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New Insights into the Drug Binding, Transport and Lipid Flippase Activities of the P-Glycoprotein Multidrug Transporter

Frances J. Sharom,[1,3] Miguel R. Lugo,[2] and Paul D. W. Eckford[1]

The MDR1 P-glycoprotein, an ATP-binding cassette (ABC) superfamily member that functions as an ATP-driven drug efflux pump, has been linked to resistance of human tumors to multiple chemotherapeutic agents. P-glycoprotein binds and actively transports a large variety of hydrophobic drugs and peptides. P-glycoprotein in reconstituted proteoliposomes is also an outwardly directed flippase for membrane phospholipids and simple glycosphinglipids. This review focuses on recent advances in our understanding of P-glycoprotein structure and function, particularly through the use of fluorescence spectroscopic approaches. Progress is being made towards understanding the structure of the transporter, especially the spatial relationship between the two nucleotide-binding domains. Exploration of the P-glycoprotein catalytic cycle using vanadate-trapped complexes has revealed that drug transport likely takes place by concerted conformational changes linked to relaxation of a high energy intermediate. Low resolution mapping of the protein using fluorescence resonance energy transfer showed that both the H and R drug-binding sites are located within the cytoplasmic leaflet. Two drugs can bind to the R-site simultaneously, suggesting that the protein contains a large flexible binding region.

KEY WORDS: ABC transporter; P-glycoprotein; MDR1; multidrug resistance; reconstitution; lipid bilayers; fluorescence spectroscopy; drug binding; lipid flippase; glycosphingolipid.

INTRODUCTION

Resistance to multiple chemotherapeutic drugs is a serious problem in cancer treatment, and has been linked to the presence of multidrug efflux pumps of the ATP-binding cassette (ABC) superfamily (Litman *et al*., 2001), especially P-glycoprotein (Pgp, MDR1 or ABCB1), MRP1 (ABCC1), and the recently discovered BCRP/ABCP/MXR (ABCG2). After 20 years of study, Pgp is the best-characterized of these proteins, and we now have a basic understanding of its structure and function. Like all ABC proteins, Pgp comprises two membrane-bound domains, each made up of six transmembrane (TM) α-helices, and two nucleotide-binding (NB) domains that hydrolyze ATP to power drug transport. Its ability to transport an astonishing variety of structurally diverse drugs, natural products, and peptides makes Pgp a fascinating protein that has challenged our view of how ATP-driven transport across membranes takes place.

[1] Department of Molecular and Cellular Biology, Science Complex Room 2255, University of Guelph, Guelph, Ontario, Canada N1G 2W1.
[2] Instituto de Biología Experimental, Facultad de Ciencias, Universidad Central de Venezuela, Caracas, Venezuela.
[3] To whom correspondence should be addressed; e-mail: fsharom@uoguelph.ca.

Abbreviations: ABC, ATP-binding cassette; FRET, fluorescence resonance energy transfer; GlcCer, glycosylceramide; LDS-751, 2-[4-(4-[dimethylamino]phenyl)-1,3-butadienyl]-3-ethylbenzo-thiazolium perchlorate; MIANS, 2-(4-maleimidoanilino)naphthalene-6-sulfonic acid; NB, nucleotide binding; NBD, nitrobenzo-2-oxa-1,3-diazole; PC, phosphatidylcholine; R123, rhodamine 123; TM, transmembrane; TMR, tetramethylrosamine.

ABC PROTEIN STRUCTURE

The presence of two NB domains in assembled ABC proteins, and the relationship between the conserved Walker A and B motifs and the unique C (signature or LSGGQ) motif, led to the suggestion that these domains form interdigitated dimers (Jones and George, 1999). Attempts to determine how this dimerization might take place, using crystallization of isolated NB subunits, led to a plethora of different high resolution structures (Jones and George, 2004; Kerr, 2002). It appears likely that the presence of the membrane-bound subunits, or blockade of the ATPase catalytic cycle, is necessary to drive the correct mode of association of the two NB domains. The first structural model to indicate that an interdigitated dimer did exist was that of Rad50cd (Hopfner *et al*., 2000), a DNA repair enzyme related to the ABC superfamily. More recently, Smith *et al*. (2002) crystallized a "sandwich dimer" of the catalytically inactive E171Q mutant of the MJ0796 NB domain. Two ATP molecules were bound at the interfaces between the two subunits of the symmetrical dimer, held in place by the Walker A and B motifs of one subunit, and the LSGGQ signature motif of the other.

