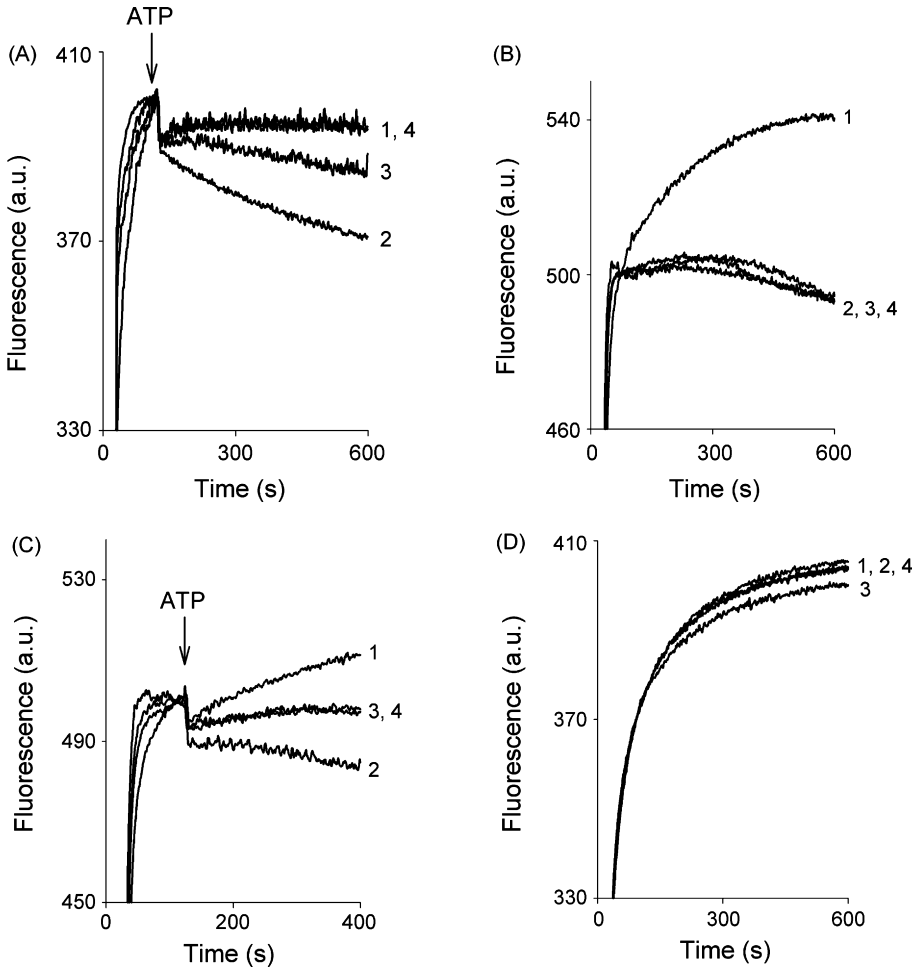

Fig. 4 – Hoechst 33342 transport in proteoliposomes. Control liposomes (trace 1) and proteoliposomes containing Wt LmrA (trace 2), E512Q LmrA (trace 3) or ΔK388 LmrA (trace 4) were prepared in 100 mM KPi buffer (pH 6.0) containing 2 mM $MgSO_4$. The (proteo)liposomes were then diluted in 100 mM KPi buffer (pH 6.0 or 7.0) containing 2 mM $MgSO_4$ with or without an ATP-regenerating system to test: (i) ATP-dependence of Hoechst 33342 transport in the absence (A) or presence (C) of a ΔpH (interior acidic), (ii) ΔpH (interior acidic)-dependence of Hoechst 33342 transport in the absence of ATP (B), and (iii) Hoechst transport in the absence of ATP or the ΔpH (D). Hoechst 33342 (0.25 μM) was added at 30 s after the fluorescence measurements were started and the fluorescence was followed as a function of time. Where required, 2.5 mM ATP was added at the *arrow*. Shown are representative traces from four independent experiments.

4-yl-labeled phosphatidylethanolamine in LmrA-containing proteoliposomes [19] which were generated by similar methods as the proteoliposomes in this investigation.

Studies on LmrA-MD (lacking the NBD) established Δ*p*-dependence of ethidium transport [9], and we recently extended the observations on ethidium-proton symport to full-length LmrA in proteoliposomes (data not shown). The current findings in proteoliposomes demonstrate the ΔpH (interior acidic)-dependence of Hoechst 33342 transport by full-length LmrA in addition to ATP-dependence (Fig. 4B). As no Hoechst 33342 fluorescence quenching was observed upon the artificial imposition of the ΔpH in empty control liposomes (Fig. 4B), the data suggest that ΔpH-dependent transport of Hoechst 33342 in the proteolipsomes is mediated by LmrA. In this context, it is interesting to note that amino acid sequence comparisons identified two transmembrane segments and a connecting loop, which are conserved between the MD of LmrA and homologous ABC transporters, and members of the Resistance-Nodulation-Cell Division family of Δ*p*-dependent multidrug transporters [22].

