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Review

Structure-function analysis of multidrug transporters in *Lactococcus lactis*

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Abstract

The active extrusion of cytotoxic compounds from the cell by multidrug transporters is one of the major causes of failure of chemotherapeutic treatment of tumor cells and of infections by pathogenic microorganisms. A multidrug transporter in *Lactococcus lactis*, LmrA, is a member of the ATP-binding cassette (ABC) superfamily and a bacterial homolog of the human multidrug resistance P-glycoprotein. Another multidrug transporter in *L. lactis*, LmrP, belongs to the major facilitator superfamily, and is one example of a rapidly expanding group of secondary multidrug transporters in microorganisms. Thus, LmrA and LmrP are transport proteins with very different protein structures, which use different mechanisms of energy coupling to transport drugs out of the cell. Surprisingly, both proteins have overlapping specificities for drugs, are inhibited by the same set of modulators, and transport drugs via a similar transport mechanism. The structure-function relationships that dictate drug recognition and transport by LmrP and LmrA represent an intriguing area of research.

Keywords: Multidrug resistance; ATP-binding cassette transporter; Secondary transporter; LmrA; LmrP; P-glycoprotein

Contents

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1. Introduction

The nutritive and therapeutic treatment of farm animals with antibiotics, amounting to half of the world's antibiotic output, has selected for drug-resistant microorganisms that contaminate the food produced [1]. Likewise, the selection of drug-resistant pathogenic microorganisms in hospitalized patients with serious infections such as pneumonia, urinary tract infections, skin infections, and bacteremia has generally been ascribed to the widespread use of antimicrobial agents [2]. Besides bacteria, parasitic protozoa are responsible for some of the most devastating and prevalent diseases of humans and domestic animals, such as malaria (*Plasmodium* spp.) and toxoplasmosis (*Toxoplasma* spp.) [3].

Microorganisms can eliminate the drug target in the cell through the alteration or replacement of molecules that are normally bound by the antibiotic [4]. Alternatively, microorganisms can reduce the intracellular concentration of drugs by: (i) synthesizing enzymes that degrade antibiotics or that chemically modify, and so inactivate, the drugs [5], (ii) eliminating entry ports for hydrophilic drugs, such as outer membrane porins in Gram-negative bacteria [6], and (iii) manufacturing drug efflux systems that export lipophilic drugs before these compounds have the chance to find their cellular targets [7,8]. Some of these drug efflux systems are fairly specific for a given drug or class of drugs, but the so-called multidrug transporters have specificity for compounds with very different chemical structures and cellular targets. Multidrug transporters can be amplified in drug-resistant pathogenic microorganisms, and can shift their drug profiles, making them a menace to drug treatment.

2. Multidrug transporters LmrA and LmrP

Lactococcus lactis has developed fascinating antibiotic resistance mechanisms. A membrane protein, LmrA, belongs to the ATP-binding cassette (ABC) superfamily and is driven by ATP-hydrolysis. LmrA contains an N-terminal membrane domain with six membrane-spanning segments followed by the ABC domain [9]. LmrA is homologous to prokaryotic ABC transporters in *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Helicobacter pylori*, *Haemophilus influenzae*, and *Mycoplasma genitalium* [10] and to the hop resistance protein HorA in the beer-spoilage bacterium *Lactobacillus brevis* [11]. LmrA is also homologous to eukaryotic ABC transporters, and is a half-molecule version of the human multidrug resistance P-glycoprotein which is a cause of multidrug resistance of tumor cells during chemotherapy [12]. Recent studies of the expression of covalently linked dimers of LmrA in *L. lactis* suggests that, by analogy with P-glycoprotein, LmrA is transport active as a homodimer [13]. Another multidrug transporter in *L. lactis*, LmrP, mediates drug extrusion by a proton motive force-driven, electrogenic proton/drug exchange reaction [14]. LmrP contains 12 membrane-spanning segments which are connected by hydrophilic loops protruding into the cytoplasmic and extracellular space, and is a member of the major facilitator superfamily [15]. LmrP is homologous to various multidrug transporters in pathogenic bacteria and to the mammalian organic cation transporter OCT1 in kidney epithelial cells [8].