Early low resolution structures of Pgp obtained by electron microscopy indicated that the NB domains were situated far apart (Rosenberg *et al*., 1997), which was not consistent with biochemical studies of the protein, or with the observation that the NB domains of other ABC proteins were interdigitated. To define aspects of the molecular architecture of Pgp, we undertook a low resolution "mapping" approach using purified protein and fluorescence resonance energy transfer (FRET). By covalently linking a fluorescent donor and acceptor to key regions of the protein, the distances separating these sites could be estimated, much like a "spectroscopic ruler." Initially, it was established that the NB domains were situated relatively close to the membrane (Liu and Sharom, 1998), consistent with the observation that ATP binding and hydrolysis at these sites is sensitive to the phase state of the lipid bilayer (Romsicki and Sharom, 1998). Further FRET studies by Qu and Sharom (2001) established that the separation of the two NB domains was consistent with the Rad50cd dimer structure, indicating that they are indeed in close contact with each other. A recent medium resolution 8 Å electron crystallographic structure of Pgp confirmed this, although secondary structural information was limited (Rosenberg *et al*., 2005).

In recent years, a few high resolution X-ray structures have been determined for complete bacterial ABC proteins, including the vitamin B12 importer, BtuCD (Locher *et al*., 2002), and the lipid A flippase, MsbA, from three different organisms (Chang, 2003; Chang and Roth, 2001; Reyes and Chang, 2005). Far from showing us how complete ABC transporters assemble in the membrane and interact with their substrates, these structures proved to be different, fuelling further controversy. No high resolution structures exist for eukaryotic ABC proteins.

THE CATALYTIC CYCLE OF PGP

Pgp-mediated drug transport requires ATP hydrolysis. Both catalytic sites can hydrolyze ATP (Urbatsch *et al*., 1995a), but if essential residues at one catalytic site are altered by mutation, ATP hydrolysis at both NB domains is abolished (Loo and Clarke, 1995). Similarly, in the presence of the P_i analog, orthovanadate (V_i), Pgp forms a stable, catalytically inactive complex in which $ADP{\cdot}Mg^{2+}{\cdot}V_i$ is trapped noncovalently in only one active site (Urbatsch *et al*., 1995b). After a single catalytic turnover at the active site, P_i dissociates, and V_i enters the active site to form the complex. The geometry of such vanadate-trapped complexes is thought to resemble that of the transient catalytic transition state formed during ATP hydrolysis (Smith and Rayment, 1996). Based on these observations, Senior *et al*. (1995) proposed that the two catalytic sites alternate in carrying out ATP hydrolysis, such that only one of them is functional at any time. Vanadate-trapping has proved to be a useful tool to explore various aspects of the catalytic cycle of Pgp. The V_i trapped complex formed using Co^{2+} instead of Mg^{2+} is stable for several hours (Qu and Sharom, 2002), allowing the K_d for drugs and nucleotides to be determined (see below).

DRUG AND NUCLEOTIDE BINDING TO PGP DURING THE CATALYTIC CYCLE

Fluorescence spectroscopy has proved to be extremely useful for quantitation of the dissociation constant, K_d, for binding of nucleotides and drugs to purified Pgp (Sharom *et al*., 2003). The low ATP-binding affinity of the transporter, coupled with the hydrophobic nature of the drug substrates, makes standard binding approaches technically difficult. Fluorescence techniques measure equilibrium binding without the necessity of separating protein-bound and free ligand. Binding has been measured in two ways; first using extrinsic fluorescence of 2-(4-maleimido-anilino)naphthalene-6-sulfonic acid (MIANS), covalently linked to a Cys residue in the Walker A motif of each NB domain (Liu and Sharom, 1996), and second, using the intrinsic Trp fluorescence of the protein (Liu *et al*., 2000). In both cases, binding of

nucleotide or drug to Pgp results in saturable quenching of the fluorescence, which can be fitted to a binding equation for estimation of the K_d value. The affinity of Pgp for ATP, ADP, and other nucleotides proved to be low, in the 0.2–0.4 mM range, while the affinity for drugs varied over a very wide range, from 37 nM for paclitaxel to 158 mM for colchicine (Sharom *et al*., 1999, 2001).

