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Drug Binding Sites on P-Glycoprotein Are Altered by ATP Binding Prior to Nucleotide Hydrolysis†

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ABSTRACT: P-glycoprotein (P-gp) confers multiple drug resistance on cancer cells by acting as a plasma membrane localized ATP-dependent drug efflux pump. Currently, there is little information on the nature of the communication between the energy-providing nucleotide binding domains (NBDs) and the drug binding sites of P-gp to generate transport of substrate. Many substrates and modulators cause alterations in ATP hydrolysis, but what effect do the various stages of the catalytic cycle have on drug interaction with P-gp? Vanadate trapping of Mg·ADP caused a reversible decrease in the binding capacity of the transported substrate [^{3}H]-vinblastine and the nontransported modulator [^{3}H]XR9576 to P-gp in CH^{r}B30 cell membranes. The non-hydrolyzable nucleotide analogue ATP-γ-S also caused a reduction in the binding capacity of [^{3}H]-vinblastine but not for the modulator [^{3}H]XR9576. This indicates that signaling to the NBDs following binding of a nontransported modulator is different to that transmitted upon interaction of a transported substrate. Second, it appears that the binding of nucleotide, rather than its hydrolysis, causes the initial conformational shift in the drug-binding site during a transport cycle.

Altered cellular accumulation of anti-cancer agents is a major factor in acquired and intrinsic resistance of tumor cells to chemotherapy (*1*−*4*). P-glycoprotein (P-gp),[1] a member of the ABC family of transport proteins, is able to confer resistance by acting as an ATP-dependent drug efflux pump in the plasma membranes of tumor cells (*5*, *6*). P-gp is distinguished from many of the ABC proteins by the large number of compounds with which it can interact, and several recent manuscripts have demonstrated that this broad substrate specificity is brought about by the presence of more than one drug-binding site (*7*−*12*). The binding sites contain defined drug specificities and are not functionally equivalent; in addition, some binding sites interact with transport substrates as well as nontransported modulators. However, the precise location of these sites is unknown, but it is clear from previous studies that an extensive network of negative allosteric communication occurs between the drug binding sites on P-gp.

P-gp uses energy from ATP hydrolysis to drive the transport of cytotoxic compounds against a concentration gradient. Consequently, one would expect that in order to instigate a transport cycle, drug binding to the protein must elicit an effect on the ATP catalytic pathway. This appears to be the case for P-gp since the basal rate of ATP hydrolysis is stimulated by the binding of several compounds such as verapamil, vinblastine, and rhodamine 123, some of which are known substrates of the protein (*11*, *13*, *14*). Further direct evidence of communication between the drug binding sites and the catalytic domains is that drug binding causes an allosteric alteration in the fluorescence spectrum of MIANS, a probe used to label the cysteine residue in the Walker A motif of the nucleotide binding domain (*15*, *16*).

The respective roles of two functional nucleotide-binding domains (NBDs) to drive drug transport by P-gp have not yet been elucidated. Are the two domains functionally distinct and is there any functionally important interaction between them? The N- and C-terminal NBDs of P-gp have been shown to have similar rates of ATP hydrolysis, and they label azido-[^{32}P]-ATP to the same extent, suggesting functional equivalence (*17*−*20*). This is in contrast to the opposing roles of the two NBDs in CFTR where ATP hydrolysis at one NBD elicits channel opening, whereas hydrolysis at the second NBD is responsible for channel closing (*21*). Mutagenesis and covalent labeling approaches demonstrate that both ATP hydrolysis and transport of drugs by P-gp require the presence of two fully functional NBDs (*22*−*25*). This evidence indicates a strong degree of cooperativity between the two NBDs, and a model proposing an alternating cycle of ATP hydrolysis has been presented. Chimeric proteins

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[1] Abbreviations: P-gp, P-glycoprotein; MDR, multidrug resistance; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP-γ-S, adenosine 5′-0-(3-thiotriphosphate); AMP−PNP, 5′-adenylimidodiphosphate; ABC, ATP binding cassette; NEM, *N*-ethylmaleimide; MIANS, 2-(4-maleimidoanilino)-naphthalene-6-sulfonic acid; NBD-7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; NBD, nucleotide binding domain; CFTR, cystic fibrosis transmembrane conductance regulator.

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with replacement of NBD1 by NBD2 displayed one important difference, namely, a markedly different pharmacological profile for stimulation of ATP hydrolysis (*26*). This indicates that P-gp has distinct routes of communication between the NBDs and the drug binding sites (*27*).

