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Review

Overexpression, purification, and functional characterization of ATP-binding cassette transporters in the yeast, *Pichia pastoris*

Jie Cai, Philippe Gros*

Department of Biochemistry and McGill Cancer Center, McGill University, Montreal, Quebec, Canada

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Abstract

The ATP-binding cassette (ABC) transporter superfamily is a large gene family that has been highly conserved throughout evolution. The physiological importance of these membrane transporters is highlighted by the large variety of substrates they transport, and by the observation that mutations in many of them cause heritable diseases in human. Likewise, overexpression of certain ABC transporters, such as P-glycoprotein and members of the multidrug resistance associated protein (MRP) family, is associated with multidrug resistance in various cells and organisms. Understanding the structure and molecular mechanisms of transport of the ABC transporters in normal tissues and their possibly altered function in human diseases requires large amounts of purified and active proteins. For this, efficient expression systems are needed. The methylotrophic yeast *Pichia pastoris* has proven to be an efficient and inexpensive experimental model for high-level expression of many proteins, including ABC transporters. In the present review, we will summarize recent advances on the use of this system for the expression, purification, and functional characterization of P-glycoprotein and two members of the MRP subfamily.

Keywords: ATP-binding cassette transporter; *Pichia pastoris*

1. Introduction

The ATP-binding cassette (ABC) transporter superfamily is a large gene family that has been highly conserved through evolution from bacteria to humans, including 51 members in human, 55 in fly, 129 in plants, and over 300 in bacteria so far. These integral membrane proteins use ATP hydrolysis to energize the transport of a large variety of molecules, ranging from proteins to ions. Many excellent reviews and books on the phylogeny, structure, function, and mechanisms of action of prokaryotic and eukaryotic ABC transporters have been published [1–8], and readers are referred to those for background information. This review will solely deal with the use of the yeast *Pichia pastoris* for expression, purification, and functional characterization of three mammalian ABC transporters, with emphasis on unique aspects of this host for expression and purification studies.

ABC transporters generally share a common structural/functional unit, consisting of one nucleotide-binding domain (NBD) associated with a membrane-spanning domain (MSD) comprising six transmembrane (TM) α-helices. In eukaryotes, a functional ABC transporter is often composed of a duplication of this unit, either as a homodimer fused in a single molecule containing two NBDs and two MSDs (e.g. P-glycoprotein), or as two heterologous half transporters (e.g. ABCB2/3, ABCG5/8). In general, eukaryotic ABC transporters are structurally organized in the order of amino (N) terminus–MSD1–NBD1–MSD2–NBD2–carboxyl (C) terminus in a single molecule (Fig. 1A), although an extra N-terminal membrane-spanning domain (MSD0) followed by a cytoplasmic linker region (L0) is found in some members of the family (Fig. 1C). The relative position of NBDs can be inversed and found amino terminally to the first TM segment (e.g. members in ABCG subfamily). On the other hand, in most ABC transporters from bacteria, NBDs, MSDs, and substrate-binding proteins (SBPs) are separate polypeptides that assemble at the membrane to form a single transporter [9,10]. All ABC proteins are defined by the presence of the hallmark consensus Walker A [$GX_4GK(S/T)$] and Walker B (hyd_4D, where hyd = hy-

* Corresponding author. 3655 Sir William Osler Promenade, room 907, Montreal, Quebec, Canada H3G 1Y6. Tel.: +1-514-398-7291; fax: +1-514-398-2603.
E-mail address: cai@med.mcgill.ca (P. Gros).

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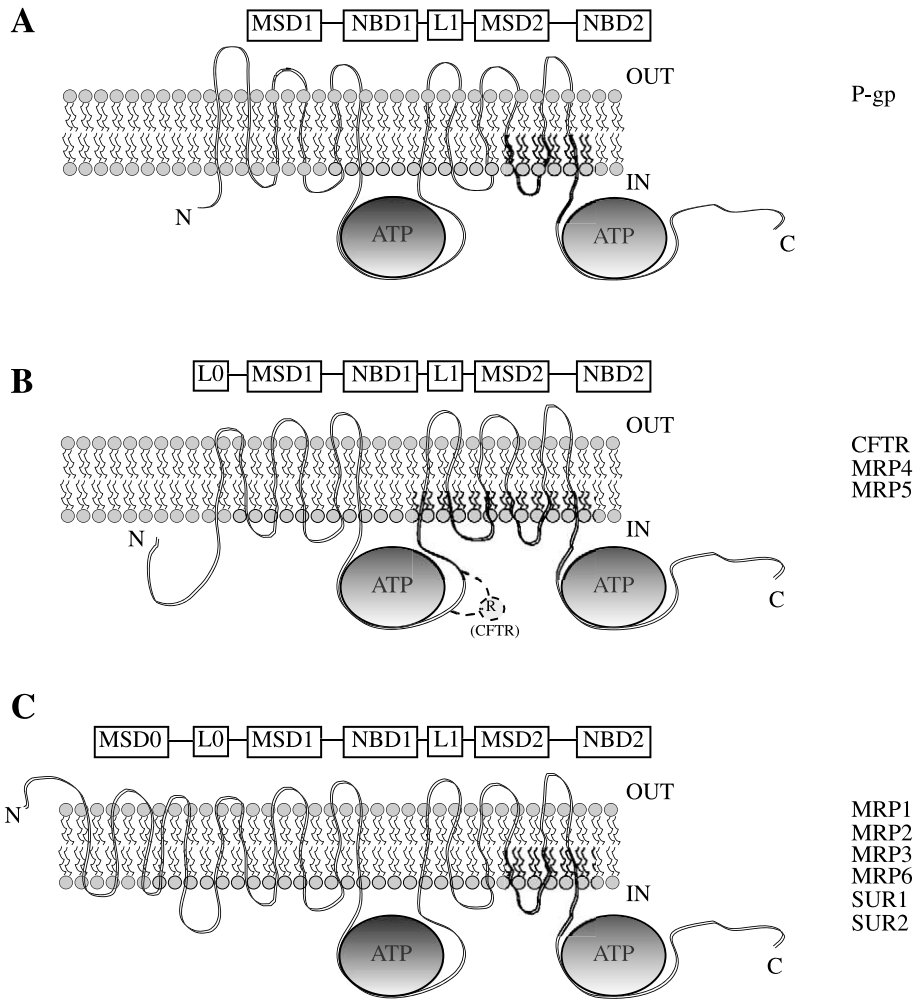

Fig. 1. Schematic diagrams of the membrane topology of ABC transporters. (A) Typical four-domain core structure (MSD1–NBD1–MSD2–NBD2) of P-glycoprotein (P-gp). (B) Membrane topology of some members of the ABCC subfamily that contain only the core structure with an extra N-terminal cytoplasmic region (L0), as in MRP4, MRP5, and CFTR. The regulatory "R" domain of CFTR in the L1 region is depicted as dashed lines. (C) Membrane topology of MRP1 and its close relatives that contain the additional N-terminal MSD (MDS0). The orientation of the lipid membrane bilayer is indicated.