We used these approaches to explore the stoichiometry and affinity of nucleotide binding to Pgp at various stages of the catalytic cycle (Qu *et al*., 2003b). Pgp binds two nucleotide molecules in its resting state, and only one molecule (at the unoccupied active site) in the vanadate-trapped state, as shown by fluorescence enhancement on binding of trinitrophenyl (TNP)-labeled ATP/ADP. Under physiological conditions, the NB domains are thus expected to be fully loaded with nucleotide. The affinity of Pgp for nucleotides remained essentially unchanged in the vanadate-trapped complex, however, loading with drugs resulted in affinity changes of 3- to 4-fold. Resting state Pgp displayed a higher affinity for ATP/ADP when loaded with drug, while the vanadate-trapped state showed decreased affinity. Drug loading thus favors ATP binding at the start of the catalytic cycle, and ADP release following ATP hydrolysis.

Substrate release from Pgp probably takes place by switching of the drug-binding site from a high to a low affinity state. Photoaffinity labeling studies had suggested that the vanadate-trapped state had very low drug-binding affinity (Ramachandra *et al*., 1998). Using purified vanadate-trapped Pgp, we used three different fluorescence spectroscopic approaches to show that this was not the case (Qu *et al*., 2003a). The binding affinity of Pgp for six different drugs was essentially unchanged following formation of the vanadate-trapped complex. A similar observation was made for another ABC protein, the TAP1/TAP2 peptide transporter (Chen *et al*., 2003). We proposed that drug is released from Pgp before formation of the vanadate-trapped complex, which suggests a mechanism involving concerted conformational changes, rather than a multistep mechanism. Most likely, a high-energy intermediate is formed during ATP hydrolysis (Senior *et al*., 1995), and relaxation of this species drives simultaneous movement of drug across the membrane. This process must be complete before formation of the vanadate-trapped state.

CHARACTERIZATION OF THE DRUG-BINDING SITES OF PGP

One mystery surrounding Pgp has been its ability to bind and transport hundreds of structurally dissimilar hydrophobic compounds, ranging from plant and bacterial natural products to linear and cyclic peptides. There is now general agreement that the binding sites are located within the TM regions of Pgp, so that drugs must first partition from the aqueous medium into the membrane to gain access to them. The transporter has been described as a "vacuum cleaner," expelling nonpolar compounds from the bilayer interior (Higgins and Gottesman, 1992). This proposal is also consistent with the observation that the membrane environment has a large modulatory effect on substrate binding to the transporter, so that the apparent affinity of Pgp for a particular drug depends on its lipid–water partition coefficient (Romsicki and Sharom, 1999).

The nature and number of drug-binding sites within Pgp has been the subject of much controversy, with suggestions of multiple drug-binding sites, or a single large flexible binding pocket. Two "functional" drug-binding sites have been identified within Pgp, based on their mutual interactions in the transport process; the H-site, which binds Hoechst 33342, and the R-site, which binds rhodamine 123 (R123) (Shapiro and Ling, 1997). An H-site drug stimulates transport of an R-site drug (and vice versa), whereas two drugs that bind to the same site give rise to mutual inhibition of transport. Fluorescence spectroscopy is an ideal tool to examine these two sites, since many of the drugs that bind to them are fluorescent. Using FRET, we mapped the location of the H-site to the regions of Pgp within the cytoplasmic membrane leaflet (Qu and Sharom, 2002), which is consistent with the idea that the transporter acts as a flippase. Using 2-[4-(4-[dimethylamino]phenyl)-1, 3-butadienyl]-3-ethylbenzo-thiazolium perchlorate (LDS-751), which interacts with the R-site, we recently established the spatial relationship of this binding site to the NB domains using FRET. The LDS acceptor was located within the cytoplasmic region of the membrane, but closer to the surface than the H-site, in the interfacial region of the bilayer (Lugo and Sharom, 2005b). The fluorescence characteristics of the bound drugs indicated that both the H and R binding sites are very hydrophobic in nature, with a polarity lower than that of chloroform (Lugo and Sharom, 2005b). This observation makes it unlikely that the drug-binding sites are open to an aqueous chamber as previously suggested (Rosenberg *et al*., 2001).