Conventional thinking suggests that communication between functional domains in a protein occurs through conformational alterations (*28*). Antibody epitope recognition changes, proteolytic accessibility alterations, ^{2}H/H-exchange kinetics and tryptophan fluorescence alterations have all been used to demonstrate that conformational changes occur in P-gp (*29*−*32*). In addition, these conformational changes appear functionally relevant since they are affected by stimuli such as nucleotide, substrate, and inhibitor compounds. Unfortunately, none of these investigations were able to localize regions of the proteins involved in the conformational changes nor did they describe the nature of these changes.

Energy obtained from the hydrolysis of ATP in the NBDs is used to drive the conformational changes required to mediate translocation of substrate. Such conformational changes may manifest as reorientation of the substrate-binding site to opposites sides of the membrane. Therefore, the NBDs must affect the nature of substrate binding to the protein during various stages of the catalytic cycle. In the present manuscript, we have investigated the communication between drug binding and the ATP catalytic cycle of P-gp using a radioligand binding approach. Equilibrium binding techniques, unlike photoaffinity labeling, allow precise quantitation of effects directly on the initial stage of drug−protein interaction. Vanadate trapping of ADP and the nonhydrolyzable ATP analogue ATP-γ-S were both able to reduce the binding capacity of the P-gp substrate [^{3}H]-vinblastine. In contrast, vanadate trapping, but not ATP-γ-S, was able to reduce the binding of [^{3}H]XR9576. The results provide evidence of a transport scheme for P-gp in which nucleotide binding, prior to hydrolysis, initiates conformational changes.

MATERIALS AND METHODS

Materials. Disodium adenosine triphosphate (Na_2ATP), sodium orthovanadate, *N*-ethyl-maleimide (NEM) and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD·Cl) were purchased from Sigma (Poole, U.K.). Maleimidylanilino-naphthalene-6-sulfonate (MIANS) was obtained from Molecular Probes. [^{3}H]-Vinblastine sulfate (12.7 Ci/mmol) and Sephadex G-50 (fine) resin were purchased from Amersham Pharmacia Biotech (Amersham, U.K.). [^{3}H]-XR9576, XR9576, XR9051, and GF120918 were synthesized and provided by Xenova Ltd.; (Slough,U.K.). Disposable spin columns (1 mL) were obtained from Bio-Rad Ltd. (Hemel Hempstead U.K.).

Cell Lines and Plasma Membrane Preparation. The P-gp-expressing drug-resistant Chinese hamster ovary cell line, CH^RB30, was cultured as previously described (*33*). Briefly, cells were grown at 37 °C in α-minimal essential medium supplemented with 30 μg mL^{-1} colchicine. Plasma membrane vesicles were isolated from CH^RB30 cells as described elsewhere (*34*), using nitrogen cavitation and sucrose density centrifugation. Membrane preparations were stored at −80 °C for up to 6 months at a protein concentration of 10−15 mg mL^{-1}.

Measurement of P-gp ATPase Activity. The basal and drug-stimulated ATPase activity of P-gp was measured using a colorimetric assay to follow release of inorganic phosphate, based on a modification of the assay first described by Chifflet (*35*). Membrane vesicles (1 μg) were incubated in ATPase buffer (150 mM NH_4Cl, 50 mM Tris pH 7.4, 5 mM $MgSO_4$, 0.02% NaN_3) in the presence of 0−4 mM Na_2ATP for a 20 min period at 37 °C. Drug effects on basal activity were measured in the presence of 2 mM Na_2ATP by adding drugs to the assay from concentrated stocks in DMSO or ethanol (final concentration of solvent not exceeding 1% vv^{-1}).

ATPase activity (nmol/min/mg membrane protein) was plotted as a function of drug concentration and fitted, using nonlinear regression, by the general dose−response equation below (*36*):

$$Y = \frac{(Y_{max} - Y_{min})}{(1 + ([B]/EC_{50})^n)} + Y_{min} \quad (1)$$

where Y_{max} is the maximal activity, Y_{min} is the minimum activity, [B] is drug concentration (M), EC_{50} is the concentration of drug which causes a 50% change in response, and n is the slope factor.

Vanadate-Induced Inhibition of P-gp. Sodium orthovanadate stock solutions (100 mM) were adjusted to pH 10 and boiled for 2 min to get rid of any polymeric species (*37*). Plasma membranes (1 mg) were incubated with 300 μM vanadate, in a total volume of 1 mL ATPase buffer at 37 °C for 30 min. The reaction was stopped by either placing the sample on ice or centrifuging 100 μL aliquots through spin-columns containing 1 mL of Sephadex G-50 (fine) preequilibrated with 50 mM Tris at pH 7.4 at 4 °C.

