Nonequivalent Nucleotide Trapping in the Two Nucleotide Binding Folds of the Human Multidrug Resistance Protein MRP1*

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Multidrug resistance protein 1 (MRP1) and P-glycoprotein, which are ATP-dependent multidrug efflux pumps and involved in multidrug resistance of tumor cells, are members of the ATP binding cassette proteins and contain two nucleotide-binding folds (NBFs). P-glycoprotein hydrolyzes ATP at both NBFs, and vanadate-induced nucleotide trapping occurs at both NBFs. We examined vanadate-induced nucleotide trapping in MRP1 stably expressed in KB cell membrane by using \( ^8\)-azido-\( ^\alpha\)-\( ^32\)P\]ATP. Vanadate-induced nucleotide trapping in MRP1 was found to be stimulated by reduced glutathione, glutathione disulfide, and etoposide and to be synergistically stimulated by the presence of etoposide and either glutathione. These results suggest that glutathione and etoposide interact with MRP1 at different sites and that those bindings cooperatively stimulate the nucleotide trapping. Mild trypsin digestion of MRP1 revealed that vanadate-induced nucleotide trapping mainly occurs at NBF2. Our results suggest that the two NBFs of MRP1 might be functionally nonequivalent.

Multidrug resistance of tumor cells is a major obstacle to cancer chemotherapy. This phenomenon is frequently associated with the expression of P-glycoprotein and multidrug resistance protein 1 (MRP1), both of which are ATP binding cassette (ABC) proteins. P-glycoprotein and MRP1 function as ATP-dependent efflux pumps that extrude cytotoxic drugs from the cells before they reach their intracellular targets, thus conferring resistance to many structurally dissimilar anticancer drugs, such as the Vinca alkaloids, colchicine, actinomycin D, etoposide, taxol, and anthracyclines (1–5). However, the mechanism of transport for MRP1 could be different from that for P-glycoprotein, because the depletion of intracellular glutathione (GSH) by buthionine sulfoximine results in a complete loss of function for MRP1 (6, 7), but buthionine sulfoximine has no effect on P-glycoprotein-mediated multidrug resistance. It has been reported that MRP1 transports GSH-S-conjugates such as leukotriene C\(_4\), glutathione disulfide (GSSG), and 2,4-dinitrophenyl-S-glutathione (8–11) and that MRP1 mediates ATP-dependent transport of vincristine, daunorubicin, and etoposide in the presence of GSH (12–14). From these findings, it has been postulated that MRP1 can actively cotransport GSH and unmodified xenobiotics as well as GSH-S-conjugates.

We have reported that MRP1 in membrane from a human MRP1 cDNA transformant can be specifically photoaffinity labeled with \( ^8\)-azido-\( ^\alpha\)-\( ^32\)P\]ATP by vanadate-induced nucleotide trapping (15). Vanadate and Mg\(^{2+}\) were required for trapping of nucleotide, and photoaffinity labeling was inhibited by the excess ADP as well as ATP. These results have suggested that a stable inhibitory complex MRP1-MgADP-Vi, an analog of the MRP1-MgADP-P transition state complex, is formed in the presence of vanadate, as suggested for P-glycoprotein (16). Vanadate-induced nucleotide trapping in P-glycoprotein has been reported to be stimulated by the transport substrates (17, 18) and to occur nonelectively in both nucleotide-binding folds (NBFs) (19). Because vanadate-induced trapping with \( ^8\)-azido-ATP in MRP1 was stimulated in the presence of GSH and GSSG as well as anticancer drugs, we assumed that these compounds directly interact with MRP1 and stimulate the formation of the transition state in the ATPase reaction of MRP1 (15). However, we have not been able to observe the cooperative effect of these compounds on vanadate-induced nucleotide trapping, which might be expected if MRP1 should actively cotransport GSH and anticancer drugs. Chang et al. (20) reported that ATPase activity of purified MRP1 was stimulated by GSH and doxorubicin and that their effects were additive; however, the stimulatory effect of GSH and doxorubicin on MRP1 ATPase was 1.7-fold at most. In this study, we examined the vanadate-induced nucleotide trapping in MRP1 at 26 °C, and found that etoposide and GSH (or GSSG) synergistically stimulate the nucleotide trapping as much as 20-fold. Interestingly, mild trypsin digestion revealed that vanadate-induced nucleotide trapping mainly occurs at NBF2.