drophobic amino acid residues) sequence motifs in each NBD, in addition to the highly conserved signature motif (LSGGQKQRIAIA) known as the ABC motif [8]. NBDs are the most conserved regions of ABC transporters, and detailed multiple sequence alignments of these NBDs from over 250 sequences have recently been published [11]. In contrast to the NBDs, sequence conservation in the MSDs of ABC transporters is modest. The exact number, position, and polarity of TM segments have been studied carefully only in a few ABC transporters, such as P-gp [12] and MRP1 [13,14], and these studies agree with hydropathy profiling, strongly supporting a 2×6 TM arrangement in the MSDs. Recently, a structure at 4.5-Å resolution of the *Escherichia coli* ABC transporter MsbA homodimer [$2 \times$ (MSD–NBD)], has been obtained by X-ray crystallography, confirming the presence of six TM helices in each MSD of the ABC transporter [15]. Interestingly, a distinct overall structure has been observed for another *E. coli* ABC transporter, BtuCD, which mediates cellular uptake of vitamin B_{12} [16]. The transporter is composed of two membrane subunits ($2 \times$ BtuC) and two ABC subunits ($2 \times$ BtuD). A crystal structure at resolution of 3.2 Å shows that, unlike most ABC transporters, the membrane subunit (BtuC) contains 10 TM α-helices instead of six. The tetramer $2 \times$ (BtuCD) forms a large translocation channel between the subunits [16]. The predicted intracellular loops that join certain pairs of TM segments in P-gp show a significant degree of sequence similarity. Although their role in the transport process remains largely unknown, their importance is highlighted by the detrimental effect of mutations in such segments (reviewed in Ref. [3]). Based on the similarity in sequence and organization of the NBDs, the human ABC transporter proteins have been classified into seven distinct subfamilies, namely ABCA to ABCG, each composed of a number of closely related genes [7] [http://humanabc.4t.com/humanabc.htm]. Mutations in many of the ABC transporters are found to be associated with various genetic disorders and pathological conditions

in humans [8]. For example, cystic fibrosis (CFTR/ABCC7) [17], Dubin–Johnson syndrome (cMOAT/MRP2/ABCC2) [18], progressive familial intrahepatic cholestasis (SPGP/BSEP/ABCB11) [19], adrenoleukodystrophy (ABCD1) [20], Stargardt's disease and age-related macular degeneration (ABCR/ABCA4) [21], Tangier disease and familial hypoalpha-lipoproteinemia (ABCA1) [22,23], and pseudoxanthoma elasticum (MRP6/ABCC6) [24] are all caused by mutations in ABC transporter genes. The substrates for ABC transporters are very diverse, and include natural product drugs (ABCB1), lipids (ABCA4, ABCA7, ABCB4), fatty acids (ABCD1–4), sterols (ABCG5, ABCG8), anionic conjugates (ABCC1–3), peptides (ABCB2, -3), cyclic nucleotides and nucleoside analogs (ABCC4, -5), and ions (ABCB6, -7, ABCC7) [http://humanabc.4t.com/humanabc.htm].

A large body of published data supports the proposal that ABC transporters transport a large variety of structurally unrelated substrates by a very similar mechanism, and that this common aspect of transport resides in regions of high degree of sequence conservation. Excellent reviews on such structure/function relationships in ABC transporters have been published [1–8], and will not be reiterated herein. Suffice it to say that studies by site-directed mutagenesis, site-specific modification, epitope mapping with photoactive ligands and studies in chimeras strongly suggest that TM segments form the substrate-binding site inside the membrane. On the other hand, the two highly conserved NBDs have been shown to bind ATP by photolabeling, and in most cases to display ATPase activity. Both processes are absolutely required for transport activity, and thought to underlie the unique substrate-induced ATPase activity seen in certain ABC transporters (see below). The current working model for ABC transporters is that ATP binding and hydrolysis by the NBDs cause structural changes in the TM-associated substrate binding sites to mediate transport of the ligand across or within the lipid bilayer. Nevertheless, a number of important questions on the structure and mechanism of action of ABC transporters have remained so far unanswered or poorly understood. Some of these include (1) a high resolution structure of one such protein, including TM segments and membrane associated regions; (2) the structure of substrate-binding sites capable of accommodating with high affinity a large number of structurally unrelated substrates; (3) the mechanism by which ATP binding and hydrolysis at either or both NBDs regulate substrate binding and transport at the MSDs; (4) contribution of each NBD to ATPase activity in the overall mechanism of transport; (5) dynamic information on structural changes during the catalytic cycle or transport process.

Large amounts of active protein in a highly pure state are required to address these issues. Several expression systems have been used to purify ABC transporter proteins in a functional state. These include bacterial [25], mammalian [26,27], baculovirus-infected insect cell [28], and yeast [29–31] expression systems. In this review, we will summarize some of our work with the yeast *P. pastoris*, review unique features of this system, and how it has been used to successfully express and study three mammalian ABC transporters, P-glycoprotein (P-gp/ABCB1), and two members of the MRP/ABCC subfamily, MRP1 (ABCC1) and MRP6 (ABCC6). These transporters are associated with the emergence of multidrug resistance in mammalian cells [2,3], fungi [32], and protozoans [33], as well as with genetic defects in human connective tissues in pseudoxanthoma elasticum (PXE) patients [24].

2. P-glycoprotein (P-gp/ABCB1)

2.1. Introduction

P-glycoprotein (P-gp/ABCB1) was discovered as being overexpressed and responsible for the multidrug resistance phenotype of tumor cells selected for high-level resistance in vitro [34] or appearing during chemotherapy in vivo. P-gp-expressing cells are resistant to a wide variety of structurally and functionally unrelated cytotoxic drugs, including natural products Vinka alkaloids, anthracyclines, taxanes, epipodophyllotoxins, and actinomycin D (reviewed in Ref. [3]). Three P-gp encoding *mdr* genes have been detected in rodents (*mdr1*, *mdr2*, *mdr3*) [35,36] while only two have been found in humans (*MDR1*, *MDR2*) [37,38]. The corresponding P-gps are functionally classified into Class I (Mdr1, Mdr3, MDR1) and Class II (Mdr2, MDR2), by the ability of Class I, but not Class II, P-gps to convey multidrug resistance upon transfection and overexpression of the corresponding cDNAs into otherwise drug-sensitive cells (reviewed in [3]). Much of the early functional studies on P-gp were carried out in mammalian cells expressing high levels of P-gp and obtained by stepwise selection and overexpression of the endogenous *MDR/mdr* genes or of a transfected *MDR/mdr* cDNA [3]. Indeed, transfection of biologically active *MDR/mdr* cDNAs into cells followed by selection for high-level drug resistance in vitro offered an early advantageous way to isolate cell clones expressing high levels of P-gp for functional studies. Studies in intact cells or in sealed membrane vesicles derived from them have established that P-gp functions as a drug efflux pump at the plasma membrane that shows amazingly broad substrate specificity. Transport is ATP- and temperature-dependent, and can be blocked by a number of structurally unrelated P-gp inhibitors, including calcium channel blockers such as verapamil, or immunomodulators such as cyclosporin A [3]. Polarized epithelial kidney cells stably transfected with *MDR/mdr* cDNAs and overexpressing Class I and Class II P-gps have also been used to monitor trans-epithelial drug transport by P-gp [39]. Studies in these cells have also demonstrated that the normal physiological function of P-gp is to act as a lipid transporter, with Class I P-gp showing broad lipid specificity, while Class II P-gp is specific for phosphatidylcholine [40]. P-gp displays the

classical four-domain structure (MSD1–NBD1–MSD2–NBD2) shared by many members of the ABC transporter superfamily (Fig. 1A) [3]. Studies with recombinant P-gps bearing discrete epitope tags inserted into different locations of the protein, followed by immunodetection in intact or permeabilized transfected cells [12], have verified the major topological features of P-gp initially predicted by hydropathy profiling [38,41]. These include 12 TM segments clustered in two MSDs, two intracellular NBDs, a large glycosylated extracellular loop, and intracellular C- and N-termini (Fig. 1A). Peptide mapping with photoactive substrate analogs [42], studies in chimeric [43–45], naturally occurring [46,47], or experimentally induced [48–53] P-gp mutants with altered substrate specificities, as well as site-specific modifications in single-cysteine mutants [54–56], suggest that the MSDs of P-gp, and of other ABC transporters, form the substrate binding site in the lipid bilayer. On the other hand, drug transport by Pgp is ATP-dependent, and P-gp directly binds ATP (reviewed in Ref. [3]). Studies by site-directed mutagenesis (for example Refs. [57,58]), site-specific modification in single Cys mutants [59], and studies in half molecules [60] show that both NBDs are essential for function, with complete cooperativity between the two NBDs for ATP hydrolysis and drug transport.