Equilibrium Radioligand Binding to CH^RB30 Membranes. Saturation binding isotherms were obtained by incubating CH^RB30 membranes (8 μg) in 100 μL of binding buffer (50 mM Tris, pH 7.4) at 22 °C with increasing concentrations of [^{3}H]-vinblastine (1−100 nM) for 2 h to reach equilibrium. In the case of [^{3}H]XR9576 (0.5−50 nM), 2 μg of membranes were incubated over a 3-hour period. Nonspecific binding was measured in the presence of 100-fold excess of XR9051 or GF120918 for [^{3}H]-vinblastine and [^{3}H]XR9576 binding, respectively, and was later subtracted from all values. Bound radioligand was separated from free ligand by rapid filtration under vacuum through a GF/F glass fiber filter followed by washing with 2 × 3 mL ice-cold wash buffer (20 mM Tris, pH 7.4, 20 mM $MgSO_4$). Filter-entrapped radioactivity was quantitated using liquid scintillation counting. The specific radioactivity bound (pmol/mg membrane protein) was plotted as a function of the free radioligand concentration using eq 2 below from which the maximal binding capacity (B_{max}) and the dissociation constant K_d, a measure of the radioligand affinity, were determined:

$$B_d = \frac{B_{max}[L]}{(K_d + [L])} \quad (2)$$

where K_d is the binding affinity (nM), B_{max} is the maximal binding capacity (pmol mg^{-1}), [L] is the radioligand concentration (nM), B_d is the amount bound (pmol mg^{-1}).

Table 1. Potencies of Various Nucleotide-Binding Domain Inhibitors to Affect ATPase Activity of P-Glycoprotein[a]

compound	inhibition of ATPase activity IC_{50} (μM)	inhibition of binding	
		[^{3}H]-vinblastine	[^{3}H]XR9576
NBD-Cl	17.8 ± 3.6	no	no
N-ethyl-maleimide	3.23 ± 0.59	no	no
MIANS	1.95 ± 0.28	no	no
ATP-γ-S	31 ± 99	yes	no
$NaVO_3$	0.74 ± 0.13	yes	yes

[a] The potencies (IC_{50}) of covalent inhibitors and nucleotide analogue on the ATP hydrolytic activity of P-glycoprotein were determined using 2 mM ATP at 37 °C with 1 μg of membrane protein. Values represent the mean ± SEM obtained from at least three independent determinations. The effects on binding of substrate [^{3}H]-vinblastine or modulator [^{3}H]XR9576 were determined as described in Materials and Methods.

Displacement of Equilibrium Drug Binding. CH^RB30 membranes (8 μg) were bound to equilibrium with 15−20 nM [^{3}H]-vinblastine in the presence of increasing concentrations of nucleotide added from 10× stock in binding buffer, or inhibitor added from 200× stock made up in either ethanol or DMSO, in a total assay volume of 200 μL for 2 h at 22 °C. Bound radioligand was separated from free by rapid filtration as described above. The specific binding remaining was plotted as a function of nucleotide or drug concentration using eq 2. From this relationship, the degree of displacement of bound radioligand and the IC_{50} of displacing agent can be determined.

RESULTS

Inhibition of P-gp-Mediated ATPase Activity by Nucleotide. P-gp in CH^rB30 membranes displays a high basal ATPase activity (151 ± 17 nmol min^{-1} mg^{-1}), which has previously been extensively characterized (*11*). This basal ATPase activity of P-gp may be altered either directly through interaction with the NBDs or indirectly via the transmembrane domains following drug binding. The efficacy and potency of the non-hydrolyzable nucleotide ATP-γ-S to inhibit P-gp-mediated ATPase activity was examined (Table 1). The ATPase activity of P-gp was completely inhibited by ATP-γ-S with a potency of IC_{50} = 310 ± 99 μM. Another direct method of inhibiting NBD activity is achieved through stable trapping of the Mg·ADP·VO_3 species during the catalytic cycle. ATP hydrolysis of P-gp was inhibited by vanadate with a potency of IC_{50} = 0.74 ± 0.13 μM.

However, ATP-γ-S may be hydrolyzed by some proteins at a rate severalfold lower than ATP. P-gp did not mediate any hydrolysis of either ATP-γ-S or AMP−PNP at 37 °C (data not shown) using an enzyme-linked assay to measure nucleotide hydrolysis (*22*). In addition, hydrolysis of ATP was reduced from a V_{max} of 151 ± 17 nmol min^{-1} mg^{-1} at 37 °C to 29.1 ± 2.1 nmol min^{-1} mg^{-1} (n = 3) at 20 °C. Since the hydrolysis of ATP-γ-S is severalfold slower than ATP, the hydrolysis of the former would be negligible at 20 °C, the temperature used for subsequent drug binding assays.

