Structure and mechanism of ATP-binding cassette exporters

Maite Rocío Arana[a] and Guillermo Alejandro Altenberg[b,*]

[a]*Instituto de Fisiología Experimental, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, CONICET, Suipacha 570, 2000 Rosario, Argentina;* [b]*Department of Cell Physiology and Molecular Biophysics, and Center for Membrane Protein Research, Texas Tech University Health Sciences Center, Lubbock, Texas 79430-6551, USA*

Abstract: The majority of proteins that belong to the ATP-binding cassette (ABC) superfamily are transporters that mediate the efflux of substrates from cells. These exporters include multidrug resistance proteins of the ABCB and ABCC subfamilies, such as P-glycoprotein (Pgp) and MRP1, respectively. These proteins are not only involved in the resistance of cancer to cytotoxic agents, but also in the protection from endo and xenobiotics, and the determination of drug pharmacokinetics, as well as in the pathophysiology of a variety of disorders. Here, we present a review of the information available on ABC exporters, with a focus on Pgp, MRP1 and related proteins. We describe tissue localization and function of these transporters in health and disease, and discuss the mechanisms of substrate transport. We also correlate recent structural information with the function of the exporters, and discuss details of their molecular mechanism with a focus on the nucleotide-binding domains,

Keywords: ATP-binding cassette; multidrug resistance; nucleotide-binding domain; dimer; hydrolysis; P-glycoprotein; MRP1; ABCB; ABCC

1. INTRODUCTION

In this review we discuss ATP-binding cassette (ABC) proteins with a focus on ABC exporters in general, and the multidrug resistance (MDR) proteins P-glycoprotein (Pgp, MDR1 or ABCB1) and multidrug-resistance 1 (MRP1, ABCC1) in particular. We did not aim to be comprehensive, but tried to focus on some recent discoveries and on the presentation of aspects not generally discussed in detail, as well as some recent finding from our research. Also, we frequently refer to publications where specific issues are addressed; these in turn contain more extensive reference lists that include the original publications.

1.1. Classification and general structure

ABC proteins are one of the largest protein families. They are widely distributed in all species, where they function primarily as transport proteins, with most mammalian ABC proteins functioning as export pumps. The human ABC transporter family includes around 50 members, which are divided into 7 sub-families according to sequence similarities (ABCA to ABCG). The Phylogenetic trees of five ABC subfamilies that include exporters are shown in Fig. 1A. ABC exporters are multispanning transmembrane proteins that are mainly localized to the plasma membrane, contributing to the reduction of intracellular concentration of their substrates. These substrates comprise a wide variety of xenobiotics such as environmental contaminants and drugs, including many currently used for the treatment of human diseases. Therefore, ABC proteins have toxicological and pharmacological functions. Several ABC exporters that have anticancer agents as substrates have been associated with cancer multidrug resistance (MDR) [1]. P-glycoprotein (Pgp, MDR1 or ABCB1), multidrug-resistance 1 (MRP1, ABCC1) and breast cancer resistance protein (BCRP or ABCG2) are examples of ABC proteins involved in MDR. ABC proteins also play physiological roles since they participate in the disposition of endobiotics and their metabolites.

Although the sequence organization and oligomeric structure of ABC exporters can vary, the basic structural/functional unit of human ABC exporters consists of two halves, each consisting of a membrane spanning domain (TMD), generally formed by six transmembrane α helices, and a nucleotide binding domain (NBD) [2] (Fig. 1B). Pgp is a typical example, where the TMD1-NBD1-TMD2-NBD2 structure is contained in a single polypeptide, with the N- and C-terminal ends localized intracellularly (Fig. 1B). MRP1 and other ABCC proteins contain an additional N-terminal transmembrane domain (TMD0), consisting of five transmembrane α helices, linked to the rest of the molecule by the L0 loop (Fig. 1B) [3, 4]. In these proteins, the N-terminal end is extracellular. BCRP is an example of a different organization. It is composed of a polypeptide with an N-terminal NBD followed by a six-transmembrane helices TMD (Fig. 1B). However, BCRP still functions as a homodimer, which each BCRP molecule being a half-transporter [5].

The hydrophobic ABC TMDs are structurally diverse, contain the binding sites for transport substrates and modulators, and form the transport pathway [6, 7]. In contrast, the NBDs, also known as ABC domains, display a highly-conserved structure [8, 9]. They are responsible for nucleotide binding and hydrolysis, providing the energy for translocation of ABC substrates across the membrane. The NBDs contain several conserved sequence motifs that are essential for nucleotide binding and hydrolysis [2, 10]. Additional details on the NBDs are presented in **sections 2.1 and 2.2**.