2.2. Expression in various systems

Characterization of P-gp ATPase activity has been carried out using partially or highly purified protein preparations from different expression systems. In general, ATPase activity is measured by standard Pi release assay or by the ability to trap photolabeled nucleotide (8-azido-[^{32}P]ADP) in a vanadate (Vi)-dependent fashion. Low-level drug-stimulated ATPase activity was initially reported in crude membrane fractions from P-gp transfected NIH-3T3 cells [61]. Using the insect cell (Sf9)/baculovirus expression system, underglycosylated recombinant P-gp can be overexpressed and readily detected by SDS-PAGE of crude membrane fractions followed by Coomassie blue staining [62]. Membrane vesicles from P-gp-expressing Sf9 cells can be used directly for transport studies, and P-gp produced in this system binds drugs [62] and shows vanadate-sensitive and drug-stimulated ATPase activity [63]. The lytic nature of the baculovirus infection/expression, although allowing overexpression of membrane proteins with cytotoxic effects, limits the amount of protein produced due to its batch mode. In addition, it is time-consuming to generate the expression constructs and to obtain sufficiently high viral titers. P-gp has also been expressed using a vaccinia virus-based system [64]. One limitation of this system is that the cloning and selection process involves multiple steps, although the level of expression of transport-competent P-gp ultimately reached in infected cells is quite high. Transient or stable expression of P-gp in HEK293 cells has been favored by some [65], and offers a reasonably simple and quick procedure to express wild-type or recombinant isoforms of P-gp, for transport studies and for biochemical characterization, including small-scale purification (see below). Finally, full-length P-gp has been expressed in *E. coli* [66]. Although bacterial expression of Pgp could constitute a rapid and relatively inexpensive way to produce large amounts of the protein, the usefulness of this system is limited by the relative instability of the full-length protein, the very short half-life, and solubility problems (inclusion bodies). Nevertheless, expression of functional human P-gp in protease-deficient *E. coli* mutants has been reported and is associated with decreased uptake of tetraphenylphosphonium (TPP) in intact cells, and increased ATP-dependent transport of TPP into inside-out vesicles from these cells [66]. The low amount of biologically active protein and the lack of eukaryotic maturation processes in this system have limited its general applicability. On the other hand, large amounts of individual NBDs of Pgp have been expressed in bacterial hosts [25]. These have been purified to homogeneity after extraction and solubilization using chaotropic agents. Although these preparations do not show ATPase activity, they have been used to study the nucleotide binding properties of the NBDs as well as their reported interaction with steroids such as RU486 [25,67].

2.3. Purification from different hosts

P-gp has been purified to homogeneity from a number of sources (Table 1). Wild-type hamster Pgp was purified from plasma membrane enriched fractions of a colchicine-resistant CHO cell line, CH^{R}B30, by a protocol involving isolation of enriched plasma membrane by sucrose density gradient, solubilization in Zwittergent 3-12, ion exchange chromatography on DEAE cellulose, and immunoaffinity chromatography using anti-Pgp monoclonal antibody C494 [26]. P-gp purified by this protocol shows ATPase activity and is transport-competent once reconstituted in proteoliposomes [26]. Hamster Pgp has also been purified from another CHO cell line (CR1R12) by a protocol based on solubilization in octylglucoside/*E. coli* lipid mixture, absorption on Reactive Red 120 agarose followed by NaCl gradient elution, and reconstitution by dialysis [27,68]. Such a preparation (1-mg purified protein from 10-mg membranes) shows a drug stimulable ATPase activity of 4.2 μmol Pi/min/mg protein, and demonstrates vanadate-induced trapping of nucleotide [27]. On the other hand, recombinant human P-gp bearing a series of histidine at the C terminus (metal binding site) and expressed in HEK 293 cells can be easily purified in small amounts by affinity chromatography. In this protocol, crude membrane extracts from Pgp-positive cells are solubilized in *n*-dodecyl-β-D-maltoside, and further purified by two successive chromatography steps on Ni-NTA resins, and eluted with imidazole (5–10 μg per 10 × 100-mm dishes of transiently transfected HEK 293 cells). Such enzyme preparations show ATPase activity of 1–1.2 μmol Pi/min/mg protein under full stimulation by verapamil or colchicine [65]. P-gp purified from *Saccharomyces cerevisiae* and reconstituted

Table 1
Comparison of expression and purification of P-glycoprotein in various expression systems

Host	Starting materials	P-gp content (%)	De	Purification methods	Final yield	Purity (%)	Lipid	Reference
CHO (CH^RB30)	3.5 mg PM 16/31 SFI	15	Zwt	DE52 IMAC	100 μg	90	bovine brain PE	[26]
CHO (CR1R12)	10 mg PM 16/31 SFI	21	OG	Reactive Red 120 agarose	1 mg	pure[a]	*E. coli* lipids	[27,68]
HEK293	20 × 10-cm dish culture	N.A.	DM	Ni-NTA	6–12 μg	80	sheep brain PE	[65]
S. cerevisiae	20 mg PM	<0.5	LPC	Ni-NTA	50 μg	90	*E. coli* lipids	[69]
KB-V1	16 mg PM	3 in DeEx	OG	CL-6B WGA	30 μg	>90	mixture[b]	[72]
Baculovirus/insect	500–600 mg PM	3–4	OG	Ni-AC	4–6 mg	80	mixture[b]	[70,71]
P. pastoris	480–600 mg microsomes	5	DM	Ni-NTA DE52	6 mg	pure[a]	asolectin or *E. coli* lipid	[31]
P. pastoris	100 mg microsomes	5	LPC	Ni-NTA avidin	700 μg	pure[a]	*E. coli* lipid	[90]

Abbreviations: AC: affinity chromatography; De: detergent; DeEx: detergent extract; DM: *n*-dodecyl-β-D-maltoside; IMAC: immunoaffinity chromatography; LPC: lysophosphatidylcholine; OG: octyl-β-D-glucopyranoside; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PM: plasma membrane; PS: phosphatidylserine; SFI: sucrose fraction interphase; WGA: wheat germ agglutinin; Zwt: Zwittergen 3-12.

[a] Contaminants were not detectable by SDS-PAGE and Coomassie blue staining.

[b] Mixture of *E. coli* lipid/PC/PS/cholesterol (w/w 60:17.5:10:12.5) at 1:8 protein/lipid ratio.

into *E. coli* lipids also exhibits verapamil-stimulated ATPase activity [69]. In addition, baculovirus-infected insect cells have also proven to be an excellent source for expression and purification of functionally active P-gp [70,71], and the yield of purified P-gp was superior to that obtained from mammalian cells [65,72]. Comparison of the expression and purification of P-gp in several expression hosts has been summarized in Table 1.

