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

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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

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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*