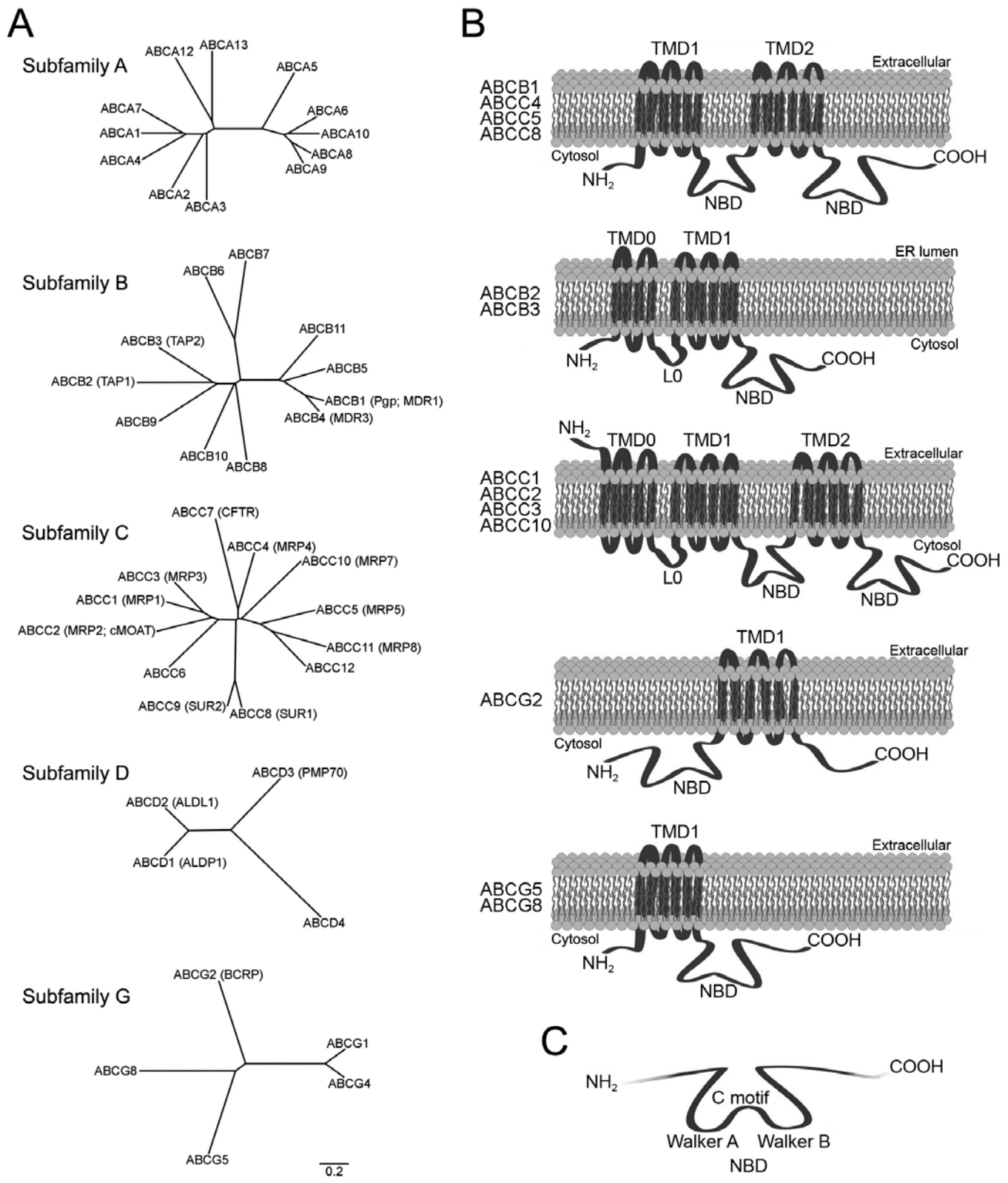

Fig. (1). **Human ABC transporters.** (A) Unrooted phylogenetic trees of five subfamilies of human ABC transport proteins. (B) Domain structure of several types of human ABC transporters. TMD: transmembrane domain; NBD: nucleotide binding domain; L0: linker or lasso domain. (C) Each NBD has highly-conserved motifs involved in nucleotide binding and hydrolysis. See **section 2.2** for details on NBDs structure and mechanism.

1.2. Localization and function of Pgp and MRPs

Pgp was the first member of the ABC family to be identified. In 1976 Juliano and Ling reported its presence in the plasma membrane of Chinese hamster ovary cells resistant to colchicine [11]. Its association with the multidrug resistant phenotype in different tumor cells conferred this family of transporters the denomination of MDR proteins [12]. It was later demonstrated that Pgp is also expressed constitutively in normal tissues such as kidney, liver, small intestine, colon, adrenal glands and lung, among others [13-18]. Knock-out mice were generated in an attempt to evaluate the physiological role of Pgp. Even though no evident physiological alterations were detected in these mice, they were more susceptible to chemical injury than wild-type mice, suggesting a major role of Pgp in xenobiotic efflux [19, 20]. The presence of Pgp in the intestinal brush border and apical cell membranes of other epithelia suggests a role of Pgp in limiting the absorption and cellular toxicity of xenobiotics by mediating the efflux of at least part of the xenobiotics that enter the cells through the apical membrane. [21]. Similar protective action has been described for Pgp in the placenta and blood-brain barrier [22, 23]. Pgp substrates are usually weakly amphipathic, rather hydrophobic, contain aromatic rings and are generally positively charged at normal pH [24-26]. A selected list of Pgp substrates is shown in Table 1. They include therapeutic drugs such as antiarrhythmic agents, calcium-channel blockers, analgesics, chemotherapeutic drugs, HIV-protease inhibitors, antihistamines, antibiotics and immunosuppressive agents [22, 27-29]. For drugs administered orally, the action of Pgp in intestine decreases their absorption and therapeutic efficacy [30]. Additionally, hepatic Pgp would contribute to the biliary elimination of drugs, shortening their lifetime [31]. Pgp-mediated decrease in intracellular concentration of chemotherapeutic agents may contribute to development of MDR of cancer cells [32]. The effects on drug pharmacokinetics and MDR are considered undesirable, in opposition to the protection exerted by Pgp against the harmful effects of environmental contaminants such as pesticides [33]. There is a great interest in understanding the mechanism by which drugs are transported by Pgp to aid in the development of drugs that selectively block Pgp, to reverse MDR, or evade recognition by Pgp, and thus to achieve more favorable pharmacokinetics.

The multidrug resistance-associated proteins comprise 9 ABCC subfamily members. They share the ABC protein core structure with Pgp, but some members (MRP1-3, 6 and 7) also have the additional TMD0 and L0 (Fig. 1B) [3]. MRPs display variable substrate specificities, membrane localization and function [3]. MRP1 was the first member of the MRP family to be identified. It was detected in a mutant lung cancer cell line exhibiting resistance to certain chemotherapeutic drugs active on the parental cell line [34]. It is expressed in normal kidney [35], intestine [36], blood-brain barrier [37], lung, testis and peripheral blood mononuclear cells [34, 38], among others. MRP2 (ABCC2) was initially cloned from rat liver and immediately identified as the canalicular transporter previously known as cMOAT (canalicular multispecific organic anion transporter) [39]. MRP2 is mainly expressed in the canalicular membrane of the hepatocyte [39], in the apical membrane of renal proximal tubules [40], in small-intestine enterocytes [41], and to a lesser extent in the blood-brain barrier [42]. MRP2 expression is also observed in tumors of several origins, and is usually associated with MDR [43, 44]. It is worth mentioning that the deficiency of MRP2 in patients with Dubin-Johnson Syndrome is responsible for the impairment of the biliary secretion independent of bile salts [45]. MRP3 (ABCC3), a basolateral-membrane isoform of MRP2, was identified in the sinusoidal membrane of the hepatic cell [46], and to a lesser extent in kidney, intestine, pancreas and gallbladder [47, 48]. It is also associated with cancer MDR [49, 50].

Table 1. Substrates of Pgp, MRP1 and BCRP. The list is not intended to be comprehensive.

P-glycoprotein: verapamil, rhodamine 123, acridine orange, azidopine, quinidine, amiodarone, calphostin C, genistein, staurosporine, progesterone, tamoxifen, cyclosporine, PSC-833, FK-506, erythromycin, rapamycin, quercetin, colchicine, vinblastine, vincristine, mitomycin C, daunorubicin, doxorubicin, teniposide, taxol, ritonavir, indinavir, dipyridamol.

MRP1: LTC4, LTD4, prostaglandin E2, calcein, folic acid, leucovorin, glutathione disulfide, arsenite, pentavalent antimonials, ritonavir, etoposide, glucuronide conjugates (*e.g.*, bilirubin, estradiol, etoposide), glutathione conjugates (*e.g.*, aflatoxin B, dinitrophenyl, ethacrynic acid, doxorubicin, cyclophosphamide, clorambucil, melphalan), sulfate conjugates (*e.g.*, estrone, dehydroepiandrosterone sulfate).

BCRP: methotrexate, doxorubicin, daunorubicin, mitoxantrone, hematoporphyrin, riboflavin, estrone-3-sulfate, and tyrosine kinase inhibitors such as genistein and gefitinib.

MRPs mediate the efflux of a wide variety of endo- and xenobiotics, with particular preference for compounds conjugated with glucuronic acid, glutathione or sulfate (see Table 1). The substrate specificity of MRP1 is extremely broad; it transports organic anion conjugates and unmodified hydrophobic compounds (*e.g.*, natural products, chemotherapeutic agents, aflatoxin B1 and other mutagenic compounds) [3]. Importantly, it transports cysteinyl leukotriene C_4 (LTC_4) with high-affinity, thus promoting its systemic release in response to inflammatory stimuli. Transport of several substrates by MRP1 depends on the presence of glutathione, and MRP1 and MRP2 have a protective role against oxidative stress [51, 52]. Bilirubin conjugates and glutathione are among the endogenous substrates of MRP2. This protein has a wide spectrum of exogenous substrates, contributing to limit the intestinal absorption of potentially toxic compounds [53]. MRP2 in the capillaries of brain endothelial cells and in the choroid plexus protects the central nervous system from xenobiotic toxicity [54, 55]. It has also been associated with MDR, as it transports a variety of chemotherapeutic agents such as methotrexate, anthracyclines (doxorubicin, epirubicin), mitoxantrone, cisplatin and etoposide (see Table 1) [56].