2.4. Functional characterization in yeast S. cerevisiae

Our laboratory has turned to yeast as a model organism to study P-gp structure and function. Yeast offers several advantages over other expression systems for rapid and efficient genetic analysis, as well as for transport studies and high-level expression of heterologous proteins. The yeast *S. cerevisiae* has an ABC transporter, designated *STE6*, that transports the mating pheromone peptide "**a**" factor; yeast cells bearing a null mutation at *STE6* are sterile and do not mate with α cells [73]. Expression of the Class I mouse P-gp *mdr3* in a *ste6* deletion mutant was shown to restore mating, suggesting that *mdr3* was functional in yeast [74,75]. Additional studies showed that P-gp could be stably expressed in the membrane fraction of yeast cells where it bound ATP and drug analogs (iodoarylazidoprazosin), and importantly, its expression at this site was concomitant to the appearance of cellular resistance to the anti-fungal macrolide peptide and cyclosporin analog FK506 and FK520 [75]. Genetic approaches have been used to identify structure/function relationship of P-gp expressed in yeast. For example, studies in chimeric molecules showed that NBD1 cannot be substituted by NBD2 in P-gp without loss-of-function, but also established that most of NBD1 could be substituted by corresponding segments of NBD2 except for two sets of discrete residues T578C and ERGA/DKGT at positions 522–525 near the Walker B motif [76]. Yeast not only permits the targeted mutagenesis of specific protein segments, but also allows the implementation of random mutagenesis protocols coupled to efficient re-cloning of mutagenized segments by homologous recombination. This approach was used to systematically mutagenize the first intracellular loop (IC1) and TM2, -3, and -4 segments of P-gp, leading to the identification of highly mutation sensitive residues essential for transport [77]. Importantly, yeast has also proven very valuable in deciphering the mechanism of transport of P-gp. Studies in inside-out membrane vesicles purified by lectin chromatography from P-gp-positive yeast cells demonstrated that P-gp can transport drugs in a temperature-, time- and ATP-dependent and osmotically sensitive fashion [78]. The yeast mutant *sec6-4* is defective in the last step of vesicular fusion and thus accumulates at the nonpermissive temperature large amounts of secretory vesicles (SV) [79]. Such SVs present a number of advantages including (1) their homogeneous size and uniform polarity, (2) they are tightly sealed and maintain a proton gradient across the membrane generated by the PMA1 vacuolar H^+-ATPase, (3) P-gp can be inserted in these SVs exposing their catalytic NBDs to the outside. Finally, temperature-sensitive mutants of PMA1 can be expressed in these SVs allowing one to monitor the effect of the ΔpH on transport function of P-gp [80]. In this system, it was directly demonstrated that mouse Class I (Mdr1, Mdr3) but not Class II (Mdr2) P-gps can mediate the accumulation of radiolabeled drugs vinblastine and colchicine in the SVs. Drug transport by P-gp was temperature- and ATP-dependent, and was not affected by membrane potential, and transport of a lipophilic cation occurred against a significant proton gradient. Likewise, vinblastine transport occurred against a continuously increasing concentration gradient [81]. These studies were the first to demonstrate that P-gp acts as a true ATP-dependent drug transporter by a mechanism clearly distinct from a proton symport or antiport mechanism, which had been previously suggested [82]. Yeast SVs were also used to demonstrate that the normal physiological role of Class II P-gp (mouse Mdr2) is a phospholipid

translocase that functions as a lipid flippase mechanism [83]. In these experiments, fluorescently labeled phosphatidylcholine (NBD-PC) containing liposomes were fused to P-gp-positive SVs, and the rate of NBD-PC translocation from the outer to the inner leaflet was measured by determining the amount of fluorescence quenched in the presence or absence of detergent. Mdr2-mediated lipid translocation was specific for PC, and was not seen for other phospholipids, such as phosphatidylethanolamine (PE); NBD-PC transport by Mdr2-positive SVs was ATP-dependent, and inhibited by verapamil [83]. Later, transport was found stimulated by the bile acid taurocholate, suggesting a mechanism for the creation of a physiological PC gradient in the canalicular domain of hepatocytes [84]. Together, these studies demonstrate that Class I and Class II P-gps are functional in yeast. In *S. cerevisiae*, the overall amount of P-gps expressed is not very large, and not sufficient for large-scale purification required for physical and structural studies or for measuring catalytic parameters of P-gp.

2.5. Overexpression and purification in yeast P. pastoris

The yeast *P. pastoris* offers a convenient alternative for such high level of protein expression. *P. pastoris* is a methylotrophic yeast capable of metabolizing methanol as a sole source of carbon. The first enzyme in the methanol utilization pathway is alcohol oxidase (encoded by *AOX1* and *AOX2* genes), which converts methanol to formaldehyde [85]. When yeast cells are grown with methanol as a sole source of carbon, the AOX1 enzyme is highly induced and accounts for 30% of total soluble protein [86]. Introduction of heterologous genes (cDNAs) at the *AOX1* locus by homologous recombination results in very high levels of mRNA and protein expression of the inserted sequence upon methanol induction. The expression system is well developed with a variety of plasmid vectors commercially available (Invitrogen). For example, the yeast plasmid vector pHIL-D2 contains sequences allowing replication and selection in bacterial (Amp®) and yeast (HIS-4) hosts. The cloning site is flanked by *AOX1* chromosomal sequences that allow for homologous recombination of the cloned gene into the *AOX1* locus of yeast chromosome. Vectors designed for secretion of recombinant proteins into culture medium have also been described (Invitrogen). Up to now, over 200 intracellular, secreted, or membrane proteins from a variety of sources, including viral, bacterial, plant, and human proteins, have been successfully expressed in *P. pastoris* [87]. Several wild-type or protease-deficient mutants of *P. pastoris* strains are available for expressing proteolysis susceptible proteins, but strains GS115 (*his4*, Mut^+) and KM71 (*arg4*, *his4 aox1*::*ARG4*, Mut^s) are most frequently used for expression from a targeted *AOX1* gene copy or from a multicopy episomal plasmid. Typically, *P. pastoris* is transformed by a lithium acetate (or chloride) procedure, followed by selection of transformants on synthetic minimum medium lacking histidine. When the GS115 strain is used, HIS^+ transformants are replica-plated on solid medium with or without methanol as a sole carbon source, and clones that show slow growing phenotype on methanol-containing medium (Mut^s), due to the disruption of the chromosomal *AOX1* locus, are selected and screened for protein expression by immunoblotting. Early studies showed that P-gp (Mdr3) could be expressed at high levels in *P. pastoris*, accounting for up to 10–15% of membrane proteins, and easily detected by SDS-PAGE and Coomassie blue staining [29]. The amount of protein produced is approximately 10 times higher than that achieved in *S. cerevisiae*, and slightly higher than that reached by baculovirus-mediated expression in insect cells (Table 1), and at a fraction of the cost. Also, *P. pastoris* cells can and have been grown and induced under fermentor conditions for very large or industrial scale production [31]. The *P. pastoris*-expressed P-gp appears properly folded as it reacts with a photoactive drug analog [29,30]; in addition, deoxycholate extracts from membrane fractions (purified by centrifugation on discontinuous sucrose density gradients, and migrating at the 16%–31% sucrose interface) of P-gp-expressing *P. pastoris* cells reconstituted in *E. coli* lipids showed ATPase activity that could be stimulated by verapamil, valinomycin, and vinblastine (maximum activity 0.20 μmol/min/mg protein), with an optimal pH at 7.7 [29]. This work suggested that P-gp was indeed active in *P. pastoris*, and opened the door to full-scale expression, purification, and functional characterization studies. For these experiments, the mouse *mdr3* cDNA was modified for optimal expression and easy purification from *P. pastoris* (pHIL-*mdr3*-H6BD). First, the 5′ untranslated region of the mRNA was deleted and replaced by a series of six adenosine residues upstream the AUG, to optimize mRNA translation in yeast [88]. Second, a series of six histidines was added at the carboxyl terminus of the protein to allow affinity purification by nickel affinity chromatography. Third, a biotin acceptor domain (BD) from oxaloacetate decarboxylase of *Klebsiella pneumoniae* [89] was fused in-frame at the C terminus of the protein. This BD domain allows for biotinylation of the protein in vitro using purified biotin ligase, which catalyses transfer of biotin from the intermediate, biotinyl-5′-AMP to the unique lysine residue in the biotin domain. The strong affinity between biotin and avidin is used to obtain highly purified and biologically active biotinylated chimeric protein by chromatography on a monomeric avidin agarose resin [90]. Using the pHIL-*mdr3*-H6BD construct, over 90% of the His^+/mut^s transformants were found to express the recombinant P-gp. Although several related protocols have been described for P-gp purification from *P. pastoris* [31,58,90], only one that uses the recombinant Mdr3-H6BD will be summarized here [90]. Two-liter culture of *P. pastoris/mdr* transformants was induced by transferring the cells to methanol-containing medium and further incubation for 48–72 h, with methanol (at 5% final concentration) replenished daily. Crude plasma membranes were prepared from these cells by lysis using a