We recently carried out a detailed biochemical characterization of the R-site (Lugo and Sharom, 2005a) with respect to its interactions with two drugs, R123 and LDS-751 (Fig. 1), which compete with each other for transport, indicating that they both bind to this site. LDS-751 displays a large fluorescence enhancement following binding to the R-site of Pgp, which allows estimation of the K_d value. It is possible to study the binding

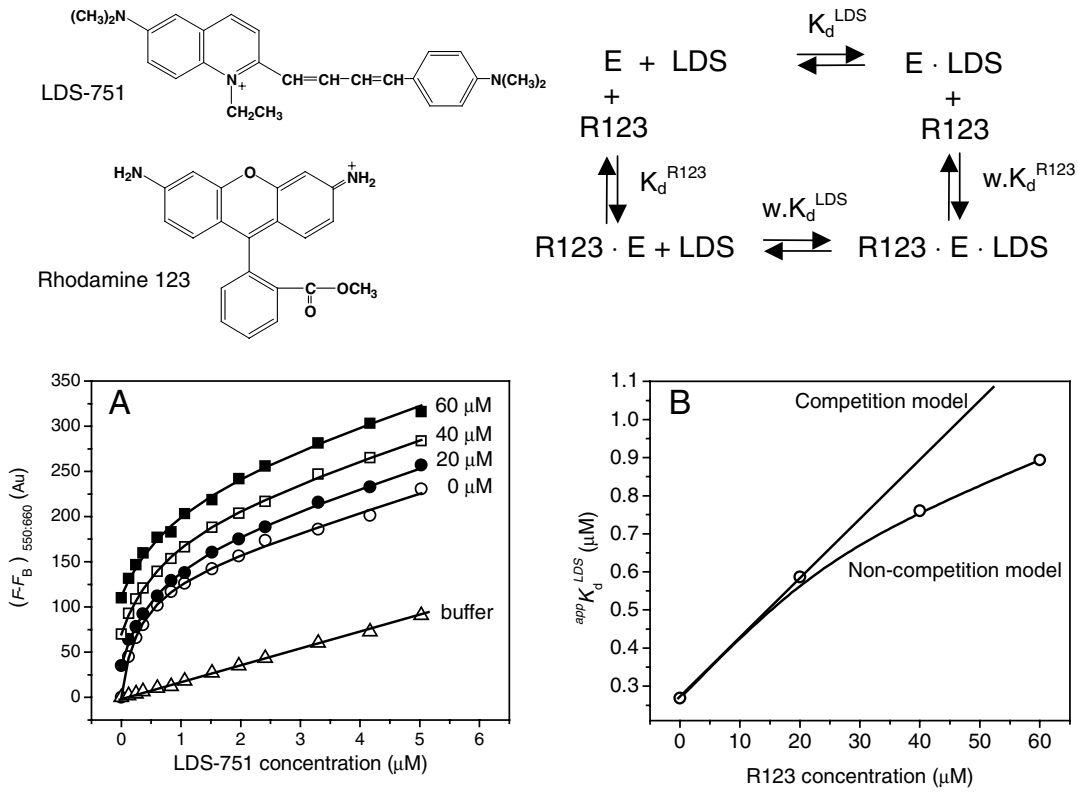

Fig. 1. Interactions of LDS-751 and R123 with purified Pgp. *Panel A*: Corrected fluorescence, $(F - F_B)$, of LDS-751 in detergent-containing buffer (△), and in the same buffer with 100 μg/mL Pgp plus the indicated concentrations of R123 (●). The solid lines represent the best fit to an equation for a single site binding model (Lugo and Sharom, 2005a). *Panel B*: Apparent dissociation constant for LDS-751 binding to Pgp (K_d^{LDS}), as a function of R123 concentration. The points represent the values of the parameters calculated from the data in Panel A. The linear and curved solid lines correspond to the dependence between both variables simulated using a competition model and fitted using a noncompetition model, respectively. The scheme at the top left shows the proposed random-binding reactions of Pgp (E) with LDS-751 and R123. The reaction is characterized by the dissociation constants for the two drugs (K_d^{LDS} and K_d^{R123}) and by the interaction factor (w). E·LDS and R123·E represent the binary complexes between Pgp and the individual drugs, while the ternary complex is depicted as R123·E·LDS (Lugo and Sharom, 2005a).