3. From cells to proteoliposomes

LmrA and LmrP were initially characterized in a number of mutants of *L. lactis* MG1363 which were isolated on the basis of resistance to high concentrations of ethidium, daunomycin, and rhodamine 6G, and which showed an increased rate of energy-dependent ethidium and daunomycin efflux compared to the wild-type [16]. Subsequently, the genes encod-

ing these transport proteins were identified and expressed in *E. coli* and *L. lactis*. Gene inactivation studies in *L. lactis* have established that ATP-dependent drug extrusion via LmrA still occurs in a *L. lactis lmrP* strain [15]. Methods have been developed to routinely overexpress histidine-tagged LmrA and LmrP in *L. lactis*, for LmrA up to levels of 30% of total membrane protein [17,18]. For this expression, a novel protein-expression system for cytotoxic proteins is used which is based on the nisin-inducible *nisA* promoter [19]. LmrA and LmrP were efficiently solubilized with dodecyl maltoside (DDM), purified by Ni-affinity chromatography, and reconstituted in DDM-destabilized preformed liposomes prepared from *E. coli* phospholipids and egg phosphatidylcholine. Interestingly, the inhibition of the activity of LmrP by detergents restricted the range of compounds that could be used for solubilization and reconstitution of the protein, because low concentrations of detergent are retained in proteoliposomes [17]. Currently, structural and functional properties of LmrA and LmrP are studied in whole cells, plasma membrane vesicles, and proteoliposomes containing the purified and reconstituted proteins.

4. Drug specificity

The drug specificity is one of the most intriguing aspects of LmA and LmrP. The number of compounds that are recognized by these proteins is vast. To compare the pharmacological properties of bacterial LmrA with those of its human homolog P-glycoprotein, LmrA was functionally expressed in human lung fibroblast cells and insect cells which previously had been used for the characterization of P-glycoprotein [20]. Lung fibroblast cells expressing LmrA protein showed a 10- to 60-fold increased resistance to a variety of natural product drugs and synthetic chemotherapeutic drugs which are typical substrates of P-glycoprotein [21]: (i) anthracyclines such as daunomycin and doxorubicin; (ii) *vinca*-alkaloids such as vinblastine and vincristine; and (iii) cytotoxic agents such as ethidium bromide, rhodamine 6G and 123, and colchicine. P-glycoprotein function can be inhibited by a large group of structurally unrelated modulators [21]. All of these modulators tested also reversed drug resistance generated by expression of LmrA in lung fibroblast cells: (i) calcium channel blockers such as verapamil and its

Table 1
Binding and transport of clinically relevant antibiotics by LmrP

Group	Antibiotic	Binding to LmrP	Transport by LmrP
Aminoglycosides	gentamycin	−	−
	kanamycin	−	−
β-Lactams	meropenem	−	−
Cephalosporins	ceftazidime	n.t.[a]	−
Lincosamides	clindamycin	+	+
Macrolides	azithromycin	+	+
	clarithromycin	+	+
	dirithromycin	+	+
	erythromycin	+	+
	roxithromycin	+	+
	spiramycin	−	−
Penicillins	ampicillin	−	−
Quinolones	ciprofloxacin	n.t.	−
	ofloxacin	n.t.	−
Streptogramins	dalfopristin	+	+
	quinupristin	+	−
Tetracyclines	tetracycline	n.t.	+
Others	chloramphenicol	−	−
	sulfamethoxazole	−	n.t.
	trimethoprim	−	−
	vancomycin	−	−

[a]Not tested.

analog CP100–356; (ii) 1,4-dihydropyridines such as nicardipine; (iii) indolizine sulfones such as SR33557; (iv) antimalarials such as quinine and quinidine; (v) immunosuppressants such as cyclosporin A; (vi) the *Rauwolfia* alkaloid reserpine; and (vii) the phenylalkylamine verapamil. Thus, the drug and modulator specificity of LmrA is very similar to that of P-glycoprotein. Interestingly, all compounds mentioned above also interact with the secondary multidrug transporter LmrP. In addition, LmrA and LmrP are able to transport antibiotics. Recently, this phenomenon has been studied in detail for LmrP (Table 1). From toxicity assays in whole cells of *L. lactis* and *E. coli*, and transport studies in membrane vesicles and proteoliposomes it was concluded that LmrP mediates the transport of various members of the lincosamides, macrolides, streptogramins, and tetracyclins [22]. These observations demonstrate the important role that multidrug transporters play in microbial antibiotic resistance.