French pressure cell (set at 20 000 psi), in the presence of a cocktail of protease inhibitors and with all steps carried out at 4 °C. Membrane fractions were separated from unbroken cells and from majority of cytosolic proteins by low- and high-speed centrifugation, and were kept at −70 °C in a storage buffer containing 20% glycerol. A 2-l culture yields, on average, between 100 and 200 mg of crude membrane proteins. For each protein purification experiment, membranes are thawed and are biotinylated in vitro for 3 h at 30 °C in a reaction mixture containing ATP, D-biotin (400 μM), and commercially available biotin ligase (12 500 U). The extent of biotinylation can be followed by immunoblotting against extravidin–peroxidase. Following biotinylation, membranes are concentrated by precipitation with $MgCl_2$, and P-gp is extracted with detergent in a buffer containing 0.6% lysophosphatidylcholine. Solubilized P-gp is then subjected to a first affinity chromatography with Ni-NTA resin, to enrich the protein and to eliminate remaining free D-biotin. For this, the protein is transferred to a buffer containing 0.1% *n*-dodecyl-β-D-maltoside, and elution of the immobilized P-gp from the resin is carried out in the presence of 80 mM imidazole [90]. The purity of this P-gp preparation is approximately 75%. Mdr3-H6BD is further purified by a second affinity chromatography on monovalent avidin–Sepharose; for this, a commercially available monovalent avidin resin is first equilibrated in buffer containing 100 nM D-biotin (to increase specific binding of the protein of interest), followed by loading of the Mdr3-H6BD imidazole eluate, and elution with 4 mM D-biotin. This simple two-step affinity chromatography protocol allows the rapid and efficient purification of highly pure biotinylated P-gp, which is devoid of contaminants detected by SDS-PAGE and by Coomassie blue staining [90] (Table 1). For reconstitution, the protein is incubated with 1% *E. coli* lipids (acetone/ether preparation) followed by dialysis to remove detergent and D-biotin. P-gp purified in this manner shows a robust ATPase activity as measured by (a) Pi release, with K_m for ATP ~ 0.7 mM, and V_{max} of 2.43 μmol Pi/min/mg protein under maximum stimulated conditions (100 μM verapamil), or (b) by Vi-induced trapping of 8-azido-[^{32}P]ADP [90]. Alternate protocols based on Ni-NTA affinity chromatography followed by DE52 ion-exchange chromatography have been described and yield protein of about 90% homogeneity which exhibits high verapamil-stimulated ATPase activity (V_{max} of 4.2 μmol min^{-1} mg^{-1}, K_m ~ 0.7 mM) [31,58]. Similar results were obtained for mouse Mdr3 purification from fermentor-grown *P. pastoris* [31,58] (Table 1), and for purification of the human MDR1 P-gp and its Cys-less mutant [91].

2.6. Biochemical and structural characterization of P-gp purified from P. pastoris

Highly purified P-gp preparations from *P. pastoris* have been used to study the catalytic cycle of P-gp, including the respective contribution of each NBD in this process. Early mutagenesis experiments have established that both NBDs are essential for function, with complete cooperativity between the two sites for ATP hydrolysis and drug transport [57,58,61,62]. Two models have been proposed for catalysis and coupling of ATP hydrolysis to transport. In the "alternate site" catalysis model [92], both NBDs are catalytically active with equal probability of hydrolysis at NBD1 and NBD2. In this model, one drug molecule is transported per ATP molecule hydrolyzed. The experiments that support this model include Vi-induced nucleotide trapping, in which equal trapping of nucleotide occurs in NBD1 and NBD2, with a complete inhibition of ATPase activity occurring with one molecule of Vi trapped per P-gp molecule [93]. Thus, when one site enters the transition state (P-gp·MgADP·Pi/Vi), the other cannot do so, implying alternate hydrolysis. In the second model, the major difference is that two ATP molecules need to be hydrolyzed for one substrate molecule to be transported [94]. This model is based on the observation that the P-gp·MgADP·Vi-inhibited P-gp has reduced affinity for drugs [94–96], and that a second ATP hydrolysis event, after release of MgADP·Vi, is required to restore normal drug binding properties [94]. Hence, drug binding induces hydrolysis of a first ATP and translocation of substrate to a low affinity site, as well as substrate release, while hydrolysis of a second ATP is required to recreate a high affinity drug site. Studies with mutants at homologous positions in the Walker A (K429R/K1072R) and B (D551N/D1196N) sequence motifs of NBD1 and NBD2 showed that alterations at either position completely inactivate the ATPase activity of P-gp. In addition, no Vi-induced trapping of 8-azido-ATP could be detected in any of the mutants, suggesting that the NBDs cannot function independently as catalytic sites in the intact molecule, but rather that cooperative interactions between the two NBDs are absolutely required for ATP hydrolysis by P-gp [58]. Similar conclusions were reached from the study of additional loss-of-function mutants at highly conserved serine residues in the Walker A motif of NBD1 (S430A) and NBD2 (S1073A) [97]. Interestingly, somewhat different conclusions were reached from the systematic study of all carboxylate residues that are highly conserved in the NBDs of all ABC transporters sequenced to date [11]. Two mutants, E552Q and E1197Q, although showing no drug-stimulated ATPase activity (measured by Pi release), showed vanadate trapping of 8-azido-nucleotide similar to wild type. This suggested that single site catalysis could occur in these mutants, with release of MgADP from the NBD being impaired in these mutants. More recent analyses of these mutants showed that drug-stimulated nucleotide trapping in E552Q was Vi-dependent and resembled the wild-type enzyme, while it is almost completely Vi-insensitive in E1197Q (Carrier and Gros, unpublished data). Furthermore, these results support a model in which the two NBDs of P-gp are not functionally equivalent, with NBD2 showing a faster turnover than NBD1 [Carrier and Gros, unpublished data]. Additional structure–function studies have been conducted in a human

MDR1 mutant lacking all endogenous cysteine residues [59], and purified from *P. pastoris* [91]. Using proteins containing different combinations of naturally occurring Cys residues, it was demonstrated that an inhibitory intramolecular disulfide bond forms between Cys-431 and Cys-1074, both located in the Walker A sequence motif of NBD1 and NBD2 [91]. This suggests that the two NBDs of P-gp are structurally very close, and that formation of such a disulfide bond in vivo could (in principle) underlie a regulatory mechanism for P-gp activity. Finally, preparations of biotinylated P-gp purified from *P. pastoris* have been used to study the interaction of P-gp with chemical inhibitors that are known to block drug transport [90]. Using a series of 1,4-disubstituted piperazine derivatives with a central chiral carbon, it was observed that all these inhibitors can stimulate P-gp ATPase activity in a dose-dependent fashion. Furthermore, it was observed that some of the compounds are also capable of inhibiting either vinblastine or verapamil stimulation of ATPase activity in an enantiomer-specific fashion, suggesting complex interactions at a single substrate binding site on P-gp [90].

Currently, there is no high-resolution structural information available for P-gp, although a 2.5-nm [98] and a 10-Å structure [99] of P-gp purified from CHO cells have been reported. The former was obtained by single particle analysis of detergent solubilized protein as well as by Fourier projection maps of small two-dimensional crystalline arrays, while the later was by electron microscopy of two-dimensional protein crystals. Three distinct conformations, namely, nucleotide-free, nucleotide-bound, and post-hydrolytic transition state, were observed during catalytic process [99]. The nucleotide-free state of P-gp has its two MSDs forming a chamber within the membrane. Nucleotide binding to the NBDs induces repacking of the MSDs and reduction in drug binding affinity. The third distinct conformation of the protein was observed in the post-hydrolytic transition state prior to the release of ADP/Pi.