EXPERIMENTAL PROCEDURES

Materials—The monoclonal antibodies MRP1r1 (21) and MRPm6 (22) were purchased from Nichirei and Chemicon, respectively. Etoposide was from Sigma and Bristol Myers Squibb. \( ^8\)-azido-\( ^\alpha\)-\( ^32\)P\]ATP was purchased from ICN Biomedicals, and \( ^{13}\)H\]estradiol 17\(\beta\)-glucuronide (E217\(\beta\)G) (44.0 Ci/mmol) was from NEN Life Science Products.

Vanadate-induced Nucleotide Trapping in MRP1 with \( ^8\)-azido-\( ^\alpha\)-\( ^32\)P\]ATP—Membranes (10 µg) were prepared from KB/MPR1, a human MRP1 cDNA transformant as described previously (15). They were mixed with GSH and etoposide in 6 µl of TEM buffer (40 mM Tris-Cl (pH 7.5), 3 mM MgSO\(_4\), 0.1 mM EDTA) containing 200 µM sodium orthovanadate and 2 mM ouabain on ice, and 10 µM \( ^8\)-azido-\( ^\alpha\)-\( ^32\)P\]ATP was added as a final component. The mixture was incubated for 1 min at 26 °C.

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The abbreviations used are: MRP, multidrug resistance-associated protein; ABC, ATP binding cassette; NBF, nucleotide-binding fold; GSH, glutathione; GSSG, glutathione disulfide; CFTR, cystic fibrosis transmembrane conductance regulator; SUR, sulfonamide receptor; E217\(\beta\)G, 17\(\beta\)-estradiol 17\(\beta\)-glucuronide; mAb, monoclonal antibody.

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The reactions were stopped by the addition of 500 μl of ice-cold TEM buffer, and free ATP was removed after centrifugation (15,000 × g, 5 min, 2 °C). The pellets were washed in the same buffer, resuspended in 8 μl of TEM buffer, and irradiated for 45 s (at 254 nm, 8.2 mW/cm²) on ice. The samples were electrophoresed on a 7% SDS-polyacrylamide gel and autoradiographed. The trapped 8-azido-[α-32P]ATP in MRP1 was measured by scanning with a radioimaging analyzer (BAS2000, Fuji Photo Film Co.). Experiments were done at least in triplicate.

**Results**

**Time Course of Vanadate-induced Nucleotide Trapping in MRP1**—We examined vanadate-induced nucleotide trapping in MRP1 stably expressed in KB cell membrane by using 8-azido-[α-32P]ATP. It has been reported that, when P-glycoprotein is incubated with ATP and vanadate, the inhibition of P-glycoprotein ATPase activity induced by vanadate is rapid, reaching completion in less than 10 s at 37 °C (25). Because we assumed that the reaction of MRP1 would be as fast as P-glycoprotein and the reaction at 37 °C would be too fast to be analyzed quantitatively, we examined the time course of vanadate-induced nucleotide trapping in MRP1 at 26 °C. When membranes from KB/MRP1 were incubated with vanadate, Mg²⁺, and 10 μM 8-azido-[α-32P]ATP, followed by UV irradiation after removing free ligands, a 190-kDa protein was specifically photoaffinity-labeled in a time-dependent manner (Fig. 1A, lanes 1–4). The 190-kDa protein was not labeled in membrane proteins from untransfected KB-3-1 (data not shown), indicating that the 190-kDa protein is MRP1. In the presence of 2 mM GSSG, which is considered to be one of the endogenous substrates for MRP1 (9), photoaffinity labeling of a 190-kDa protein was stimulated (Fig. 1A, lanes 5–8) and showed a strong time dependence up to 2 min (Fig. 1B).

**Effects of Etoposide and Glutathione on Nucleotide Trapping in MRP1**—We examined the effects of GSH, GSSG, and etoposide on the nucleotide trapping in KB/MRP1 membranes incubated at 26 °C for 1 min with 10 μM 8-azido-[α-32P]ATP (Fig. 2). KB/MRP1 cells showed the highest resistance to etoposide among the anticancer drugs examined (15). GSSG, GSH, and etoposide significantly stimulated nucleotide trapping in a concentration-dependent manner. The nucleotide trapping is stimulated 4.3- and 2.2-fold by 2 mM GSSG and GSH, respectively, and 3.7-fold by 1 mM etoposide.