Inhibition of P-gp-Mediated ATPase Activity and Substrate Binding by Covalent Inhibitors. MIANS, NEM, and NBD-Cl are reactive with the cysteine residues located in each Walker A motif of P-gp (*25*, *38*). The result of this interaction is a complete inhibition of ATP hydrolysis by P-gp, through either the inhibition of ATP binding at the NBD or the destabilization of the ATP molecule at the active site. MIANS (IC_{50} = 1.95 ± 0.28 μM) and NEM (IC_{50} = 3.23 ± 0.59 μM) displayed similar potencies to inhibit P-gp ATPase activity (Table 1). The derivatizing agent NBD-Cl displayed a lower potency for this inhibition with an IC_{50} = 17.8 ± 3.6 μM but was also able to completely abolish ATP hydrolysis.

Table 2. Effects of Vanadate Treatment on the ATP Hydrolytic Activity of P-Glycoprotein[a]

	V_{max} (nmol min^{-1} mg^{-1})	K_m (μM)
control	151 ± 17	0.62 ± 0.10
trapped	29 ± 4	0.15 ± 0.03
recovered	128 ± 15	0.37 ± 0.06

[a] Plasma membranes from CH^rB30 cells were trapped with 300 μM vanadate in the presence of 2 mM ATP. Recovery from vanadate trapping was done at 37 °C as described in Materials and Methods. ATPase activity was measured at 37 °C for 25 min with 1 μg of membrane protein and 0.1−3 mM ATP. Activity parameters were determined from nonlinear regression of the Michaelis−Menten equation. All values represent the mean ± SEM of five independent experiments.

[^{3}H]-Vinblastine binding to P-gp in CH^rB30 membranes displayed high affinity (K_d = 16.1 ± 2.2 nM, n = 5), with a capacity of 59 ± 8 pmol mg^{-1} membrane protein. The covalent inhibitors MIANS, NEM, and NBD-Cl were also examined for their ability to alter the equilibrium binding of [^{3}H]-vinblastine to P-gp (Table 1). In the concentration range 10^{-8} to 3 × 10^{-4} M, none of these covalent inhibitors were able to displace the binding of either 30 nM [^{3}H]-vinblastine or 3 nM [^{3}H]XR9576 binding to P-gp despite their ability to abrogate ATP hydrolysis. This is in contrast to the reported ability of substrates (e.g., vinblastine) to alter the fluorescence of MIANS and NBD-Cl-labeled P-gp, through conformational interactions (*15*). The lack of effect observed here may be due to the poorer efficacy of these agents to allosterically affect binding events at the transmembrane domain.

The Ability of Vanadate To Modulate Basal ATPase Activity of, and Drug Binding to, P-gp. Vanadate at a concentration of 300 μM was able to reduce the maximal basal velocity of ATP hydrolysis by P-gp (Table 2) from 151 ± 17 (K_m = 0.6 ± 0.1 μM) to 29 nmol min^{-1} mg^{-1} (K_m = 0.15 ± 0.03). This inhibition of ATPase activity was stable and long-lived, which is in agreement with a previous report (*22*). Removal of unbound nucleotide and vanadate followed by incubation at 37 °C for 1 h was sufficient to reactivate P-gp in CH^rB30 cell membranes. This removal of vanadate resulted in almost complete recovery of the P-gp ATPase activity with a maximal velocity of 128 ± 15 nmol min^{-1} mg^{-1} (K_m = 0.4 ± 0.1 μM). There was no significant acceleration of the recovery with inclusion of either ATP (0.25−2 mM) or drugs that stimulate ATP hydrolysis, such as verapamil (data not shown).

Vanadate inhibition provides a valuable tool to investigate drug-binding events with P-gp at a late stage of the catalytic cycle. Vanadate trapping of P-gp had significant effects on the equilibrium binding of both the substrate [^{3}H]-vinblastine and the nontransported modulator [^{3}H]XR9576 (Table 3). The vanadate-trapped P-gp had a significantly reduced (P < 0.01) binding capacity for [^{3}H]-vinblastine (15 ± 2 pmol

Table 3. Effects of Vanadate Trapping on the Equilibrium Binding Properties of [^{3}H]-Vinblastine and [^{3}H]-XR9576[a]

	[^{3}H]-vinblastine		[^{3}H]-XR9576	
	B_{max} (pmol mg^{-1})	K_d (nM)	B_{max} (pmol mg^{-1})	K_d (nM)
membranes	58.7 ± 8.3	16.1 ± 2.2	211 ± 37	4.6 ± 0.5
trapped	15.2 ± 2.1	23.6 ± 5.4	63.9 ± 6.8	1.5 ± 0.1
recovered	71.5 ± 16.1	23.0 ± 4.7	179 ± 35	7.2 ± 2.1