The Breast Cancer Resistance Protein (BCRP; ABCG2) was originally cloned from a human breast cancer cell line [57], and it transports a variety of chemically-unrelated substrates (see Table 1). Because of its expression in the canalicular membrane of hepatocytes and the apical membranes of enterocytes and renal proximal-tubule cells, it affects absorption/secretion and therefore bioavailability of many drugs [58]. Even though it has a minor role in renal uric acid excretion, its dysfunction is associated with hyperuricemia, gout, kidney disease and hypertension [59]. In addition, it is expressed in brain capillary endothelial cells, participating in the blood-brain barrier function [22], and plays a protective role in blood-placental and blood-testis barriers, and in the breast [22, 60]. Its expression in immature myeloid and leukemia cells suggests a role in cancer MDR [60]. BCRP transports chemotherapeutic drugs such as mitoxantrone, doxorubicin and daunorubicin [57], and it has been associated with secretion into the milk of essential compounds such as the vitamin riboflavin [61]. BCRP has also been associated with secretion of other compounds, including therapeutic drugs and toxins [62].

Fig. 2 summarizes the localization and function of representative ABC transporters in two model epithelia and in tumor cells. Fig. 2A shows examples of ABC transporters associated to the apical and basolateral membranes of the hepatocyte. Those in the apical membrane mediate biliary elimination of endobiotic metabolites such as bilirubin glucuronide, as well as xenobiotics that reach the liver through the hepatic artery (systemic origin) or portal vein (*e.g.*, after oral ingestion). The ABC transporters of the basolateral membrane mediate excretion of their substrates to the sinusoidal blood, which can be ultimately eliminated from the body by the kidneys. Basolateral transporters (*e.g.*, MRP1 and MRP3) are usually expressed at low levels, but can be induced when biliary secretory function is affected [63] or after treatment with specific drugs [64]. Fig. 2B illustrates the membrane barrier function of apical-membrane transporters in intestine, where they can limit absorption of ingested xenobiotics. A similar protective function of the central nervous system can be attributed to apical transporters in the blood-brain barrier. Finally, Fig. 2C shows how overexpression of ABC transporters in tumor cells leads to decreased concentration of chemotherapeutic agents, to produce MDR.

1.3. Substrate transport by ABC exporters

Although there are studies on many mammalian ABC exporters, here we focus on some general aspects of the transport by Pgp because of its importance and the fact that it is one of the most studied mammalian ABC exporters. Interest in the mechanism of transport by Pgp started early on after its discovery because of the relationship between Pgp and MDR [65, 66]. Multidrug-resistant cells that express Pgp or MRP1 display an energy-dependent decrease in intracellular concentration of substrates [4, 65, 66]. This has been generally ascribed to ATP-dependent pumping of the drugs out of the cells (Fig. 3B) [4, 65, 66]. However, since most Pgp substrates are liposoluble, aromatic and positively charged at physiologic extracellular pH [24, 25], and elevated intracellular pH is a frequent finding in Pgp-expressing multidrug resistant cells [67-69], it has also been proposed that the reduced intracellular concentration of Pgp substrates is secondary to the increased intracellular pH [67-69]; if the concentration of uncharged (lipophilic) moiety of the drug is similar on both sides of the plasma membrane (D in Fig. 3A and left panel of Fig. 3B), increased intracellular pH would lower the intracellular concentration of the protonated moiety, and thus the total intracellular concentration of the drug (Fig. 3). Along a similar reasoning, a combined indirect role of Pgp through cell-membrane depolarization and elevated cell pH has also been proposed [70, 71]. For a direct effect of cell-membrane voltage on drug distribution the charged drug should be permeable. However, a variety of experiments, including studies in cells and with purified Pgp clearly support the view that Pgp is a transport ATPase (Fig. 3B, right panel) [66, 68, 69]. An interesting twist is a recent report that shows that the Pgp bacterial homologue MsbA not only couples substrate transport to ATP hydrolysis, but also to the transmembrane electrochemical H^+ gradient [72]. This kind of coupling does not require protonatable substrates and could account for the efflux of cationic, anionic, and neutral substrates by Pgp. Confirmation of this finding and extension to other ABC exporters could have important implications for our understanding of the ABC mechanism of transport. It will be critical to perform studies under well-controlled conditions

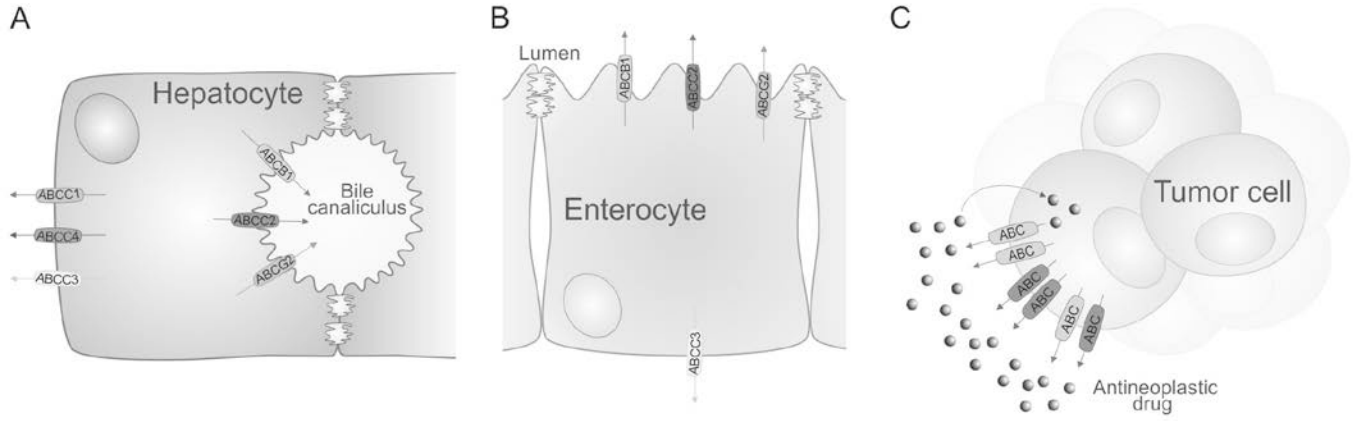

Fig. (2). Localization and function of some human ABC transporters. (A) ABC transporters present in normal hepatocytes. (B) ABC transporters present in normal intestinal epithelium. (C) Representation of the increased expression of ABC transporters in tumor cells and its relationship to cancer multidrug resistance.