In contrast to the inhibitory effect of the ΔK388 and E512Q mutations on ATP-dependent Hoechst 33342 transport by LmrA, these mutations did not affect this activity in the presence of the imposed ΔpH only. In the simultaneous presence of ATP and the ΔpH (Fig. 4C), the Hoechst 33342 transport activities of the E512Q and ΔK388 LmrA mutants were clearly above control, but were reduced compared to their activities in the presence of the ΔpH only (Fig. 4B). The results can be explained by the notion that the conformational changes in the MDs of dimeric LmrA required for substrate transport, are coupled to conformational changes in the NBDs of the transporter. Evidence for this conformational coupling comes from the observation that the *in vitro* basal rate of ATP hydrolysis of LmrA can be stimulated up to threefold in the

presence of substrates such as the 1,4-dihydropyridine verapamil [7]. Coupling between conformational changes in the NBD and MD of LmrA has also been demonstrated by infrared spectroscopy and trytophan fluorescence quenching measurements [23]. For E512Q LmrA, the reduced ATPase activity compared to Wt, and hence, reduced rate at which the catalytic cycle proceeds at the NBDs of the mutant transporter, would slow down the rate of conformational changes at the MD during the ΔpH-dependent transport of Hoechst 33342, reminiscent of the findings in inside-out membrane vesicles (Fig. 3). For the ΔK388 LmrA, the binding of ATP but subsequent lack of significant hydrolysis of the nucleotide at the NBD would limit the rate of ΔpH-dependent transport of Hoechst 33342. This effect was more pronounced in proteoliposomes (Fig. 4B and C) than in inside-out membrane vesicles (Fig. 3).

Our observations on ΔpH-dependent Hoechst 33342 transport by ΔK388 and E512Q mutants in proteoliposomes provide an explanation for recent work by Van Den Berg Van Saparoea et al. [24] who concluded that the LmrA-mediated transport of Hoechst 33342 in inside-out membrane vesicles was not affected by mutations that reduce its ATPase activity. However, it should be noted that these authors observed a clear reduction in Hoechst 33342 transport in inside-out membrane vesicles containing E512Q LmrA compared to Wt LmrA (Fig. 2A, Ref. [24]), and that these researchers failed to detect the residual ATPase activity of this mutant (Fig. 1, Ref. [24]). By analogy to work on the F_0F_1 H^+-ATPase [25], the glutamate equivalent to E512 in LmrA has been proposed to act as the catalytic base in ATP hydrolysis in ABC proteins. Mutation of this carboxylate by the corresponding amide variously affects ATP hydrolysis depending on the ABC protein: in MJ1267 and MJ0796 [26] and BmrA [27] no steady state ATP activity could be detected whereas in contrast, and similar to LmrA, a significant residual ATPase activity was determined for HlyB [28] and Mdl1 [29]. The latter findings might be compatible with the notion that ABC proteins hydrolyse ATP by substrate-assisted catalysis rather than general base catalysis [28], and might reflect differences in the stability of the ATP-sandwich NBD dimer that has been observed by X-ray crystallography for this type of mutant [30].

Detailed knowledge of the biochemical properties of LmrA and homologous multidrug transporters in prokaryotic and eukaryotic cells will be essential, if we aim to understand their molecular mechanisms and physiological roles, and if we aim to manipulate or bypass their transport activities in a clinical setting. Our experiments clearly establish the ATP-dependence and ΔpH-dependence of LmrA-mediated Hoechst 33342 transport in (proteo)liposomes in which (i) purified LmrA was the only ATP binding/hydrolyzing enzyme, (ii) a well-defined ΔpH (interior acidic) was imposed artificially, and (iii) Hoechst 33342 fluorescence changes in the presence and absence of LmrA proteins could be directly compared under identical experimental conditions. Hence, recent conclusions regarding the bioenergetics of LmrA-mediated transport by Van Den Berg Van Saparoea et al. [24] and Lubelski et al. [31] should be taken with caution. In contrast to ethidium, which is a permanently charged monovalent cation [9], Hoechst 33342 is a bis-benzimidazole dye, which can be present in cationic, neutral or zwitterionic forms in the physiological pH range [32]. Therefore, in contrast to the previous studies on LmrA-MD showing ethidium-proton symport [9,10], the current investigations on Hoechst 33342 transport by LmrA in membrane vesicles and proteoliposomes do not discriminate between ATP/ΔpH-dependent uniport of Hoechst 33342, or more complex symport or antiport mechanisms involving Hoechst 33342, protons and, perhaps, other ions. We recently tested the drug transport properties of LmrA in intact cells with the Δ*lmrA* Δ*lmrCD* genotype, and we will report these interesting observations elsewhere.

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