[a] Plasma membranes from CH^{r}B30 cells were treated with 300 μM vanadate and allowed to recover from this inhibition as described in Materials and Methods. The equilibrium binding properties of [^{3}H]-vinblastine (1−100 nM) and [^{3}H]XR9576 (0.1−40 nM) were determined by a rapid filtration method following a 2−3 h incubation at 22 °C. Binding capacity (B_{max}) and affinity (K_d) of the hyperbolic relationships were obtained from nonlinear regression. Data represent mean ± SEM from five independent experiments.

mg^{-1}) when compared with its nontrapped state (58 ± 8 pmol mg^{-1}). There was no effect on the affinity of the interaction, and the removal of vanadate resulted in recovery of the binding capacity to 71 ± 16 pmol mg^{-1}. The complete recovery of binding indicates that a reversible molecular change, rather than a detrimental effect on the structure of the [^{3}H]-vinblastine binding site on P-gp, occurred during vanadate trapping. Similarly with [^{3}H]XR9576, vanadate trapping of P-gp caused a reduction in the binding capacity from 211 ± 37 to 64 ± 7 pmol mg^{-1} with no effect on the affinity of binding for the modulator. Reversal of the decreased binding capacity to [^{3}H]XR9576 was also observed following removal of vanadate from P-gp. These results indicate that vanadate trapping causes a conformational effect on P-gp that alters the number of drug binding sites with the high-affinity conformation. Such a change manifests as an apparent decrease in the binding capacity to the radioligand.

Nucleotide-Induced Alteration of Drug Binding to P-gp. The vanadate trapping data shown above indicate an alteration in drug binding sites at a late stage of the catalytic cycle. Do earlier stages of this cycle modify the drug binding sites of P-gp? This question was examined by comparing the influence of the nucleotides ATP-γ-S, AMP−PNP, ADP, and AMP on radioligand binding. The effects of ATP-γ-S, AMP−PNP, ADP, and AMP (10^{-5}−10^{-2} M) on the equilibrium binding of 15−20 nM [^{3}H]-vinblastine to P-gp in CH^{r}B30 membranes is shown in Figure 1. ATP-γ-S caused a dose-dependent decrease in the binding of [^{3}H]-vinblastine to P-gp to 40% of the amount bound in the absence of nucleotide, with a potency of IC_{50} = 0.14 ± 0.05 mM. AMP−PNP caused a similar decrease in the binding of [^{3}H]-vinblastine displaying a potency of IC_{50} = 0.37 ± 0.09 mM. The binding of [^{3}H]-vinblastine was only reduced by 30% with ADP at a potency of 0.16 ± 0.06 mM, and the effects of AMP were negligible at the concentrations used. In contrast to the results obtained with [^{3}H]-vinblastine, none of the nucleotides tested were able to affect the binding of [^{3}H]XR9576 to P-gp at the concentrations used (data not shown). This difference in effect may be due to the different affinities of the two radioligands at their respective binding sites or the fact that vinblastine is a transport substrate of P-gp, whereas XR9576 acts purely as a nontransported modulator.

The drug displacement assays described above provide information concerning the effect of nucleotides on drug

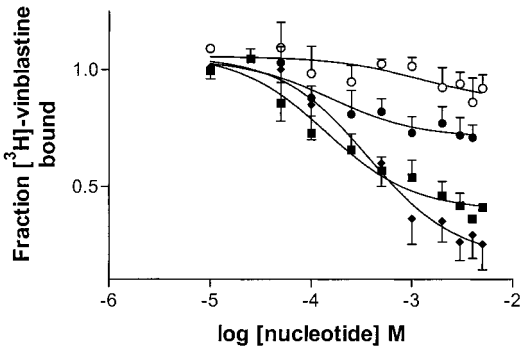

FIGURE 1: Nucleotide displacement of [^{3}H]-vinblastine binding. Displacement of the equilibrium binding of [^{3}H]-vinblastine was performed by the nucleotides AMP (○) and ADP (●) and the non-hydrolyzable ATP analogues ATP-γ-S (■) and AMP−PNP (◆). CH^{r}B30 membranes (8 μg) were incubated with [^{3}H]-vinblastine (20 nM) in the presence of nucleotides (0.01−5 mM). The amount bound was expressed as a fraction of the total specific binding in the absence of nucleotide and the general dose−response curve was fitted by nonlinear regression. The degree of displacement and the relative potencies were determined from the curve fitting. Data points represent the mean ± SEM (standard error of the mean) obtained from at least 3 to 5 independent observations.