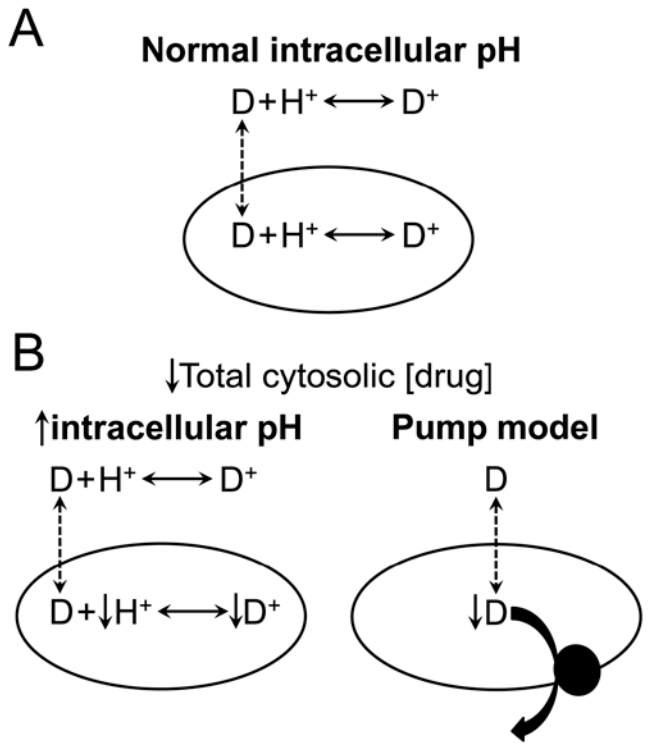

Fig. (3). Two potential mechanisms for reducing the free cytosolic concentration of Pgp substrates. (A) Passive distribution of a weak base Pgp substrate where the uncharged drug (D) is freely permeable and the charged drug (D^+) is not. The oval shape represents a cell and the dashed line indicates passive diffusion. (B). Left panel: effect of increasing cytosolic pH on the passive distribution of a weak base Pgp substrate. See panel A for details. Right panel: accepted model for the decrease in intracellular drug accumulation by Pgp. The black circle represents Pgp and the thick arrow indicates ATP-dependent pumping of the drug.

because cells expressing Pgp can display a number of abnormalities, including decreases in cell membrane voltage and Na^+,K^+-ATPase activity, and increases in ionic currents, nucleoside transport and membrane trafficking, as well as alterations in Na^+/H^+ exchanger activity, Ca^{2+} metabolism and lipid composition [68]. These multiple effects of Pgp expression could be explained by alterations in the levels of regulatory endogenous substrates and/or interaction of Pgp with other proteins. For example, more than a decade ago, we found that Pgp regulates swelling-activated Cl^- channels and demonstrated different, but additive effects in response to phosphorylation of Pgp by PKA and PKC [73]. The effects seem to be the result of phosphorylation of serines in the linker between the two homologous halves; possibly serine 661 by PKC and serine 683 by PKA [73]. A regulatory role of Pgp through association to other proteins such as annexin A2 has also been proposed [74]. In this context, sulfonylurea receptors (SUR1, ABCC8; SUR2, ABCC9) associate with Kir6.x channels to form ATP-sensitive K^+ channels (K_{ATP}) [75-77].

A transport issue that is very hard to address experimentally and is still unresolved is the origin of the transported substrate. The general view is that the influx of Pgp substrates through the membrane occurs predominantly by solubility-diffusion across the phospholipid moiety of the plasma membrane. The prevalent view of the efflux mechanism is that substrates access the Pgp binding sites from the lipid bilayer itself [78], and could occur by Pgp working as a flippase, moving substrates from the inner to the outer leaflet of the membrane. Flipping of lipid substrates from the inner to the outer leaflet has been demonstrated for several ABC proteins, including Pgp [26]. The quantitative importance of the flippase mechanism vis-à-vis more traditional transport mechanisms is still unclear, and may vary among substrates and ABC exporters. The nature of the Pgp and MRP1 substrates differs. While Pgp substrates are predominantly hydrophobic [24, 25], most MRP1 substrates are amphipathic, with many being organic anions that are frequently conjugated to glutathione, glucuronic acid or sulfate [4, 79]. These differences have a structural correlate with the Pgp and MRP1 structures that suggest that Pgp can transport substrates from the lipid bilayer whereas MRP1 likely takes them from the cytosol (see **section 2.3**). The possibility that Pgp transports substrates from the membrane has resulted in the "vacuum cleaner hypothesis" [78]. This hypothesis is based on the hydrophobicity of many Pgp substrates [24, 25, 66], the demonstration by fluorescence energy transfer that in Pgp expressing cells essentially all doxorubicin and rhodamine 123 are associated to Pgp [78], and the contention that unidirectional influx is reduced in MDR cells [80, 81]. Two predictions of this transport model are that the unidirectional influx of Pgp substrates into the cells should be reduced by Pgp and that Pgp inhibition should cause not only a decrease in efflux, but also an increase in influx into the interior of the cells. These predictions are expected because some of the substrate partitioning into the membrane would be extruded by Pgp from the membrane, without reaching the cell interior. However, the fact that the unidirectional influx of rhodamine 123 is independent of Pgp function, even in cells with very high Pgp expression, does not support the hypothesis that substrates are extruded by Pgp directly from the membrane [82]. Nevertheless, it is possible that the contribution of extrusion from the membrane is more significant for other substrates based on their cytosol/membrane partition coefficient and accessibility to the binding site from the cytosol *vs*. membrane.

2. STRUCTURE AND MECHANISM

2.1. NBD structure

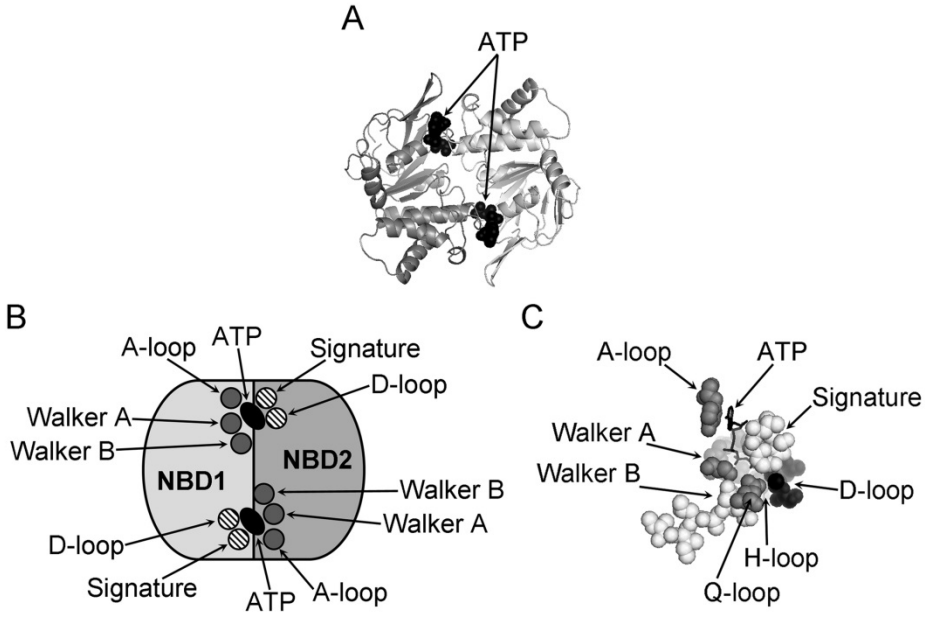

Fig. (4). Structure of the ATP-bound NBD dimer. (A) ATP-bound dimer. NBDs are shown in ribbon representations, each in a different tone of gray. (B) Schematic representation of the ATP-binding sites in the ATP-bound NBD dimer. (C) The nucleotide-binding site. Sphere representation. Most of the structure was removed to facilitate viewing. The figures were based on the structure of a catalytically-inactive MJ0796 mutant (PDB PDB1L2T). See ref. [85] for details.

While poorly-conserved TMDs are tuned to the varied functions of the ABC proteins (ion channel, transporter of lipids, peptides, organic chemicals, etc.), the common nucleotide binding and hydrolysis functions rest on the structurally-conserved NBDs. It is well established that NBDs can form nucleotide-bound dimers in a head-to-tail arrangement, where two ATP molecules are sandwiched between the monomers (Fig. 4A) [83]. As mentioned above, the NBDs contain several conserved sequences that are important for nucleotide binding and hydrolysis. Each of the two nucleotide-binding sites in the NBD dimer is formed by the Walker A motif, Walker B motif, A loop, H loop and Q loop of one NBD, and the D loop and signature motif of the other NBD. The formation of each nucleotide-binding site by motifs from both NBDs in the ATP-bound dimer is illustrated in Fig. 4B. The Walker A motif is involved in binding of nucleotide phosphates and the Walker B, together with the Q loop, is involved in Mg^{2+} and water coordination at the catalytic site. The A loop contains a conserved aromatic residue that interacts with the adenine ring of ATP, whereas the signature motif and D and H loops are involved in the coordination of the ATP γ phosphate (Fig. 4C).