P-gp preparations purified from *P. pastoris* are also started to be used for structural studies. Nucleotide-induced conformational changes in P-gp and its NBD mutants (in Walker A and B motifs from NBD1 and NBD2) were recently analyzed by limited trypsin digestion of proteins purified from *P. pastoris*, followed by epitope mapping using monoclonal antibodies that recognize both or either NBDs in P-gp [100]. A unique trypsin digestion profile indicative of trypsin resistance was observed under conditions of Vi-induced trapping of nucleotides. Under such conditions the trypsin sensitivity profiles of double P-gp mutants K429R/K1072R and D551N/D1196N and of single mutants K429R, K1072R, and D1196N were very similar and clearly distinct from the wild-type protein. In contrast, under Vi-induced nucleotide trapping conditions, the D551N mutant showed a trypsin sensitivity profile similar to that of wild type. This raised the possibility that structural and functional differences exist between the two NBDs, including different sensitivity to Vi and hence possible differences in catalytic activity [100]. More recently, structural information on P-gp purified from *P. pastoris* was obtained by attenuated total reflection Fourier Transform Infrared (ATR-FTIR) spectroscopy, including conformational changes induced by nucleotide binding and hydrolysis [101]. In these experiments, also included for study were Walker B mutants, E552Q and E1197Q, that have impaired ATPase activity due to their inability to release ADP and/or Pi, but that appear to undergo single site turnover [11] (Carrier and Gros, unpublished data). P-gp reconstitution into proteoliposomes (inside-out orientation) was achieved by mixing the purified protein with detergent-destabilized asolectin liposome, followed by detergent removal and sucrose gradient fractionation. The reconstituted wild-type P-gp proteoliposomes exhibited drug-stimulated ATPase activity, and P-gp was transport-competent [101]. The secondary structure of wild-type P-gp evaluated by IR spectrum was found to contain 46% α-helices, 18% β-sheets, 8% β-turns, and 28% random structures. In the case of wild-type P-gp, comparison of H/D exchange rates for nucleotide-free, and MgATP-, or MgATP + Vi-bound P-gp shows that a major conformational change occurs in the protein (conformation B) upon inhibition by Vi. This conformational change is also seen in the E552Q mutant analyzed in a similar fashion but, in contrast to wild-type P-gp, in this mutant conformation B is observed both in the presence (MgATP + Vi) and absence of Vi (MgATP alone). On the other hand, this structural change is never observed in the E1197Q mutant under any of the conditions tested. This apparent difference clearly indicates an asymmetric structure and possibly different functions of the two NBDs of P-gp in the catalytic cycle [101]. Finally, our laboratory has recently created a P-gp mutant devoid of tryptophan residues [102]. In mammalian cells, this mutant retains the ability to convey multidrug resistance, albeit at a reduced level when compared to the wild-type protein. This mutant was expressed and successfully purified from *P. pastoris*. Therefore, single tryptophan insertion constructed on this Trp-less backbone and purified from *P. pastoris* should prove very useful to obtain, by fluorescence spectroscopy, dynamic information on structural changes associated with drug or nucleotide binding and hydrolysis.

3. MRP1/ABCC1

3.1. Introduction

MRP1/ABCC1 was the first identified member of the MRP/ABCC subfamily, and was discovered as a gene overexpressed in the P-gp-negative multidrug resistant lung tumor cell line H69AR [103]. Subsequent transfection studies demonstrate that overexpression of MRP1 does confer multidrug resistance in otherwise sensitive cultured cells by an energy-dependent drug efflux mechanism [104,105]. Although sequence analyses clearly identity

MRP1 as part of the ABC transporter family, significant differences in their predicted NBDs, as revealed by multiple sequence alignment, have placed MRP1 and a cluster of other ABC transporters in a separate subgroup, ABCC [2,5] [http://humanabc.4t.com/humanabc.htm]. The MRP/ABCC subfamily contains at least 12 members, including MRP1 to MRP9, the chloride channel CFTR, and the sulfonylurea receptors SUR1 and SUR2 [6,106,107]. The NBDs of this sub-group of proteins are characterized by the absence of 11–14 amino acid residues between Walker A and Walker B motifs of NBD1, compared to the corresponding region of P-gp and other ABC transporters. In addition, the two NBDs of the MRP/ABCC subfamily are more divergent than those of P-gp [2,5]. The apparent evolutionary distance between the two NBDs of the MRP/ABCC subgroup is paralleled by a nonequivalent role of the two NBDs in the catalytic action of the protein, as supported by a large body of published biochemical data (see below). In addition, hydropathy profiling of the MRP/ABCC subgroup identifies the presence of a unique amino terminal membrane associated region (MSD0) in addition to the classical (MSD1–NBD1–MSD2–NBD2) core structure shared by many ABC transporters (Fig. 1C). Epitope mapping and topology studies have established that this additional amino terminal membrane region is most likely composed of five TM segments, which would place the amino terminus of the protein extracellularly on a glycosylated segment [13,108]. This extra N-terminal MSD0 is present in some members of the MRP subfamily, including MRP1, -2, -3, -6, and SUR1 and SUR2 (Fig. 1C), and absent in others, like MRP4, -5, and CFTR (Fig. 1B) [5,6]. The topology of the newly discovered MRP7, MRP8, and MRP9 has not been well characterized.

The importance of the MRP/ABCC family in normal physiology is highlighted by the discovery of disease-causing mutations in several inherited genetic disorders, including cystic fibrosis (CFTR/ABCC7) [17], Dubin–Johnson syndrome (cMOAT/MRP2/ABCC2) [18], pseudoxanthoma elasticum (MRP6/ABCC6) [24,109,110], and familial persistent hyperinsulinemic hypoglycemia of infancy (SUR1/ABCC8) [111]. In addition, increased expression of MRP1/ABCC1 in multidrug resistant cancer cells in vitro, as well as in tumor isolates in vivo, suggests major clinical relevance and has generated much interest in this gene and protein for potential intervention in treatment of drug resistant cancers with specific inhibitors [2,5]. MRP1 shows many similarities with P-gp but also displays unique and distinguishing structural (see above) and functional characteristics that have helped identification of structure/function relationships in both proteins. The drug resistance profile of MRP1-overexpressing cells has been investigated extensively, and found to be similar but not identical to that of multidrug resistant P-gp. Indeed, overexpression of P-gp and MRP1 confers cellular resistance to many of the same cytotoxic drugs, including anthracyclins, Vinka alkaloids, and epipodophyllotoxins, and mitoxantrone. In general, the level of drug resistance imparted by P-gp towards these drugs seems to be higher than those conferred by MRP1 (reviewed in Refs. [2,5,6]). More importantly, each protein confers resistance to a unique set of compounds such as colchicine and taxol for P-gp [3,4], and arsenite and antimony for MRP1 [2,3,5,6]. Both MRP1 and P-gp transport drugs at the expense of ATP, and convey drug resistance by a seemingly related drug efflux mechanism. An important difference, however, is that while P-gp can transport unmodified cationic lipophilic cytotoxic drugs directly, MRP1 can transport organic anions, such as drugs conjugated with glutathione, glucuronide, and sulfate, and uniquely co-transports certain unmodified drugs together with glutathione (GSH) [2,5,6]. In addition to multiple anticancer drugs, MRP1 is known to transport physiologically important substrates, such as bilirubin glucuronide, glucuronide, and sulfate-conjugated bile salts, glutathione disulfide (GSSG), 17-β-estradiol, and the GSH-conjugates of prostaglandin A_2 [5,112]. One well-characterized physiological substrate of MRP1 is cysteinyl leukotriene LTC_4, which is an important mediator of inflammatory responses. LTC_4 is the highest affinity substrate of MRP1 identified so far [5]. Interestingly, MRP1 was recently shown capable of translocating lipid molecules, regulating lipid asymmetry in certain types of physiological membranes such as those of erythrocytes [113,114]. Therefore, MRP1 may share with P-gp a similar lipid flippase mechanism of action.