properties of LDS-751 in the presence of R123, and examine the interactions between them. In the presence of various concentrations of R123, the parameters of LDS-751 fluorescence were altered (Fig. 1A). Fitting to an equation indicated that the dependence of the K_d for LDS-751 on R123 concentration followed a noncompetition, rather than a direct competition model (Fig. 1B). Thus, each drug occupies a separate region within Pgp, forming the ternary complex R123·Pgp·LDS-751, as shown in the scheme in Fig. 1, top right. Using single and double fluorescence titrations with these two drugs, we showed that they bind to Pgp in random order, and have a reciprocal 5-fold negative effect on each other's binding ($w = \sim 5$ in the scheme in Fig. 1). In other words, the presence of R123 reduces the LDS-751 binding affinity by 5-fold, and vice-versa. Thus, in contrast to the mutual exclusion expected for two R-site drugs, both of them are able to bind simultaneously to Pgp. In the case of the multidrug-binding transcription repressor, QacR, X-ray crystallographic studies showed that two drugs can bind to different, but partially overlapping "mini-pockets" inside a large flexible drug-binding site (Schumacher *et al*., 2004). Pgp may contain a similar binding site, where steric interference between the two drugs would account for the observation of a reciprocal negative, noncompetitive binding interaction.

TRANSPORT AND LIPID FLIPPASE FUNCTIONS OF PGP

Transport of drugs by Pgp reconstituted into proteoliposomes was first demonstrated by Sharom *et al*. (1993). Experiments followed the uptake of radiolabeled drug into the vesicle lumen after addition of ATP and a

regenerating system to the exterior using rapid filtration. Transport of several different drugs and a series of hydrophobic peptides was characterized, and results showed that ATP hydrolysis was required to build up a 6- to 8-fold gradient of substrate across the bilayer membrane (Sharom *et al*., 1993, 1996). Competition for drug transport has been a useful tool for identifying Pgp substrates, and quantitating their interaction with the protein (for example, the lipid-based antitumour agent, mitelfosine (Rybczynska *et al*., 2001) and sesquiterpene plant natural products (Munoz-Martinez *et al*., 2004). Although this approach is very useful for characterization of several aspects of the transport process, it is essentially an equilibrium technique, too slow to allow measurement of initial rates.

We later developed a real-time fluorescence-based assay for transport of tetramethylrosamine (TMR) into Pgp proteoliposomes, which had a high enough time resolution for initial rate determination (Lu *et al*., 2001). The dependence of TMR transport on both ATP and TMR concentrations was found to obey Michaelis-Menten kinetics, with K_M values close to the K_d values measured for binding of these substrates to the purified protein. Using this technique, we also directly demonstrated the ATP-driven generation of a substrate concentration gradient, which was dependent on the vesicle internal volume, and its subsequent collapse following addition of V_i or Pgp inhibitors. When the protein was reconstituted into bilayers of lipids with defined acyl chains, the rate of transport was found to reach a maximum at the gel-to-liquid crystalline phase transition temperature (Lu *et al*., 2001). A similar pattern was observed for the lipid–water partition coefficient of organic compounds, suggesting that the rate of Pgp-mediated drug transport might be regulated by the level of drug partitioning into the lipid bilayer.