**Synergistic Effects of Etoposide and Glutathione on Nucleotide Trapping in MRP1**—The unmodified xenobiotics have been suggested to be actively cotransported with GSH (12, 13). If this is true, then GSH and xenobiotics can be expected to cooperatively affect ATP hydrolysis of MRP1. To explore this possibility, we examined the effect of etoposide on the nucleotide trapping in the presence of GSH. Fig. 3A shows that etoposide at 0.5 mM and 1 mM stimulates the nucleotide trapping about 20-fold in the presence of 2 mM GSH. Interestingly, in the presence of 2 mM GSSG, etoposide also stimulates the nucleotide trapping to the similar extent. The combination of 2 mM GSH and 2 mM GSSG did not stimulate nucleotide trapping more than 2 mM GSSG alone did (data not shown). No stimulatory effect on the nucleotide trapping with etoposide was noted with 2-mercaptoethanol or dithiothreitol, which are other reducing agents (data not shown).
Western blotting (Fig. 4B) indicates that the 120-kDa fragment, produced by the mild trypsin digestion, was recognized by monoclonal antibody (mAb) MRP1, and the 75- to 80-kDa fragments were recognized by mAb MRPM6 as reported previously (21). MRP1 and MRPM6 have been reported to recognize the N-proximal half of MRP1 containing NBF1 and the C-proximal half containing NBF2, respectively (21). Photoaffinity labeling, done in parallel, showed that the labeled 75- to 80-kDa fragments comigrated with the fragments recognized by MRPM6 (lane 5). These results suggest that NBF2 of MRP1 is preferentially photoaffinity-labeled.

**Preferential Vanadate-induced Nucleotide Trapping at NBF2 of MRP1**—To confirm that the 75- to 80-kDa fragments, containing NBF2, were preferentially photoaffinity-labeled, the decrease of photoaffinity-labeled undigested MRP1 and the increase of labeled tryptic fragments were measured. KB/ MRP1 membranes were incubated with 10 μM 8-azido-[α-32P]ATP and vanadate in the presence of GSH and etoposide at 26 °C and were photoaffinity-labeled by UV irradiation after removing free ligands. They were then incubated with various concentrations of trypsin for 15 min at 20 °C (Fig. 5).

The photoaffinity-labeled band of undigested MRP1 decreased and those of tryptic fragments increased with increasing concentrations of trypsin (Fig. 5B). By incubating with 0.25 μg/ml trypsin, the photoaffinity-labeled band of undigested MRP1 decreased 24 ± 6%, and those of the 120- and 75- to 80-kDa tryptic fragments increased 3 ± 2% and 20 ± 4%, respectively. This indicates that most of 8-azido-[α-32P]ATP is trapped in the 75- to 80-kDa fragments containing NBF2 after incubation in the presence of GSH and etoposide.

**8-Azido-ATP Supports Active Transport of E217βG**—To confirm that 8-azido-ATP supports the active transport activity of MRP, we examined ATP-dependent E217βG uptake into membrane vesicles prepared from KB/MRP1 (Fig. 6). E217βG has been reported to be a good substrate for MRP1 (27). [3H]E217βG uptake into membrane vesicles prepared from KB/MRP1 was observed in the presence of 8-azido-ATP as well as ATP, but not in the presence of AMP or ADP. The ATP-dependent uptake of [3H]E217βG into membrane vesicles prepared from KB-3-1 host cells was less than one-tenth of that with membrane vesicles prepared from KB/MRP1. These results suggest that 8-azido-ATP is hydrolyzed by MRP1 and supports active transport by MRP1.