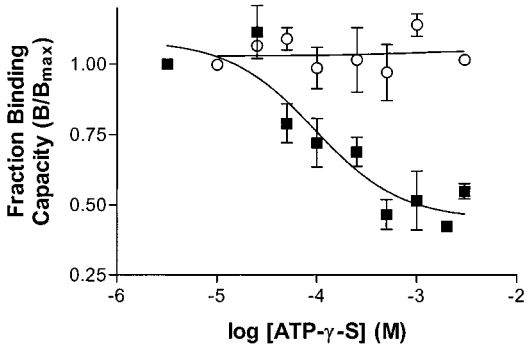

FIGURE 2: Effect of ATP-γ-S on the binding capacity of [^{3}H]-vinblastine and [^{3}H]XR9576. Complete saturation isotherms were performed for [^{3}H]-vinblastine (1−100 nM) and [^{3}H]XR9576 (0.2−40 nM) in the presence and absence of various concentrations of ATP-γ-S. Binding capacity was determined for each saturation isotherm from nonlinear regression and plotted against the ATP-γ-S concentration. The extent and potency of the effects of ATP-γ-S on [^{3}H]-vinblastine (■) and [^{3}H]XR9576 (○) were determined from nonlinear regression of the general dose−response equation. Data represent the mean ± SEM obtained from 3 to 5 independent observations.

binding, but only at the particular concentration of radioligand used. However, they do not yield information about the nature of interaction occurring; therefore, the effects of ATP-γ-S on the complete saturation binding curves of [^{3}H]-vinblastine and [^{3}H]XR9576 was examined to gauge whether the displacement results outlined above were due to changes in binding site affinity or capacity. There was no significant effect of ATP-γ-S on [^{3}H]XR9576 binding at any of the concentrations used (Figure 2). In contrast, the binding of ATP-γ-S to P-gp did have profound effects on the binding capacity (B_{max}) of [^{3}H]-vinblastine which was reduced dose-dependently to 50% of the value in the absence of nucleotide. The potency of ATP-γ-S to halve the number of [^{3}H]-vinblastine binding sites on P-gp was 923 ± 40 μM (n = 3). The decrease in binding capacity for [^{3}H]-vinblastine on P-gp reflects a conformational shift in the substrate binding site away from the high-affinity configuration.

These results clearly demonstrate that it is the binding of nucleotide, rather than its hydrolysis, which brings about the initial conformational change in the vinblastine-binding site during transport.

DISCUSSION

Primary active transporters such as P-gp are able to translocate substrates by transmitting energy from the hydrolysis of nucleotide to the drug binding domain(s). This transmission of energy is most likely in the form of a conformational change leading to reorientation of the drug binding site or its exposure to the opposite side of the membrane (*28*). It is well established that signals can be transmitted between the transmembrane and NBD domains, since many substrates of P-gp cause an increase in ATP hydrolysis following their binding to the protein (*11, 13, 14*). However, little is known about communication in the opposite direction; that is, how do the NBDs alter or change the drug-binding site to initiate transport? In the present manuscript, we provide the first evidence that nucleotide binding, prior to any hydrolysis, is able to cause a dramatic change in the properties of the transported substrate-binding site.

The vanadate-trapped intermediate form of P-gp is analogous to the P-gp·Mg·ADP·P_i complex and due to its stability, allows investigation of P-gp function immediately after ATP hydrolysis (*22, 39*). This vanadate-trapped intermediate of P-gp has previously been shown to display impaired photolabeling by iodo-aryl-azido-prazosin (*8*). By using quantitative radioligand binding techniques, we have demonstrated that the impaired binding of vinblastine and XR9576 is brought about by an apparent decrease in the number of binding sites. Binding sites on many transport and receptor proteins are known to exist in equilibrium between two conformations, a low- and a high-affinity form (*40, 41*). The reduced substrate and nontransported modulator binding observed in vanadate-trapped P-gp may be due simply to an occlusion of the high-affinity binding site, or possibly a reorientation of this site to a low-affinity form following nucleotide occupation of an NBD. Both of these switches will manifest as apparent reductions in the number of drug binding sites.