It was proposed some time ago that Pgp might operate as a drug "flippase," moving hydrophobic molecules from the inner to the outer leaflet of the plasma membrane (Higgins and Gottesman, 1992). If the transporter maintains a difference in drug concentration between the two bilayer leaflets, equilibration with the aqueous phases on each side of the membrane would produce the observed substrate concentration gradient. This idea was supported by the discovery that the MDR3 isoform (which shares >75% sequence similarity with MDR1) functions as a phosphatidylcholine (PC) flippase in liver canalicular cells (Ruetz and Gros, 1994). Several studies indicated that MDR1 could translocate phospholipids from the inner to the outer leaflet in intact cells, but the most convincing evidence was provided by experiments using Pgp reconstituted into proteoliposomes. Using a fluorescence quenching assay that allows direct measurement of the transbilayer distribution of nitrobenzo-2-oxa-1,3-diazole (NBD)-labeled lipid probes (Fig. 2A), Romsicki and Sharom (2001) showed that reconstituted Pgp carried out ATP-driven flipping of a variety of phospholipids. Pgp translocated NBD-labeled PC, phosphatidylethanolamine and phosphatidylserine species with acyl chains that were long, short, saturated or unsaturated. Insertion of NBD-PC into only one bilayer leaflet resulted in translocation of almost 40% of the NBD-lipid in the presence of ATP over a 20 min period, essentially equilibrating the lipid between the two leaflets (Fig. 2B), and providing a dramatic demonstration of the flippase activity of the protein (Eckford and Sharom, 2005).

More recently, we showed that Pgp can also rapidly flip NBD-derivatives of sphingomyelin, and simple glycosphingolipids including glucosylceramide (GlcCer) (Fig. 2C) and galactosylceramide (Eckford and Sharom, 2005). Flipping of lactosylceramide was substantially slower, suggesting that addition of a second polar sugar residue to the headgroup presents a significant barrier to movement across the membrane. Flipping of both phospholipids and glycosphingolipids was vanadate-sensitive and required hydrolysis (as opposed to binding) of ATP. Drugs and modulators inhibited flipping of both phospholipids and glycosphingolipids in a saturable manner, and an excellent correlation was found between the efficiency of inhibition and the K_d for binding of the drug to Pgp (Eckford and Sharom, 2005; Romsicki and Sharom, 2001), which suggests that lipid translocation and drug transport take place through the same path in the protein. Thus, Pgp appears to be a broad specificity outwardly directed flippase for phospholipids and simple glycosphingolipids. Biosynthesis of glycosphinglipids requires translocation of GlcCer from the cytoplasmic to the lumenal surface of the Golgi, where more sugar residues are added. Pgp may operate alongside an energy-independent flippase activity that exists in the Golgi membrane (Buton *et al*., 2002).

FUTURE DIRECTIONS

The use of purified Pgp in reconstituted lipid bilayer systems, together with the application of fluorescence spectroscopic approaches, has opened up new avenues for exploration of the architecture and function of the Pgp molecule. FRET mapping studies with more fluorescent drugs may reveal the nature of the different subsites within the drug-binding region of the protein, and how they are interrelated, both structurally and functionally. Further understanding of how ATP hydrolysis