5. Transport models

Several transport models have been postulated for LmrP, LmrA and P-glycoprotein pump function to explain the broad specificity for chemically unrelated compounds. Drug translocation may involve substrate transport from the cytoplasm to the exterior (conventional transport hypothesis [23]) which would require flexibility of an 'enzyme-like' drug recognition site. However, a property common to most LmrP, LmrA and P-glycoprotein substrates is their ability to intercalate between the phospholipids of biological membranes. This notion has led to the suggestion that these transporters recognize drugs within the membrane (vacuum cleaner and flippase hypotheses [14,24–26]). Drug recognition within the membrane is supported by a number of observations: (i) photo-affinity analogs of P-glycoprotein substrates label P-glycoprotein predominantly in or near the transmembrane helices 4–6, and 11–12 [27–29], (ii) point mutations resulting in the alteration in drug specificity of P-glycoprotein are frequently found in transmembrane helices [12], (iii) acetoxymethyl esters of several fluorescent probes accumulate less in P-glycoprotein or LmrA-expressing cells, despite the fact that the ester moieties are rapidly cleaved by intracellular esterases and the resulting carboxylates are not substrates for P-glycoprotein and LmrA [26,30], (iv) the kinetics of ATP-dependent transport of Hoechst 33342 by P-glycoprotein and LmrP, and of TMA-DPH by LmrA and LmrP in membrane vesicles is consistent with transport of the compounds from the inner, but not from the outer leaflet of the lipid bilayer [14,26,31,32]. This transport mechanism is likely to be a more general mechanism for membrane transporters with hydrophobic substrates. The human *MDR2* gene-encoded P-glycoprotein transports phosphatidylcholine from the cytoplasmic leaflet of the bile canicular membrane of hepatocytes into the bile [33,34]. In addition, the *E. coli* hemolysine transporter HlyB most likely binds the transport signal sequence of hemolysine, when the signal sequence forms an amphiphilic helix that binds to the cytoplasmic leaflet of the plasma membrane [35,36]. Thus, a main determinant of specificity for multidrug transporters will be the ability of drugs to be intercalated into the lipid bilayer. The subsequent interactions between drug molecules and drug binding sites on the transport proteins will be the second determinant of drug specificity.

6. Multiple drug binding sites

There is increasing evidence for the presence of more than one drug binding site on LmrP and LmrA. For LmrP, transport studies in membrane vesicles of *L. lactis* suggest that some drugs inhibit LmrP-mediated Hoechst 33342 transport through competition with Hoechst 33342 for binding to the same drug binding site on LmrP, whereas other drugs inhibit LmrP-mediated Hoechst 33342 transport non-competitively, through binding to a drug binding site different from the Hoechst 33342 binding site [37]. Similar observations have been reported for the staphylococcal proton motive force-dependent multidrug export protein QacA [38]. Competition studies showed that the QacA-mediated export of ethidium is competitively inhibited by monovalent cations, and non-competitively inhibited by divalent cations, which suggests that monovalent and divalent cations bind at separate sites on the QacA protein. Thus, LmrP and QacA each contain at least two distinct drug binding sites. Recently, a 3-dimensional

(3D) structure analysis of the *B. subtilis* transcriptional regulator BmrR [39], and site-directed mutagenesis studies on the *E. coli* multidrug resistance protein MdfA [40] have revealed that a negatively charged glutamate residue in a hydrophobic environment plays a key role in the cation selectivity of these proteins. Similarly, the three negatively charged residues in putative transmembrane segments of LmrP (D142 in TM5, E327 in TM10, and E388 in TM12) may play a role in the binding of cationic drugs by LmrP. For LmrA expressed in plasma membranes of *Spodoptera frugiperda* insect cells [20] and P-glycoprotein expressed in plasma membranes of Chinese hamster ovary B30 cells [41–43], kinetic analysis of drug dissociation revealed the presence of two non-identical, allosterically linked drug binding sites in both proteins.

7. Concluding remarks

Multidrug resistance in eukaryotic and prokaryotic cells is an increasingly complex clinical and public health problem. Prevention and control strategies will require the application of epidemiological and behavioral approaches, as well as research technologies aimed at the basic mechanisms of drug resistance. Understanding the molecular basis of drug recognition and transport by multidrug transporters will ultimately require the elucidation of their structures to high resolution. As yet there is no structural information of sufficient detail available. A recent report on the structure of P-glycoprotein at 2.5 nm resolution showed a monomeric molecule with discrete domains [44], giving a promising basis for the future. For LmrA and LmrP, milligram quantities of highly purified protein can be obtained rather easily. These results give us a very good starting point for protein crystallization procedures.

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