2.2. Molecular mechanism of the ABC NBDs

It is thought that ATP hydrolysis by the NBDs is catalyzed *via* a general base mechanism where the carboxylate of the Walker B catalytic glutamate polarizes the hydrolytic water that attacks the ATP γ-phosphate. However, it has also been proposed that the Walker B glutamate works as part of a complex that stabilizes the transition state rather than serve as a catalytic base [84]. An aspartate replaces the catalytic glutamate in MRP1 NBD1 and other ABCC proteins. This aspartate side chain may not be long enough to interact with the hydrolytic water. The MRP1 NBD2 signature motif also deviates from the consensus LSGGQ sequence [4]. Therefore, whereas the functional unit of many prokaryotic ABC proteins and most human ABC exporters consists of two identical or very similar NBDs (*e.g.*, Pgp), in a few human exporters one of the NBDs is catalytically defective, and the functional roles of the two NBDs may differ (*e.g.*, MRP1, CFTR) [4, 85]. Even though the two nucleotide-binding/hydrolysis sites of Pgp are very similar, the available evidence points to an asymmetric interaction with nucleotides, with ATP hydrolysis occurring at one of the two sites, and not simultaneously, supporting a model for alternating site catalysis [86]. A recent structural study on a Pgp mutant (partial linker deletion) that does not display substrate-stimulated ATPase activity suggests that NBD1 is the preferred ATP-binding site [87]. Consistent with the notion of asymmetry, 1 mol of nucleotide *per* mol of Pgp is trapped by vanadate, which stabilizes a post-hydrolysis intermediate state [86]. This and other studies suggest that the ATP-bound NBD dimer is asymmetric, with only one ATP bound tightly, committed to hydrolysis ("trapped").

Computational simulations suggest that ATP hydrolysis at one site is sufficient to induce opening of the dimer [88, 89], but a number of molecular models have been proposed [83, 86, 90-95]. We recently addressed the issue of the number of ATP molecules that are hydrolyzed *per* NBD hydrolysis cycle using lanthanide (or luminescence) resonance energy transfer (LRET). We demonstrated that there is association/dissociation of the NBDs during that hydrolysis cycle, and found that the rates of dissociation of ATP-bound NBD dimers containing two ATP binding sites and either two or one hydrolysis competent site(s) were identical [96, 97]. The NBDs dissociation rate was the same as the rate of hydrolysis, and therefore it seems that the power stroke for NBD dimer dissociation requires hydrolysis of only one ATP (Fig. 5B). Therefore, a single hydrolysis event can drive dissociation even when the NBD dimer is formed by two identical NBDs. These observations suggest that the molecular mechanism of proteins such as Pgp and MRP1, which differ in the number of ATP hydrolysis-competent sites, is not necessarily different. It is presently unknown whether the conformational change that follows hydrolysis of one ATP can power substrate transport. A schematic representation showing the NBD association/ dissociation during the ATP hydrolysis cycle is shown in Fig. 5A.

One obvious question that follows is about the need of two ATP-binding sites in ABC proteins. Using LRET on isolated NBDs, we have compared dimerization of NBDs that form dimers with two sites that can bind and hydrolyze ATP (wild type) with dimerization in mutants where the affinity for ATP of one of the binding sites was reduced [98]. The mix of wild-type and mutant NBDs produced stable dimers under conditions where both binding sites are occupied (high ATP concentration). However, no stable dimers were formed under conditions where only one of the binding sites was occupied (low ATP concentration). Hence, it appears that occupation of the two nucleotide-binding sites in ABC proteins is necessary to produce an ATP-sandwich dimer of sufficient stability to allow for ATP hydrolysis at one of the sites, which in turn drives dimer dissociation.

An interesting aspect of the ABC NBDs that has not been explored in detail is the nature of the nucleotide triphosphate that is bound and hydrolyzed by the NBD dimer. Although it has been known for many years that at least some ABC proteins can hydrolyze nucleotide triphosphates different from ATP, detailed studies are few [99-102]. It was recently found that the prototypical thermophile NBD MJ0796 can bind and hydrolyze a number of natural nucleotide triphosphates, including ATP, CTP, GTP and UTP [102]. Considering that the concentration of different nucleotide triphosphates varies among cells and under different conditions, with GTP, CTP and UTP cytosolic concentrations in the 300-600 μM range, it is quite likely that ATP is not the only nucleotide that occupies the NBD nucleotide-binding sites under physiological conditions. It is also likely that dissociation of NBD dimers can follow hydrolysis of nucleotide triphosphates different from ATP, especially under conditions such as ATP depletion (*e.g.*, ischemia).

2.3. Structure of ABC exporters

The ABC superfamily includes many different exporters, but here we focus on the ABCB member Pgp and the ABCC member MRP1. The available Pgp structures correspond to nucleotide-free inward-facing conformations and display the two "halves" arranged in a pseudo two-fold symmetry (Fig. 6) [103-106]. Pgp spans ~135 Å perpendicular to the membrane plane and ~70 Å in membrane plane, with the NBDs separated by several tens of Å. A large internal cavity formed between the two TMDs is accessible to the

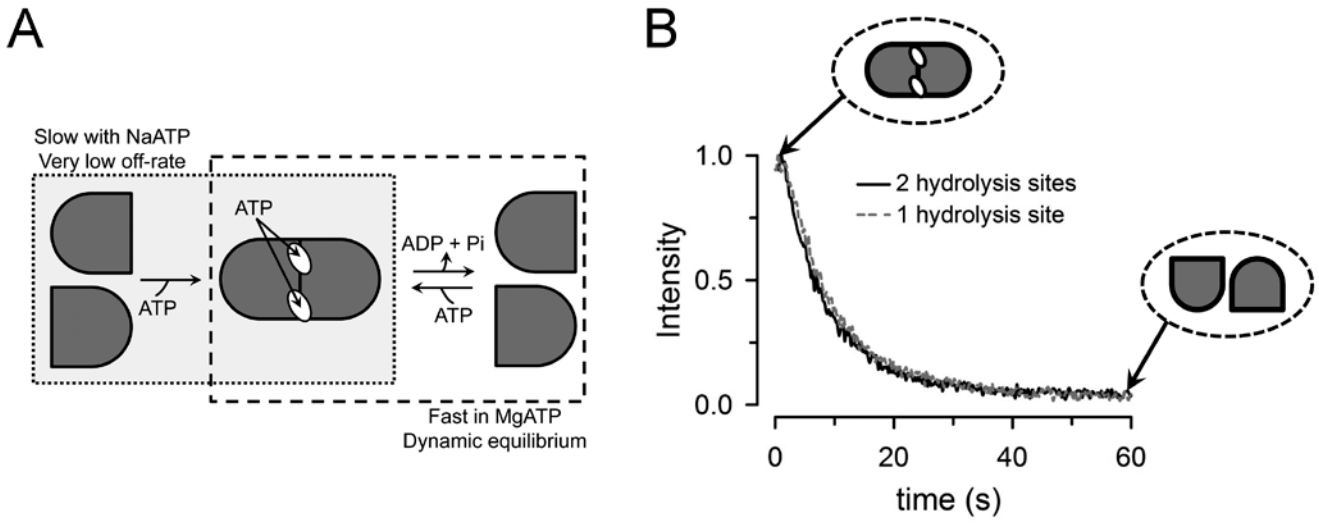

Fig. (5). Association/dissociation of NBDs. (A) Schematic representation of the ATP-dependent dimerization of NBDs, and the monomer/dimer dynamic equilibrium during ATP hydrolysis. (B) Rates of NBD dissociation elicited by ATP hydrolysis by dimers with one and two ATP binding sites competent for hydrolysis. For these experiments one of the NBDs in the ATP-bound dimer was labeled with an LRET donor and the other with an LRET acceptor. At time = zero Mg^{2+} was added to start ATP hydrolysis and the rate of dimer dissociation was followed from the decrease in LRET intensity. See refs. [98], [99] and [121] for details. Panel (B) has been modified from ref. [98], with permission from the American Society for Biochemistry and Molecular Biology.