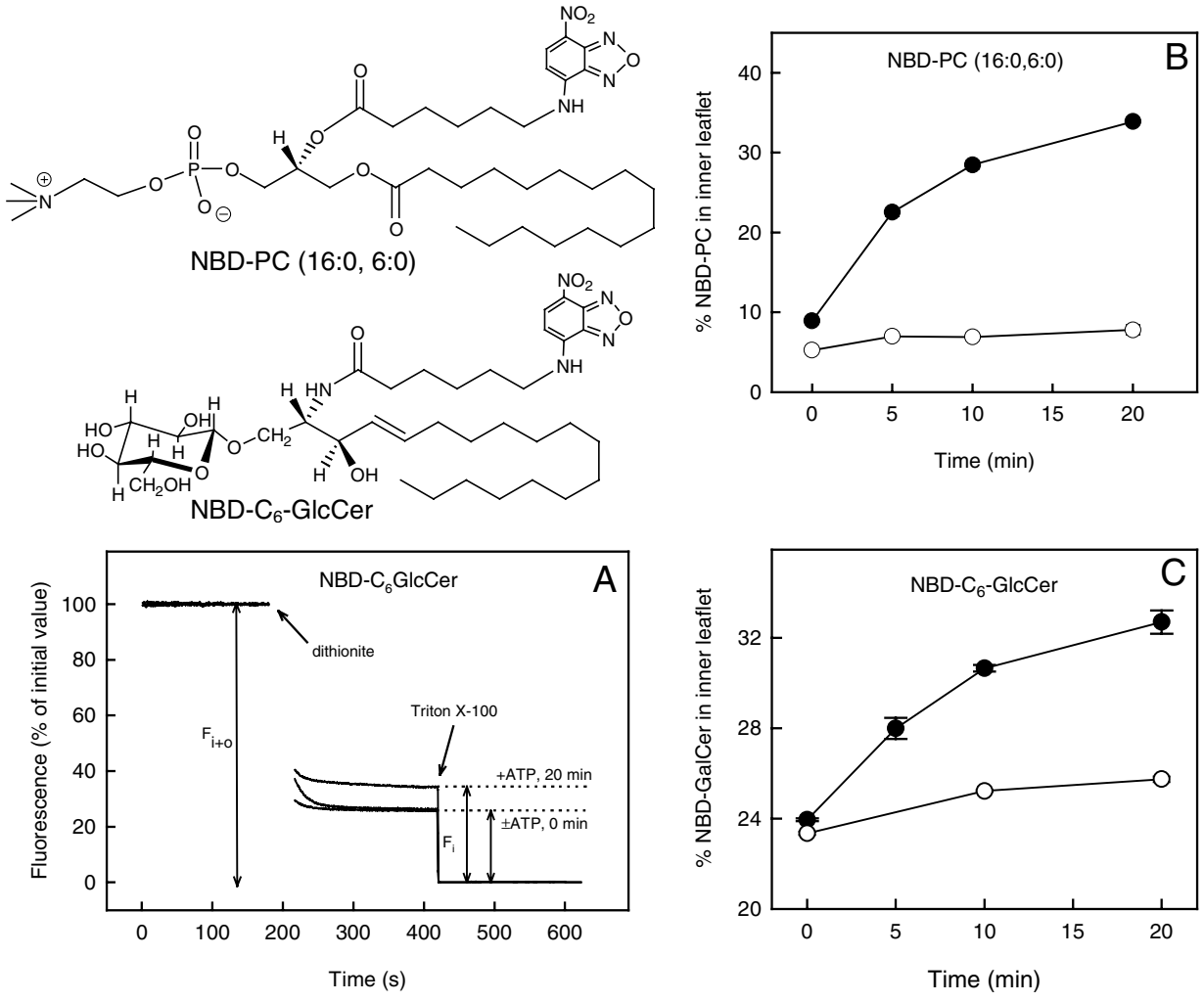

Fig. 2. Phospholipid and glycosphingolipid flippase activity of reconstituted Pgp. Two fluorescent NBD-labeled lipid species that are translocated from one side of the bilayer to the other are shown. *Panel A*: Fluorescence assay for determining the bilayer distribution of an NBD-labeled membrane lipid (in this case, NBD-C_6-GlcCer) incorporated at a concentration of 0.3% into egg PC proteoliposomes containing reconstituted Pgp. Experiments were conducted in the absence or presence of 1 mM ATP and a regenerating system to energize Pgp. After establishing a stable baseline, membrane-impermeant dithionite was added to quench NBD-GlcCer fluorescence in the outer membrane leaflet only. Subsequent addition of the detergent Triton X-100 permeabilized the bilayers, and resulted in quenching of NBD-GlcCer in the inner leaflet. Vertical arrows represent the total fluorescence of NBD-GlcCer in both the inner and outer leaflets (F_{i+o}), and the fluorescence of NBD-GlcCer in the inner leaflet in the absence and presence of ATP (F_i). *Panel B*: Time course of translocation at 37°C of NBD-PC (16:0, 6:0) inserted asymmetrically into the outer leaflet of egg PC proteoliposomes containing Pgp, with (●) or without (○) 1 mM ATP and the regenerating system. *Panel C*: Time course of translocation at 37°C of 0.3% NBD-GlcCer in egg PC proteoliposomes containing reconstituted Pgp. Proteoliposomes were incubated either with (●) or without (○) 1 mM ATP and the regenerating system (see Eckford and Sharom, 2005).

powers transport may be obtained by studying stable complexes of Pgp with other agents trapped in the NB domain, which may resemble different points along the catalytic cycle. Finally, understanding the mechanism by which Pgp flips membrane phospholipids and glycosphingolipids may provide clues as to whether drugs are also moved between leaflets of the bilayer, and how drug transport takes place.

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