**DISCUSSION**

We demonstrated in the present study that MRP1 is specifically photoaffinity-labeled with 8-azido-[α-32P]ATP in KB/MRP1 membranes. The photoaffinity labeling of MRP is dependent on vanadate and Mg2+ and is inhibited by the excess ADP as well as ATP. These properties of vanadate-induced nucleotide trapping is similar to those of P-glycoprotein (25). 8-Azido-ATP supports active transport of E217βG into plasma membrane vesicles from KB/MRP1 (Fig. 6). 8-Azido-ATP also supports active transport of leukotriene C4 with similar affinity (28). These results suggest that a stable inhibitory complex, MRPMgADP-Vi, an analog of the MRP1MgADP-Pi, transition state complex, is formed in the presence of vanadate after ATP hydrolysis, as suggested for P-glycoprotein (16). However, more experiments are needed to establish whether there is a good correlation between vanadate-induced nucleotide trapping and ATP hydrolysis by MRP.

The vanadate-induced nucleotide trapping of MRP1 with 8-azido-[α-32P]ATP is stimulated by GSH, GSSG, and etoposide, and, importantly, is synergistically stimulated by both GSH and etoposide and by GSSG and etoposide. These results suggest that glutathione and etoposide interact with MRP1 at different sites as proposed (26) and that those bindings coop-
Membrane vesicles prepared from KB-3-1 or membrane vesicles. 

Analyzing.

\[ \text{ATP} \rightarrow \text{ADP} + \text{P}_i \]

Incubated with 10 \( \mu \text{M} \) (lanes 1, 3, 5, 7) or 100 \( \mu \text{M} \) (lanes 2, 4, 6, 8) 8-azido-[\( \alpha^{32}\text{P} \)]ATP in the presence of 200 \( \mu \text{M} \) orthovanadate and 3 \( \mu \text{M} \) MgSO\(_4\). The reactions were done in the absence (lanes 1 and 2) or presence of 1 \( \mu \text{M} \) etoposide (lanes 3 and 4), 2 \( \mu \text{M} \) GSSG (lanes 5 and 6), and 2 \( \mu \text{M} \) GSH and 1 \( \mu \text{M} \) etoposide (lanes 7 and 8) at 26 \( ^\circ \text{C} \) for 1 min. After free ATP was removed, the proteins were irradiated with UV and digested with 2.0 \( \mu \text{g/ml} \) trypsin for 15 min at 20 \( ^\circ \text{C} \). B, Western blot with mAb MRPr1 (lanes 1 and 2) and with mAb MRPr6 (lanes 3 and 4) of membrane proteins from KB/MRP1 digested with 2.0 \( \mu \text{g/ml} \) trypsin before (lanes 1 and 3) and after (lanes 2 and 4) vanadate-induced nucleotide trapping. Lane 5, photoaffinity labeling under the condition of A, lane 7. The tryptic 120-kDa fragment containing NBF1 and the 75–80-kDa fragments containing NBF2 are indicated.

FIG. 5. Preferential vanadate-induced nucleotide trapping at NBF2 of MRP1. A, membrane proteins (20 \( \mu \text{g} \)) from KB/MRP1 were incubated with 10 \( \mu \text{M} \) 8-azido-[\( \alpha^{32}\text{P} \)]ATP, 200 \( \mu \text{M} \) orthovanadate, and 3 \( \mu \text{M} \) MgSO\(_4\) in the presence of 2 \( \mu \text{M} \) GSH and 1 \( \mu \text{M} \) etoposide for 3 min at 26 \( ^\circ \text{C} \) and UV irradiated after removing free ATP. Membrane proteins were digested with 0 \( \mu \text{g/ml} \) trypsin (lane 1), 0.25 \( \mu \text{g/ml} \) trypsin (lane 2), 1 \( \mu \text{g/ml} \) trypsin (lane 3), and 2 \( \mu \text{g/ml} \) trypsin (lane 4) for 15 min at 20 \( ^\circ \text{C} \) and the tryptic fragments were analyzed. B, relative photoaffinity labeling of undigested MRP1 (●), the tryptic 120-kDa fragment containing NBF1 (▲), and the 75–80-kDa fragments containing NBF2 (○) were calculated relative to the photoaffinity labeling of undigested MRP1 without trypsin digestion.

FIG. 6. 8-Azido-ATP supports the uptake of [\( ^3\text{H} \)]E217βG into membrane vesicles. Membrane vesicles prepared from KB-3-1 or KB/MRP1 were incubated with 57 \( \mu \text{Ci} \) [\( ^3\text{H} \)]E217βG in the presence of no nucleotide (lanes 1 and 6), 5 \( \mu \text{M} \) AMP (lanes 2 and 7), 5 \( \mu \text{M} \) ADP (lanes 3 and 8), 5 \( \mu \text{M} \) ATP (lanes 4 and 9), or 5 \( \mu \text{M} \) 8-Azido-ATP (lanes 5 and 10) for 30 min. Experiments were done in triplicate and expressed relative to the value with KB/MRP1 in the presence of ATP.