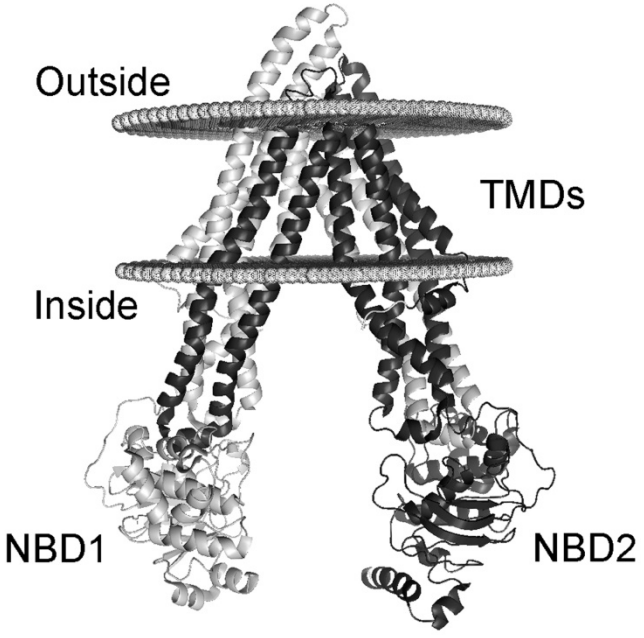

Fig. (6). Pgp structure. Ribbon representation of the mouse Pgp in the apo state (based on PDB 4M1M). The discs delimit the membrane, and outside and inside correspond to the extracellular and intracellular sides, respectively. See ref. [105] for details.

cytoplasm and the inner leaflet of the membrane. The two membrane openings are formed by transmembrane helices 4 and 6 and 10 and 12. These helices have side chains that could be small enough for tight packing in the outward-facing (ATP-bound) conformation. The drug-binding cavity within the lipid bilayer seems sufficiently large to accommodate large substrates and/or several substrates simultaneously, or one substrate bound to more than one site [103, 107]. Approximately 80% of the residues in the drug-binding pocket are hydrophobic/aromatic. Whereas the upper half of the drug-binding pocket contains predominantly hydrophobic and aromatic residues, the lower half has more polar and charged residues that may bind hydrophobic weak bases [103, 107]. Binding studies have identified several sites that interact negatively with each other, in competitive or non-competitive manner, or that display positive cooperative effects [108-111]. Two sites generally known as H-site (selective for Hoechst 33342 and calcein-AM) and R-site selective for rhodamine 123) seem to be involved in drug transport [112]. Unfortunately, the locations of these binding sites in the structure have not been identified.

The MRP1 transporter displays a core structure similar to that of Pgp, with a large transmembrane vestibule between the two pseudo symmetric helical bundles that opens to the cytoplasm and penetrates halfway into the lipid bilayer (Fig. 7) [79]. The two NBDs are 30 **Å** down into the cytoplasm, and separated from each other [79]. TMD0 contains five transmembrane α-helices, similar to SUR1 [76, 77, 79]. The TMD0 of SUR1, SUR2 and TAP mediates protein-protein interactions (SUR-Kir6 and TAP-tapasin) [75, 113], but the role of this domain in MRP1 is still unknown. The L0 linker sequence is conserved in all ABCC members, including those that do not have TMD0, and the CFTR and MRP1 L0 structures are very similar [79, 114]. Experimental studies point to an important role of L0 in the folding and trafficking of ABCC proteins [115]. Part of the L0 linker forms an extended structural motif between the membrane and the cytosol that packs tightly against transmembrane helices 7, 15 16 in MRP1, whereas an amphipathic helix inserts diagonally into the inner leaflet of the membrane [79]. In support of an important function of this amphipathic helix, co-expression of L0 rescues the folding-defective MRP1 mutant lacking TMD0-L0 [115, 116], an effect that is not observed for L0 without the amphipathic helix [116]. The MRP1 NBDs in the apo structure are unevenly separated, and the interactions with the TMDs vary between the two

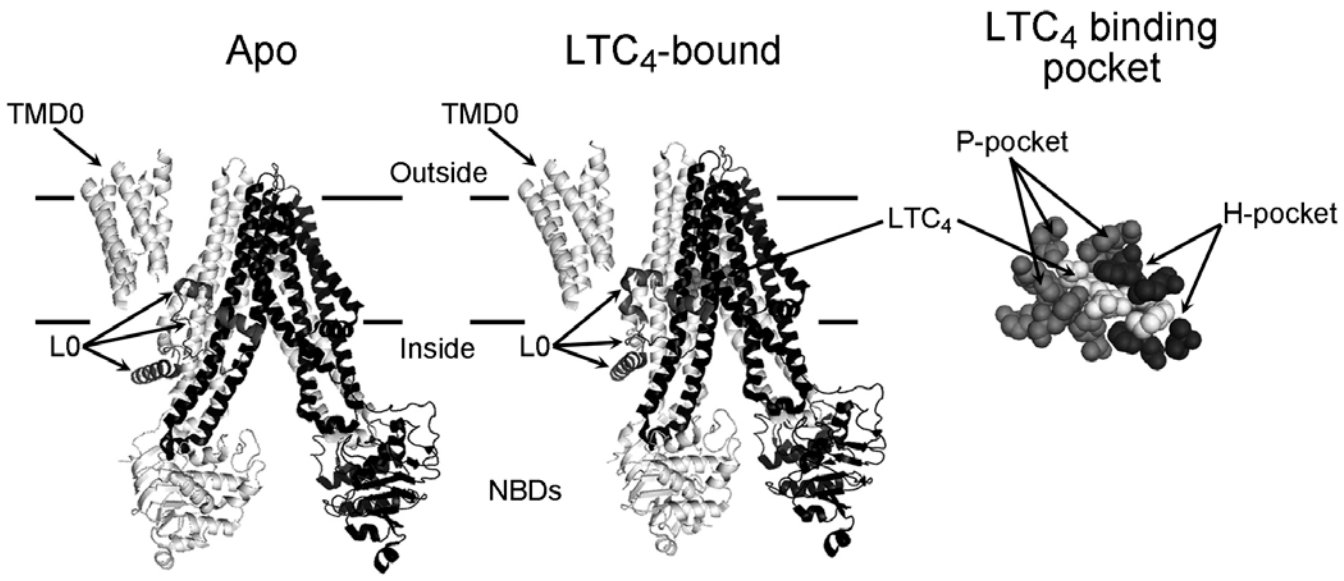

Fig. (7). MRP1 structure. Ribbon representation of the bovine MRP1 in the apo (PDB 5UJ9) and LTC_4-bound states (PDB 5UJA). The lines correspond to the membrane boundaries. Details of the LTC_4 binding pocket (sphere representation) are also shown with most of the structure removed to facilitate viewing. See reference [81] for details.