Biochemical and functional characterization of MRP1 has been mostly carried out in drug-selected mammalian cells overexpressing either an endogenous or a transfected copy of the *MRP1* gene, and in Sf9 insect cells overexpressing a corresponding recombinant baculovirus (reviewed in Refs. [2,4–6]). Like P-gp, photolabeling studies have shown that MRP1 can directly interact with drug substrates, and that TM segments play a key role in such interactions. Indeed, MRP1 can be photolabeled by analogs of leukotriene C_4 (LTC_4) [115], as well as by photoactive analogs of a quinoline (IACI) [116] and Rhodamine (IAARh123) [117]. Studies with the latter two compounds have identified photolabeling sites in the second (MSD1) and third (MSD2) large membrane associated portions of the protein [118]. In membrane fractions, MRP1 can be photolabeled by 8-azido-[α-^{32}P]ATP in the presence of magnesium and Vi [119]. Vanadate-induced nucleotide trapping in MRP1 can be stimulated by anticancer drugs and by glutathione, a first indication that MRP1 may possess substrate-stimulated ATPase activity [119]. Studies with MRP1 purified and reconstituted in liposomes have confirmed that MRP1 has intrinsic ATPase activity, which is vanadate-sensitive, and can be further stimulated by drug substrates, glutathione and LTC_4 [120,121], although large differences in K_m and V_{max} were reported in these studies. Both NBD1 and NBD2 are required for ATP hydrolysis by MRP1, as mutations in the Walker A and B motifs in either site drastically reduce ATP hydrolysis and transport by the protein [121,122]. Moreover, MRP1 ATPase activity can be significantly stimulated by certain nucleotide diphosphates [123]. Additional photolab-

eling studies of wild-type, mutant variants, and half molecules of MRP1 with 8-azido-[α-^{32}P]ATP or 8-azido-[γ-^{32}P]ATP strongly suggest that the two NBDs are not functionally equivalent in MRP1 but may allosterically interact. Indeed, it appears that MRP1 exclusively binds to 8-azido-[α- or γ-^{32}P]ATP at NBD1 while hydrolysis preferentially takes place at NBD2 [121,122,124].

Structural information on MRP1 is limited and has just started to emerge. The secondary structure and possible conformational changes caused by interaction with substrates and nucleotides have been investigated by infrared spectroscopy (ATR-FTIR) [125]. Ligand binding did not affect the MRP1 secondary structure but increased accessibility to solvent, suggesting conformational change similar to that seen with P-gp analyzed in a similar fashion [101]. Using acrylamide quenching of natural tryptophan fluorescence to monitor conformational changes, it was observed that binding of MRP1 drug substrates (and not others) favors a conformational change upon ATP binding, suggesting that drug substrate-induced conformational changes may be a requirement for the transport process [125]. A 22-Å structure of MRP1 was recently obtained by EM analysis of 2-D crystals [126], and suggests many similarities with P-gp [126]. These include a monomeric toroidal ring structure with a large stain-filled central region, and two pseudosymmetric domains, presumably corresponding to the two NBDs and located at the cytoplasmic face. Reconstitution of MRP1 into crystalline protein/lipid arrays allowed detection of dimeric protein, although it is uncertain whether MRP1 is dimeric in native membrane [126].

3.2. Overexpression and functional characterization in *P. pastoris*

The systematic study of common and distinct aspects of drug transport by MRP1 and by P-gp would be greatly facilitated by an experimental system where large amounts of biologically active protein can be produced for purification. Likewise, obtaining structural information on MRP1 in a dynamic fashion during transport or ATP hydrolysis also requires large amounts of purified protein. Following the successful expression, characterization, and purification of P-gp from overexpressing *P. pastoris* yeast cells, the expression of MRP1 in this yeast was attempted. An experimental strategy similar to that used for P-gp was implemented [127]. The human *MRP1* cDNA was modified by the deletion of all 5′ and 3′ untranslated sequences, with insertion of six consecutive adenosine residues immediately upstream the ATG initiation codon to enhance translation efficiency [88]. A hemagglutinin HA epitope tag and six consecutive histidine residues were added in-frame at the C terminus for identification of the recombinant protein in yeast and to facilitate future purification procedures, respectively [127]. The recombinant MRP1 protein was successfully expressed from the pHIL-D2 plasmid construct in two *P. pastoris* strains, GS115 (*his4*, Mut^{+}) and KM71 (*arg4*, *his4 aox1*::*ARG4*, Mut^{s}). Immunoblotting experiments indicated that MRP1 was expressed as a 165-kDa underglycosylated protein species, which is of significantly faster electrophoretic mobility than its counterpart overexpressed in drug-resistant HeLa/*MRP1* transfectants [14]. Comparative immunoblotting with crude membrane fractions indicated that the *P. pastoris/MRP1* membranes expressed about 30 times more MRP1 protein per weight than their mammalian HeLa/*MRP1* counterpart. For large-scale preparation of membrane fractions to be used for biochemical characterization, 2-l cultures of *P. pastoris* cells were induced with methanol for 72 h, after which the cells were collected and lysed using a French pressure cell. Crude membranes were isolated by successive centrifugation. The ATP binding and ATP hydrolysis properties of MRP1 were analyzed using 8-azido-[α-^{32}P]-ATP, under binding (4 °C) or hydrolysis (37 °C) conditions. In contrast to P-gp, ATP binding to the protein was found to be dependent on the presence of Mg^{2+} ions, and sensitive to EDTA; binding was specific and was competed by excess of both cold ADP and ATP. Under hydrolysis conditions, vanadate could induce nucleotide trapping into MRP1, which was temperature-dependent and required the presence of Mg^{2+} ions. Vanadate-induced trapping of nucleotide into MRP1 could be stimulated by drugs such as vinblastine and vincristine, and by LTC_4. These results strongly suggested that MRP1 was functional in yeast membranes, and could carry out substrate-stimulated ATP hydrolysis [127]. In addition, a transport assay for MRP1 using [^{3}H]-LTC_4 was designed. In this assay, vesicles are created from enriched membrane fractions and incubated with ligand in the presence or absence of an ATP regenerating system. The effect of MRP1 on accumulation of [^{3}H]-LTC_4 was measured over time by a standard rapid filtration assay. MRP1 induced time-, concentration-, and ATP-dependent accumulation of [^{3}H]-LTC_4 in membrane vesicles, establishing that MRP1 expressed in *P. pastoris* is indeed functional [127]. Finally, interactions between MRP1 protein and its potential substrates were investigated in *P. pastoris* membranes using a photoaffinity labeling approach. It was determined, as for MRP1 expressed in mammalian cell membranes [118], that MRP1 expressed in *P. pastoris* binds a photoactive derivative of the fluorescent MRP1 substrate Rhodamine 123, [^{125}I]-IAAR. Binding was found to be specific and saturable, and could be effectively competed by several known MRP1 substrates/modulators, including LTC_4 and MK571 [127]. Taken together, these results proved that *P. pastoris* is an efficient expression system for overexpression and biochemical characterization of MRP1. Although experimental data for purification of MRP1 from *P. pastoris* have not yet been documented, thus making direct comparison of the yield of pure MRP1 protein between *P. pastoris* and other expression systems unavailable, the high level of the recombinant protein produced in *P. pastoris* provides an optimistic future for large-scale purification and reconstitution studies and for structural analysis.

