Conformational changes of P-glycoprotein by nucleotide binding

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P-glycoprotein (Pgp) is a membrane protein that transports chemotherapeutic drugs, causing multidrug resistance in human cancer cells. Pgp is a member of the ATP-binding cassette superfamily and functions as a transport ATPase. It has been suggested that the conformation of Pgp changes in the catalytic cycle. In this study, we tested this hypothesis by using limited proteolysis as a tool to detect different conformational states trapped by binding of nucleotide ligands and inhibitors. Pgp has high basal ATPase activity; that is, ATP hydrolysis by Pgp is not rigidly associated with drug transport. This activity provides a convenient method for studying the conformational change of Pgp induced by nucleotide ligands, in the absence of drug substrates which may generate complications due to their own binding. Inside-out membrane vesicles containing human Pgp were isolated from multidrug-resistant SKOV/VLB cells and

treated with trypsin in the absence or presence of MgATP, Mg-adenosine 5'-[β,γ -imido]triphosphate (Mg-p[NH]ppA) and MgADP. Changes in the proteolysis profile of Pgp owing to binding of nucleotides were used to indicate the conformational changes in Pgp. We found that generation of tryptic fragments, including the loop linking transmembrane (TM) regions TM8 and TM9 of Pgp, were stimulated by the binding of Mg-p[NH]ppA, MgATP and MgADP, indicating that the Pgp conformation was changed by the binding of these nucleotides. The effects of nucleotides on Pgp conformation are directly associated with the binding and/or hydrolysis of these ligands. Four conformational states of Pgp were stabilized under different conditions with various ligands and inhibitors. We propose that cycling through these four states couples the Pgp-mediated MgATP hydrolysis to drug transport.

INTRODUCTION

P-glycoprotein (Pgp) is a plasma membrane protein which belongs to the superfamily of ATP-binding cassette (ABC) transporters [1] or membrane traffic ATPases [2]. Overexpression of Pgp in cancer cells causes multidrug resistance (MDR) by pumping drugs from the inside to the outside of cells [3–7]. Analysis of the amino acid sequence suggests that Pgp consists of two homologous halves, each containing a hydrophobic transmembrane (TM) domain and a hydrophilic ATP-binding domain. There are six putative TM segments within each TM domain, predicted from hydropathy plot analyses [8–10]. However, recent studies have generated controversial models on the topological folding of Pgp and on the number of TM segments of Pgp located in the membrane bilayer [11–18]. One example of an alternative model of Pgp topology is shown in the inset to Figure 1.

Pgp has been shown to have high basal ATPase activity [19–22]. This suggests that the hydrolysis of MgATP by Pgp may not be rigidly associated with the drug transport process. Measurements of the K_m (MgATP) of Pgp vary from 0.6 mM to 1.4 mM [20–23]. It has also been shown that both halves of Pgp bind ATP [24]. The ATPase activity of Pgp is inhibited by vanadate [25] and by covalent modification with *N*-ethylmaleimide (NEM) [21,26,27].

It has been suggested that Pgp undergoes a conformational change during its catalytic cycle. Four conformational states of Pgp in the catalytic cycle have been postulated [28] (see also Figure 1). Recently, using IR spectroscopy, Sonveaux et al. [29] suggested that in the presence of verapamil, the tertiary, but not the secondary, structure of Pgp may change upon binding and hydrolysis of MgATP. In this paper, we report detailed studies of changes in Pgp conformation elicited by nucleotides. Limited

trypsin digestion of Pgp was used to deduce the conformational states of Pgp induced by the binding of different nucleotide ligands. We performed these experiments in the absence of drug substrates to eliminate potential complications by drug substrates which, in themselves, may affect the Pgp conformation [30]. The proteolysis approach has been used successfully to investigate ligand-induced conformational changes of various transport ATPases, such as Na, K-ATPase [31,32], bacterial MalK transporter [33], F_1 -ATPase [34,35] and the ArsA anion pump [36]. The binding of MgATP to these proteins altered their trypsin-digestion profile, which was used to decipher the conformational changes generated by nucleotide binding. Since the ATPase activity of Pgp has been shown to depend on lipid composition and is affected by detergent [27,30], we used isolated membrane vesicles both to preserve the ATPase activity of Pgp and to avoid potential artifacts introduced by solubilization and reconstitution procedures. We found that the trypsin-digestion profile of Pgp was altered by nucleotide binding. Four Pgp conformational states were stabilized under different conditions.

EXPERIMENTAL

Materials

Monoclonal antibodies (mAbs) MD-7, C219, C494 and the multidrug-resistant cell-line SKOV/VLB were gifts from Dr. Victor Ling (The British Columbia Cancer Center, Vancouver, Canada). α -MEM (minimal essential medium) medium for maintaining the SKOV/VLB cells was from Gibco-BRL (Gaithersburg, U.S.A.). Peroxidase-conjugated goat anti-(mouse IgG), the enhanced chemiluminescence (ECL) reagent, and PVDF membranes were purchased from Sigma (St. Louis, U.S.A.), Amersham (Arlington Heights, U.S.A.) and Bio-Rad (Hercules, U.S.A.), respectively. Trypsin (analytical grade iso-

Abbreviations used: ABC, ATP-binding cassette; BAEE, *N* α -benzoyl-L-arginine ethyl ester; DTT, dithiothreitol; ECL, enhanced chemiluminescence; mAb, monoclonal antibody; MDR, multidrug resistance; NEM, *N*-ethylmaleimide; Pgp, P-glycoprotein; p[NH]ppA, adenosine 5'-[β,γ -imido]triphosphate; TM, transmembrane.