MRP1 halves [79]. The TMD/NBD2 interface resembles the ‘‘ball-and-socket’’ joint described for other ABC transporters [79, 103]. This structure, formed by a cytoplasmic helix from the TMD (ball) and a cleft on the NBD2 surface (socket), is not present in the TMD/NBD1 interface [79]. Compared to ABCB protein, ABCC members have a deletion of the 10-13 NBD1 residues (13 for MRP1) that form the socket, which may make the NBD1/TMD interaction weaker than the NBD2/TMD interaction.

LTC_4 binding results in the movement of both transmembrane bundles inward through rigid body rotations around the binding site [79]. The small displacements at the LTC_4-binding site (~3 **Å**) are propagated over 50 **Å** to bring the NBDs ~12 **Å** closer to each other. In the apo MRP1 structure the NBDs are twisted relative to each other, whereas in the LTC_4-bound structure they are closer and better aligned [79]. These changes may account for the stimulation of MRP1 ATPase activity by LTC_4, since they are somewhat analogous as those described in the response of the maltose transporter to association with the substrate-loaded binding protein [117].

A fundamental puzzle regarding multidrug resistance is how a single transporter binds and transports many structurally dissimilar molecules. Current structural data suggest that this is accomplished in different ways by Pgp and MRP1. Pgp substrates are generally hydrophobic and/or weak bases [24, 25], and polyspecificity seems to arise from the presence of a flexible multi-binding site that is hydrophobic, with some acidic patches [103, 107]. Most MRP1 substrates are organic acids [4], and polyspecificity depends on a flexible single bipartite binding site that has a positively-charged pocket (P-pocket) and a hydrophobic pocket (H-pocket) [79]. The H-pocket can accommodate hydrophobic moieties of different sizes and also has some polar side chains that could interact with polar groups of hydrophobic substrates. Amphipathic substrates such as methotrexate and substrates conjugated to sulfate or glucuronic acid are likely to interact with the P- and H-pockets.

Another difference between Pgp and MRP1 is that only in Pgp the helices lining the membrane opening to the cytoplasmic side are flexible and could form a gate that allows access of substrates of different sizes from the inner leaflet [103, 104]. In contrast, the MRP1 translocation pathway is shielded from the membrane bilayer [79].

Structures of CFTR have been solved for the dephosphorylated state in the absence of ATP (low permeability, mostly closed channel) and the phosphorylated, ATP-bound CFTR (active channel) [114, 118]. In the dephosphorylated state the regulatory R domain wedges between the NBDs preventing their dimerization. As expected, major conformational changes lead to activation by phosphorylation, including disengagement of the R domain, NBD dimerization, and an opening of the putative Cl- pathway into the cytosol (although the other side of the pathway remained unexpectedly closed).

In addition to the structures of the Cl^- channel CFTR, the structures of several additional structures of ABC exporter pumps became available recently, including those of the cholesterol transporter heterodimer ABCG5/ABCG8, BCRP and ABCA1 [5, 114, 118-120]. The recent structure of human BCRP in the nucleotide-free state [5] shows overall similarity with the cholesterol transporter ABCG5/ABCG8 [120]. It is a homodimer in the inward-open conformation, with a deep, slit-like binding pocket that opens to the cytosolic side and inner leaflet of the membrane. The drug-binding pocket could accommodate a variety of molecules, and the presence of two cholesterol molecules with their hydroxyl groups pointing into the cavity was inferred from the structural data. The cholesterol binding area is well conserved in ABCG cholesterol transporters [5]. Although cholesterol is not a BCRP substrate, it regulates its activity [121], and BCRP transports estrone-3-sulfate, a structurally-related sterol. The deep binding pocket reaches more than halfway across the membrane and is separated from a second smaller cavity located below the extracellular loop 3 [5]. This cavity is not present in ABCG5/ABCG8 due to differences in the structure of the extracellular loop 3 [5, 120]. The smaller BCRP cavity is less hydrophobic than the binding pocket, and probably displays lower affinity for substrates, which could promote their release. In the proposed transport model, binding of nucleotides and substrate will switch the protein from the inward- to the outward-facing conformation, which would move the substrate to the now accessible smaller cavity [5]. As for other ABC protein models, ATP hydrolysis with release of the nucleotides will reset the transporter, completing the active cycle. An interesting aspect of the BCRP and ABCG5/ABCG8 structures is the contact between the NBDs in the absence of nucleotides [5, 120], in sharp contrast with the apo structures of the ABCB proteins Pgp and MsbA [103, 104, 122].

The structure of the phospholipid and cholesterol exporter ABCA1 in the apo state has also been solved recently [119]. The related processes of lipid export by ABCB1 and loading of lipids to apolipoprotein A-I control the formation of high-density lipoprotein (HDL), which is essential for the reverse transport of cholesterol from tissues to the liver. Mutations of ABCA1 cause Tangier disease, a rare genetic disorder that presents with severe decrease in HDL levels and high incidence of premature cardiovascular disease, as well as less severe familial HDL deficiency syndromes [123]. ABCA1 displays an overall ABC core structure similar to that of Pgp, but with small regulatory R domains following each NBD and two large extracellular domains between the first two transmembrane helices of each TMD [119]. The two R domains are in close contact and may stabilize the interactions between the NBDs, which are in contact in spite of the absence of nucleotides [119]. Different from other ABC exporters, in the inner leaflet area TMD1 and TMD2 are in close contact to each other through a narrow interface, without a discernible central cavity [119]. Since the apo ABCA1 structure displays an outward-facing conformation in the absence of nucleotide, an outward-facing only molecular mechanism was proposed [119], as for the lipid-linked oligosaccharide flopping by the ABC exporter PglK (no alternating access) [124]. A polar cluster on one side of TMD1, near the cytoplasmic side, would participate in the transfer of the lipids polar head across the hydrophobic membrane. This polar cluster may bind the lipids polar head in the inner leaflet of the membrane, and the TMDs’ conformational changes driven by the NBDs’

work would result in the delivery of the lipid polar head to an unidentified binding site close to the outer leaflet. The extracellular domains of ABCA1 form a large flame-shaped structure that includes a base, a tunnel, and a lid. The two extracellular domains are packed together through extensive interactions at the base and tunnel [119]. The tunnel, formed primarily by the first extracellular domain, is likely involved in the interactions with apolipoprotein A-I, and its predominantly hydrophobic hollow interior could participate in the delivery and temporary storage of lipids.

In summary, analysis of the structures of Pgp and MRP1 points to different molecular bases for the polyspecificity of these proteins, and the major variations among the available ABC exporter structures are likely to translate into differences in the molecular mechanisms of operation.

2.4. Molecular mechanism of ABC exporters

There is general agreement that the two NBDs associate to form a nucleotide-bound dimer that is competent for hydrolysis [83, 93, 125, 126]. However, our understanding of the mechanism of ABC exporters is very limited and the available models are controversial [83, 86, 90-95]. It is generally accepted that ABC exporters work by alternating the access to their transmembrane central cavity between both sides of the membrane. Formation of a tight ATP-bound NBD dimer is coupled to rearrangements in the TMDs with a transition from an inward-facing, open conformation (dissociated NBDs or loosely-associated NBDs), with the binding pocket accessible to the inner leaflet/cytoplasm, to an outward-facing, closed conformation (tight ATP-bound NBD dimer), with the binding pocket accessible to the extracellular side. Bound substrates can dissociate from this outward-facing conformation into the external medium. Finally, NBD dissociation or dimer opening follows ATP hydrolysis, which resets the exporter for the next cycle. A schematic representation of the mechanism of Pgp for the case of large conformational changes at the NBD-NBD interface is shown in Fig. 8A, whereas Fig. 8B shows two sets of models of the NBDs catalytic cycle: one proposing complete NBDs dissociation (monomer-dimer models) and the other continuous contact between the NBDs (constant-contact models).