4. MRP6/ABCC6

4.1. Introduction

Several members of the MRP/ABCC subfamily function as glutathione conjugate (GS-X) pumps that play key physiological roles. For example, MRP2 (cMOAT) is expressed in the canalicular domain of hepatocytes where it transports a wide range of anionic conjugates into the biliary space [128], and mutations in *MRP2* cause Dubin–Johnson syndrome in humans [18]. Transfection of *MRP2* causes resistance to natural product drugs and to cisplatinum [129,130]. Likewise, MRP3 transports glucuronide conjugates and methotrexate [131], and MRP3 overexpression in transfected cells causes low-level resistance to VP16 and teniposide [132]. On the other hand, MRP4 and MRP5 can transport cyclic nucleotides cGMP and cAMP [133–135], and overexpression of either protein causes resistance to the antiviral nucleoside analog 9-(2-phosphonyl methoxyethyl)adenine (PMEA), and to anticancer thiopurines, 6-mercaptopurine (6-MP) and thioguanine [133,135,136].

A poorly characterized but clinically relevant MRP family member is MRP6 [137,138]. The human *MRP6* gene maps immediately adjacent of *MRP1* on human chromosome 16p13 [139]. The human *MRP6* encodes a protein of 1503-amino-acid residues, which share 45% identity and 55% similarity with *MRP1*, its most closely related *MRP* homologue, suggesting that both genes may have emerged from a recent gene duplication event. Expression of *MRP6* mRNA was found increased in certain drug-resistant cell lines, but was always concomitant to increased expression and amplification of the neighbouring *MRP1* gene [139]. Despite close sequence identity with MRP1, MRP6 does not appear capable of conferring drug resistance in transfected mammalian cells (Cai and Gros, unpublished data). Studies in membrane vesicles from rMrp6-expressing Sf9 cells indicate that unlike MRP1 and MRP2, rMrp6 does not transport glucuronide, sulfate, or glutathione conjugates [138]. So far, the only identified transport substrate for rMrp6 is the cyclic pentapeptide endothelin-1 receptor antagonist BQ123 (cyclo[D-Trp-D-Asp-L-Pro-D-Val-L-Leu]), though endothelin-1 itself is not a substrate [138].

Recently, human *MRP6* (*ABCC6*) was found mutated in patients with pseudoxanthoma elasticum (PXE) [24,109,110]. PXE is a inherited disorder of connective tissues, characterized by calcification of elastic fibers in skin, arteries, and retina that results in dermal lesions with associated loss of elasticity, arterial insufficiency, and retinal bleeding leading to macular degeneration [140]. Mutational analysis of *MRP6* in PXE patients identified large and small deletions, splicing mutations, nonsense mutations, and a number of missense mutations [24,109]. Interestingly, most of the single amino acid substitutions mapped in NBD2 of MRP6. Recently, a rare MRP6 mutation (R1268Q) in a PXE patient was found to be associated with type IV hyperlipidemia and hypoalphalipoproteinemia, suggesting that MRP6 may be a determinant of plasma lipoproteins [141]. Expression studies of *MRP6* mRNA by RT-PCR in tissues affected in PXE patients have revealed low *MRP6* expression in skin, retina, vessel walls, and placenta [24]. The human MRP6 protein is expressed at the basolateral membrane of kidney proximal tubules as well as in liver hepatocytes. However, no MRP6 protein expression is detected in sections of human skin and retina [110], the tissues affected in PXE patients. In rat tissues, the Mrp6 protein was detected in lateral and, to a lesser extent, canalicular membranes of hepatocytes [138]. Interestingly, a recent study revealed that human MRP6 was able to mediate ATP-dependent transport of at least two anionic glutathione conjugates, *N*-ethylmaleimide *S*-glutathione (NEM-GS) and LTC_4, and this transport activity was inhibited by probenecid, benzbromarone, and indomethacin [142], suggesting some functional similarity with MRP1 and MRP2. Moreover, three PXE mutations were found to abolish the transport activity of MRP6, suggesting that loss of transport function in these mutants was the direct cause for the PXE phenotype [142]. Despite this initial functional study, the substrate specificity, mechanism of transport, and role of MRP6 in PXE still remain largely unknown.

4.2. Overexpression and functional characterization in *P. pastoris*

To get an insight into the catalytic activity of Mrp6, our group has introduced the rat *mrp6* cDNA into *P. pastoris*, and several clones that express high levels of Mrp6 recombinant protein were isolated [143]. Membrane proteins isolated from rat Mrp6-overexpressing *P. pastoris* cells were photoaffinity labeled with 8-azido-[α-^{32}P]ATP followed by UV cross-linking. The results showed that, under nucleotide binding conditions, Mrp6 is able to bind ATP in a Mg^{2+}-dependent and EDTA-sensitive fashion, with an affinity apparently greater than MRP1. ATP binding by Mrp6 can be supported by other divalent cations, such as Ni^{2+}, Co^{2+}, and Mn^{2+}, in a manner similar to that seen for MRP1. However, under hydrolysis conditions, characteristics of nucleotide trapping induced by transition state analogs into Mrp6 showed important differences when compared to MRP1, with respect to divalent cation requirement and selectivity for transition state analogs. For example, Vi can induce trapping of 8-azido-nucleotide in MRP1 in the presence of a number of divalent cations, including Mg^{2+} [127]. In contrast, Vi-induced nucleotide occlusion in rMrp6 occurs only in the presence of Ni^{2+}. On the other hand, stimulation of nucleotide trapping by Mrp6 can be observed in the presence of another transition state analog, beryllium fluoride (BeF_x). Such differences in nucleotide trapping properties of MRP1 and Mrp6 suggest possible structural differences between the NB sites of the two proteins [143].

One important yet intriguing question on Mrp6 function is, what substrates does it transport if not anticancer drugs? Mrp6 can transport the anionic cyclopentapeptide and endo-

thelin-receptor antagonist BQ-123 in vitro, which is also a substrate of Mrp2 [138]. The affinity for BQ-123 ($K_m \approx 17$ μM) by Mrp6 is about sevenfold higher compared to that by Mrp2 ($K_m \approx 124$ μM), suggesting that Mrp6 may be involved in hepatocellular transport of cyclic oligopeptides [138]. Other known substrates for Mrp2, including GS-DNP and LTC_4, are apparently not transported by Mrp6. This result is in apparent contrast to those recently obtained with the human MRP6 protein [142]. The dual localization of Mrp6 in lateral and canalicular membranes of hepatocytes is also difficult to reconcile with an exclusive role in biliary excretion [138]. In an effort to characterize substrate-binding properties of Mrp6, a photoaffinity labeling approach was implemented using membrane preparations from Mrp6-expressing *P. pastoris* cells and [^{125}I]-IAAR (iodoarylazido Rhodamine 123) as a potential ligand [143]. It was observed that Mrp6 could bind the Rhodamine 123 analogue in a fashion similar to MRP1. [^{125}I]-IAAR binding to Mrp6 is concentration-dependent and saturated at approximately 4 μM. It is interesting to note that [^{125}I]-IAAR binding to Mrp6 is competed by several drugs, including vinblastine, doxorubicin, and MRP1 modulator, MK571, and is stimulated by the only known substrate of Mrp6, BQ123. Moreover, the binding appears unaffected by LTC_4, the high affinity substrate of MRP1, as well as by the anticancer drug VP16 [143]. These results suggest important similarities and unique differences in the ligand binding properties of MRP1 and Mrp6 [127,143]. In addition, these photoaffinity labeling studies using *P. pastoris*-expressed Mrp6 provide a rational basis for the systematic testing of candidate substrates for Mrp6. More broadly, the ATP binding and hydrolysis assays described for *P. pastoris*-expressed Mrp6 can be used to further characterize the molecular basis of the apparent loss-of-function mutations seen in PXE patients.

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