Dissociation of P_i from P-gp following ATP hydrolysis in the catalytic cycle is proposed to provide the energy required to mediate active transport (*23*). Therefore, the altered binding characteristics of the vanadate-trapped P-gp intermediate indicate that a major change in the binding site occurs prior to free-energy release from the protein. Further evidence in favor of a binding site alteration prior to harnessing energy from hydrolysis is that binding of the nonhydrolyzable ATP analogue (ATP-γ-S) causes a decrease in the binding capacity of P-gp for the transported substrate vinblastine. Since nucleotides bind to the NBDs (*18, 39*) and transported substrates bind to the membrane spanning domains (*8, 42*−*44*), this alteration in binding capacity is brought about by a noncompetitive or allosteric alteration of the binding site. Binding to the NBDs by nucleotides such as ADP and AMP, or covalent attachment of NBD-Cl, NEM, or MIANS, was unable to elicit significant changes in drug binding. Therefore, a specific conformational alteration due to triphosphate nucleotide binding, rather than simple chemical occupation of an NBD, is required to modify the binding site occupied by transported substrate. Allosteric interactions between drugs on P-gp have been demonstrated to result in abrogation of binding capacity (*11, 45*). In contrast, the allosteric effect of ATP-γ-S only caused a reduction in the binding capacity of P-gp for vinblastine to 50% of the original value, and the partiality of this effect may be related to the requirement for two interacting NBDs. It has been demonstrated that the NBDs of P-gp operate in an alternating cycle (*22, 24*), and this cooperativity may also extend to modulation of drug binding sites during a complete transport cycle.

It is clear that in order to initiate transport of substrate by P-gp, the communication between the drug binding sites and the NBDs is very specific. There is a precise requirement for the triphosphate nucleotide to alter drug binding, but this allosteric alteration is only targeted to a particular type of drug, i.e., those that are transported. This requirement is evidenced by the lack of effect of ATP-γ-S on binding of the nontransported modulator XR9576. XR9576 is a high-affinity modulator of P-gp that is not transported; however, it does bind at the same site as Hoechst 33342, which is a transport substrate of P-gp (*46*). In addition, ATP hydrolysis is completely and potently inhibited by XR9576 (*12*), indicating some communication between this drug and the nucleotide binding sites. Does this difference in effect of ATP-γ-S on substrate versus modulator binding reveal any mechanistic details on P-gp? The simplest explanation is that the NBDs do not transmit to the XR9576 binding site; however, this is unlikely since *prior* trapping of the protein with vanadate did reduce binding capacity for the modulator. Modulators such as XR9576 may prevent binding of nucleotide, or alternatively, occupancy of a binding site by XR9576 renders it less sensitive to the allosteric actions of nucleotide. The latter proposal is supported by the significantly slower dissociation rate for the XR9576−P-gp complex compared with that of the vinblastine−P-gp complex described previously (*12*).

Drug transport across a membrane requires reorientation or exposure of substrate binding sites to alternate sides of a bilayer. Concomitant with this "movement" of binding site is a switch in binding site characteristics from high to negligible or low affinity, thus enabling dissociation of transported substrate. The results of this investigation show that the stimulus for altering conformation of the substrate-binding site is the binding of nucleotide prior to its hydrolysis. Nucleotide binding-mediated initiation of function is also characteristic of the F_1F_0−ATPase (*47*) and in the movement of actin filaments in muscle (*48*). Molecular details on the location of regions involved in communication between binding sites and NBDs on P-gp are required to completely unravel these complex interactions.

REFERENCES

1. Dalton, W. S., Grogan, T. M., Meltzer, P. S., Scheper, R. J., Durie, B. G., Taylor, C. W., Miller, T. P., and Salmon, S. E. (1989) *J. Clin. Oncol. 7*, 415−424.
2. Choi, K., Chen, C.-J., Kriegler, M., and Roninson, I. B. (1988) *Cell 53*, 519−529.
3. Ferry, D. R. (1998) *Int. J. Clin. Pharmacol. Ther. 36*, 29−40.
4. Woodhouse, J. R., and Ferry, D. R. (1995) *Ann. Med. 27*, 157−167.