The idea that association/dissociation of the NBDs occurs with NBD-NBD separations of several tens of Å as part of the physiologic Pgp cycle has been challenged by the nucleotide-bound, outward-facing structure of Sav1866 [90]. The interactions between the two subunits of this homodimeric Pgp-related ABC exporter do not support the view of a significant separation of the NBDs during the transport cycle. However, there can be mechanistic differences between the modes of operation of Pgp and Sav1866. Contrary to Sav1866, Pgp displays a clear preference for the inward-facing conformation [87, 127-129] that could result from the larger hydrophobicity of the drug-binding pocket and increased charge density of the NBDs interface [130]. Nevertheless, the conclusions based on the Sav1866 structure agree with electron microscopy structures of Pgp [131, 132] and our recent spectroscopy studies in MsbA that point to close proximity of the NBDs in the absence of ATP [127, 133].

There are very few studies performed on ABC proteins during ATP hydrolysis. Our recent studies on MsbA suggest that the NBDs of this ABC exporter dissociate during hydrolysis when the protein is in detergent, at 37^{o}C [133]. However, the results obtained with the protein reconstituted into a lipid bilayer suggest that the NBDs never separate as much as suggested by the crystal structures [127]. It also seems that MsbA is more compact in a lipid bilayer compared to the protein in detergent. In conclusion, it seems that the large separation of NBDs in crystal structures and those estimated from double electron-electron resonance spectroscopy do not represent physiologic conformations [103, 104, 134, 135]. Comparison of MsbA in detergent and in lipid nanodiscs suggests that the conformational changes during the ATP hydrolysis cycle are smaller than previously thought, with a maximum NBD separation of only ~10 Å [127]. It appears that the NBDs have the ability to dissociate completely following ATP hydrolysis, but factors such as the lipid bilayer and bound substrates can restrict the separation between the NBDs, facilitating re-association and increasing the ATPase rate.

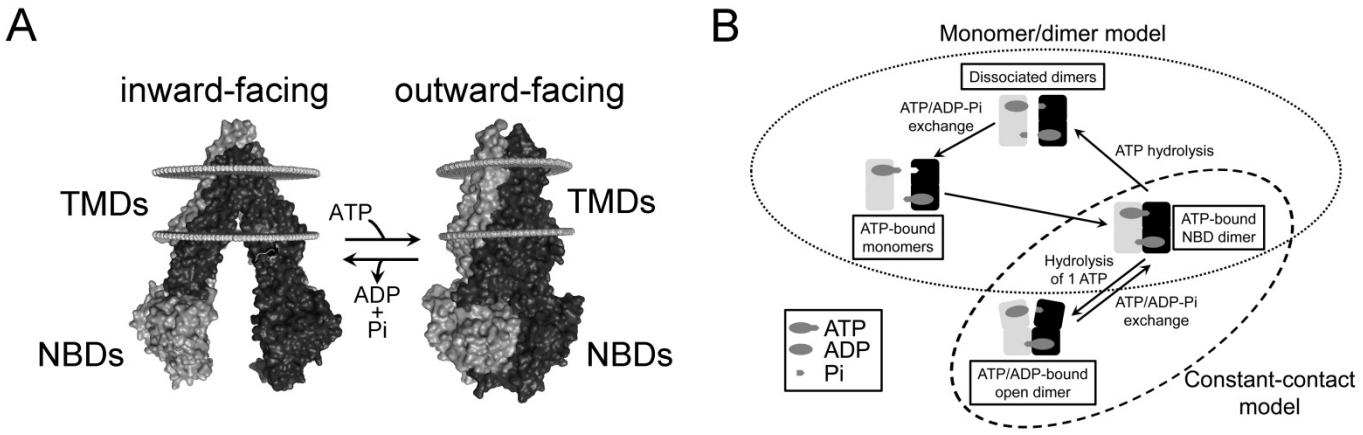

Fig. (8). NBD-NBD interactions during the ABC exporter hydrolysis cycle. (A) Model of ABC exporter operation based on Pgp crystal structures. (B) Schematic representation of the NBD interactions of two model of the catalytic cycle of ABC exporters. Panel (B) has been modified from ref. [128], with permission from the American Society for Biochemistry and Molecular Biology.

CONCLUSION

ABC proteins are present in all kingdoms of living organisms. ABC exporters in general, and Pgp and MRP1 in particular, play important roles in human physiology, pathophysiology, blood-tissue barriers, drug pharmacokinetics and drug resistance. The core structure of ABC exporters consists of two TMDs not conserved among ABC members, which determine the protein function (ion channel, regulator, exporter), and two conserved NBDs. The NBDs associate in the presence of ATP to form dimers with two ATP molecules sandwiched at the NBD-NBD interface. The NBD dimer has two ATP binding sites, and whereas in most ABC exporters both sites can hydrolyze ATP (*e.g.*, Pgp), in some (*e.g.*, MRP1) only one can. While binding of two ATPs is necessary to form a stable dimer competent for ATP hydrolysis, hydrolysis of only one of the bound ATPs is sufficient for dissociation or opening of the tight ATP-bound dimer.

Conformational changes on the NBD side of the ABC exporters seem to drive major conformational changes of the TMDs. These changes switch the accessibility of the substrate-binding pocket between the inside and outside, which is coupled to substrate efflux. Although there is agreement on the alternate-accessibility model, the magnitude and nature of the changes at the NBD side are not well defined. Evidence has accumulated, however, for coupling between substrate binding and conformational changes on the NBDs side. It appears that substrate binding to the TMDs decreases the NBD-NBD distance, and perhaps improves the alignment of the NBDs' ATP-binding residues, priming the domains to form the ATP-bound dimer. These changes are likely involved in the stimulation of the ATPase activity by substrates.

Comparison of the structures of Pgp and MRP1 provides important clues for our understanding of their substrate selectivities and the bases for their distinct polyspecificities. Pgp substrates are hydrophobic and/or weak bases, and polyspecificity is explained by a flexible hydrophobic multi-binding site that has a few acidic patches. MRP1 substrates are mostly organic acids, and its polyspecificity is due to a single bipartite binding site that is flexible and displays positive charge.

*Address correspondence to this author at the Department of Cell Physiology and Molecular Biophysics, Texas Tech University Health Sciences Center, Lubbock, TX 79430-6551, USA; Tel/Fax: +1-806-743-2531, +1-806-743-1512; E-mail: g.altenberg@ttuhsc.edu

LIST OF ABBREVIATIONS

ABC = ATP-binding cassette

BCRP = breast cancer resistance protein or ABCG2

CFTR = cystic fibrosis transmembrane conductance regulator, ABCC7

L0 = linker or lasso domain of ABCC proteins

LRET = lanthanide (or luminescence) resonance energy transfer

MDR = multidrug resistance

MRP1 = multidrug resistance protein 1 or ABCC1

NBD = nucleotide-binding domain

Pgp = P-glycoprotein, ABCB1 or MDR1

SUR = sulfonylurea receptor (SUR1, ABCC8; SUR2, ABCC9)

TMD = transmembrane domain

TMD0 = N-terminal domain of some ABCC proteins

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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