GSSG is transported by MRP1 (29), whereas transport of GSH is controversial (6, 29, 30). Lower stimulation of vanadate-induced nucleotide trapping in MRP1 by GSH compared with GSSG suggests that GSH per se is not a good substrate for MRP1. Synergistic stimulation of vanadate-induced nucleotide trapping by etoposide and GSH suggests that their simultaneous binding to MRP1 cooperatively induces conformational changes at the catalytic NBF. Because GSSG is as effective as GSH for stimulating vanadate-induced nucleotide trapping in the presence of etoposide, and because GSSG would not interact with etoposide even in a noncovalently associated form, etoposide may be recognized as an unmodified form by MRP together with glutathione as proposed (6, 30). We have examined the effects of doxorubicin and vincristine on vanadate-induced nucleotide trapping in MRP. However, they did not show such strong synergistic effects with glutathione as etoposide showed (data not shown). The reason why etoposide shows such a strong synergistic effect with glutathione remains to be identified.

In P-glycoprotein, two NBFs appear to be functionally equivalent (16). Both NBFs can hydrolyze ATP, and vanadate-induced nucleotide trapping occurs in both NBFs probably nonselectively (19) and sequentially (16, 31). Mild trypsin digestion of MRP1 reveals that vanadate-induced nucleotide trapping occurs mainly at NBF2 under any conditions examined: under a basal condition, in the presence of GSSG alone, etoposide alone, and etoposide and GSH. There could be several possible explanations for the preferential vanadate-induced nucleotide trapping at NBF2: the first is that NBF1 has lower ATP binding affinity than does NBF2. If that is the case, NBF1 might not be photoaffinity-labeled with 10 \( \mu \text{M} \) 8-azido-[\( \alpha^{32}\text{P} \)]ATP. However, NBF2 was preferentially photoaffinity-labeled even with 100 \( \mu \text{M} \) 8-azido-[\( \alpha^{32}\text{P} \)]ATP. The second explanation is that MRP1 hydrolyzes ATP only at NBF2; the third is that ATP hydrolysis of MRP1 occurs selectively at NBF2 first, when substrates bind to MRP, and then at NBF1. If the first catalytic turnover occurs selectively at NBF2 of MRP1, it would produce an intermediate containing ADP at NBF2, and then vanadate would bind to this intermediate to form a stable inhibitory complex, MRP1-MgADP-Vi at NBF2. This would not allow any further reaction at NBF1, and no vanadate-induced nucleotide trapping would occur at NBF1. The fourth explanation would be that NBF1 of MRP1 also hydrolyzes ATP but a stable inhibitory complex, MRP1-MgADP-Vi, is not formed at NBF1. It is not clear yet what causes the preferential photoaffinity labeling of NBF2. However, these results suggest that the two NBFs of MRP1 are functionally nonequivalent.

Nonequivalent features between two NBFs of ABC proteins have been reported for SUR1 and cystic fibrosis transmembrane conductance regulator (CFTR). SUR1 is a subunit of the pancreatic β-cell K\(_{\text{ATP}}\) channel, which plays a key role in the...
regulation of glucose-induced insulin secretion. SUR1 may not be a transporter or a channel but may function only as a switch that monitors intracellular ATP/ADP concentrations. SUR1 binds ATP strongly at NBF1 even in the absence of Mg$^{2+}$ and binds MgADP at NBF2 (17, 32). Recently, we suggested that NBF2 of SUR1 may hydrolyze ATP (32, 33). CFTR is an ATP-dependent chloride channel and malfunction in cystic fibrosis. NBF1 of CFTR was preferentially labeled with 8-azido-ATP in the absence of vanadate. The addition of vanadate increased photoaffinity labeling and resulted in the labeling of both NBFs of CFTR (34).

In conclusion, vanadate-induced nucleotide trapping in MRP1 is strongly and synergistically stimulated by the presence of etoposide and glutathione. Interestingly, vanadate-in-

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