5. Van Veen, H. W., and Konings, W. N. (1997) *Seminars Cancer Biol. 8*, 183−191.
6. Ambudkar, S. V., Dey, S., Hrycyna, C. A., Ramachandra, M., Pastan, I., and Gottesman, M. M. (1999) *Annu. Rev. Pharmacol. Toxicol. 39*, 361−398.
7. Ayesh, S., Shao, Y.-M., and Stein, W. D. (1996) *Biochim. Biophys. Acta 1316*, 8−18.
8. Dey, S., Ramachandra, M., Pastan, I., Gottesman, M. M., and Ambudkar, S. V. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 10594−10599.
9. Greenberger, L. M., Lisanti, C. J., Silva, J. T., and Horwitz, S. B. (1991) *J. Biol. Chem. 266*, 20744−20751.
10. Ferry, D. R., Russell, M. A., and Cullen, M. H. (1992) *Biochem. Biophys. Res. Commun. 188*, 440−445.
11. Martin, C., Berridge, G., Higgins, C. F., and Callaghan, R. (1997) *Br. J. Pharmacol. 122*, 765−771.
12. Martin, C., Berridge, G., Mistry, P., Higgins, C. F., Charlton, P., and Callaghan, R. (1999) *Br. J. Pharmacol. 128*, 403−411.
13. Pascaud, C., Garrigos, M., and Orlowski, S. (1998) *Biochem. J. 333*, 351−358.
14. Orlowski, S., Mir, L. M., Belehradek, J., and Garrigos, M. (1996) *Biochem. J. 317*, 515−522.
15. Liu, R., and Sharom, F. J. (1996) *Biochemistry 35*, 11865−11873.
16. Romsicki, Y., and Sharom, F. J. (1999) *Biochemistry 38*, 6887−6896.
17. Loo, T. W., and Clarke, D. M. (1994) *J. Biol. Chem. 267*, 7750−7755.
18. Al-Shawi, M. K., and Senior, A. E. (1993) *J. Biol. Chem. 268*, 4197−4206.
19. Senior, A. E., Al-Shawi, M. K., and Urbatsch, I. L. (1995) *FEBS Lett. 377*, 285−289.
20. Urbatsch, I. L., Al-Shawi, M. K., and Senior, A. E. (1994) *Biochemistry 33*, 7069−7076.
21. Gadsby, D. C., and Nairn, A. C. (1994) *Trends Biochem. Sci. 19*, 513−518.
22. Urbatsch, I. L., Sankaran, B., Weber, J., and Senior, A. E. (1995) *J. Biol. Chem. 270*, 19383−19390.
23. Urbatsch, I. L., Sankaran, B., Bhagat, S., and Senior, A. E. (1995) *J. Biol. Chem. 270*, 26956−26961.
24. Loo, T. W., and Clarke, D. M. (1995) *J. Biol. Chem. 270*, 22957−22961.
25. Al-Shawi, M. K., Urbatsch, I. L., and Senior, A. E. (1994) *J. Biol. Chem. 269*, 8986−8992.
26. Beaudet, L., and Gros, P. (1995) *J. Biol. Chem. 270*, 17159−17170.
27. Beaudet, L., Urbatsch, I. L., and Gros, P. (1998) *Biochemistry 37*, 9073−9082.
28. Van Winkle, L. J. (1999) *Biomembrane Transport*, Academic Press, San Diego.
29. Sonveaux, N., Shapiro, A. B., Goormaghtigh, E., Ling, V., and Ruysschaert, J. M. (1996) *J. Biol. Chem. 271*, 24617−24624.
30. Sonveaux, N., Vigano, C., Shapiro, A. B., Ling, V., and Ruysschaert, J. M. (1999) *J. Biol. Chem. 274*, 17649−17654.
31. Wang, G., Pincheira, R., and Zhang, J.-T. (1998) *Eur. J. Biochem. 255*, 383−390.
32. Mechetner, E. B., Schott, B., Morse, B. S., Stein, W. D., Druley, T., Davis, K. A., Tsuruo, T., and Roninson, I. B. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 12908−12913.
33. Kartner, N., Riordan, J. R., and Ling, V. (1983) *Science 221*, 1285−1288.
34. Lever, J. E. (1977) *J. Biol. Chem. 252*, 1990−1997.
35. Chifflet, S., Chiesa, U. T. R., and Tolosa, S. (1988) *Anal. Biochem. 168*, 1−4.
36. De Lean, A., Munson, P. J., and Rodbard, D. (1978) *Am. J. Physiol. 235*, E97-E102.
37. Goodno, C. C. (1982) *Methods Enzymol. 85*, 116−123.
38. Senior, A. E., Gros, P., and Urbatsch, I. L. (1998) *Arch. Biochem. Biophys. 357*, 121−125.
39. Urbatsch, I. L., Beaudet, L., Carrier, I., and Gros, P. (1998) *Biochemistry 37*, 4592−4602.
40. Kenakin, T. (1996) *Trends Pharm. Sci. 17*, 190−191.
41. Burgen, A. S. (1981) *Fed. Proc. 40*, 2723−2728.
42. Bruggeman, E. P., Germann, U. A., Gottesman, M. M., and Pastan, I. (1989) *J. Biol. Chem. 264*, 15483−15488.
43. Greenberger, L. M. (1993) *J. Biol. Chem. 268*, 11417−11425.
44. Safa, A. R. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7187−7191.
45. Ferry, D. R., Malkhandi, J. P., Russell, M. A., and Kerr, D. J. (1995) *Biochem. Pharmacol. 49*, 1851−1861.
46. Shapiro, A. B., and Ling, V. (1997) *Eur. J. Biochem. 250*, 130−137.
47. Boyer, P. D., Cross, R. L., and Momsen, W. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2837−2839.
48. Bagshaw, C. R., and Trentham, D. R. (1973) *Biochem. J. 133*, 323−328.

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