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Figure 1 Models of different Pgp conformational states trapped by nucleotide binding

Four conformational states are presented as I, II, III and IV. Conformation I is the unbound state. Upon binding of MgATP or Mg-p[NH]ppA, conformation I is converted into conformation II. Hydrolysis of MgATP will generate the conformation III, which can be stabilized using vanadate. Upon release of the phosphate, the conformation is changed from III to IV. High concentrations of MgADP can also drive the production of conformation IV. In all the conformational changes, Mg^{2+} is required. Two different schematic topologies of a Pgp domain are presented in the inset. Model A is predicted on the basis of the hydropathy plot [8], whereas model B was observed in both *in vitro* [11] and *in vivo* [17] studies. Designated by arrows, a, b and c indicate the possible trypsin digestion sites which release peptides Y (a–b) or Z (b–c).

lated from bovine pancreas) was obtained from Boehringer Mannheim (Indianapolis, U.S.A.). All other chemicals were purchased either from Sigma or Fisher Scientific (Pittsburgh, U.S.A.). The pH of stock solutions containing nucleotides was adjusted to 7.0 before use.

Trypsin activity assay

The trypsin activity was assayed using *N*-(α -benzoyl)-L-arginine ethyl ester (BAEE) as the substrate, as described previously [37]. Briefly, about 40 BAEE units of trypsin was incubated in the presence or absence of 5 mM nucleotide ligands or inhibitors for 5 min at room temperature, before mixing with the BAEE substrate cocktail containing BAEE (final concentration 0.15 mM), 40 mM sodium phosphate, pH 7.6 and 0.06 mM HCl. The A_{253} was recorded immediately after mixing and continued for 5 min on a Shimadzu UV-160 spectrophotometer. The remaining enzyme activity after nucleotide treatment was calculated on the basis of the following relationship:

% Remaining activity =

$$100 \times \frac{(\Delta A_{253}/\text{min test}_{(+nuc)} - \Delta A_{253}/\text{min blank})}{(\Delta A_{253}/\text{min test}_{(-nuc)} - \Delta A_{253}/\text{min blank})}$$

where $\Delta A_{253}/\text{min test}_{(+nuc)}$ is the rate of A_{253} change catalysed by nucleotide-treated trypsin, $\Delta A_{253}/\text{min test}_{(-nuc)}$ is the rate of A_{253} change catalysed by mock-treated trypsin, and $\Delta A_{253}/\text{min blank}$ is the rate of A_{253} change in the absence of trypsin. The data used for calculations were within the linear range of ΔA .

Limited trypsin treatment of Pgp in inside-out membrane vesicles

Membranes were isolated from SKOV/VLB cells according to the method described previously by Riordan and Ling [38]. Sealed inside-out vesicles were prepared as previously described by Zhang et al. [17]. The membrane orientation was determined as described previously [39]. Protein concentration was estimated

using Bio-Rad Protein Assay reagent. Limited trypsin digestion of inside-out membrane vesicles was performed at 37 °C for 2 h using 30 μg of membrane proteins with 0.24 BAEE unit of trypsin. To investigate the effects of ligands on trypsin digestion of Pgp, the membranes were preincubated with various ligands, as indicated in the Figure legends, for 30 min at room temperature before trypsin digestion was performed. After 2 h incubation with trypsin, the reaction was stopped by adding PMSF and soybean trypsin inhibitor to final concentrations of 10 mM and 200 $\mu\text{g}/\text{ml}$ respectively. The membrane fraction was separated from trypsin by centrifugation at 4 °C. The final membrane pellet was immediately solubilized in SDS/PAGE sample buffer and used for electrophoresis. For experiments lacking Mg^{2+} , EDTA was included to remove any contaminating Mg^{2+} .

Western-blot analysis

Western blot was performed as described previously [17]. Briefly, untreated or trypsin-treated membranes of SKOV/VLB cells were subjected to SDS/PAGE. The fractionated proteins were then transferred to a PVDF membrane and probed with primary mAbs MD-7, C219 or C494 at 1 $\mu\text{g}/\text{ml}$, followed by peroxidase-conjugated anti-(mouse IgG) (1:2500 dilution). The signal was detected by ECL using an ECL detection kit (Amersham, Arlington Heights, U.S.A.).

RESULTS

Using isolated inside-out membrane vesicles of MDR cells (SKOV/VLB), we have shown previously that the loop (loop 8) linking TM8 and TM9 is resistant to trypsin digestion, possibly due to its atypical extracellular location [17] (see also inset to Figure 1). As shown in Figure 2(A), limited trypsin digestion of Pgp generated two major peptide fragments (C and Z, respectively) which were detected by a Pgp-specific mAb MD-7 (Figure 2A, lane 2). The mAb MD-7 has an epitope in loop 8 [40]. Peptide C represents the C-terminal-half fragment generated by trypsin digestion at site a (see Figure 1) which precedes the

Figure 2 Trypsin digestion of Pgp in inside-out membrane vesicles

(A) Effects of MgATP on trypsin digestion of Pgp. Membrane proteins (30 μ g) were incubated in the absence of MgATP (lane 2) or pretreated with 5 mM MgATP (lane 3) for 30 min at room temperature, followed by digestion with 0.24 BAEE unit of trypsin at 37 °C for 2 h. The reaction was stopped by using PMSF and trypsin inhibitor. The membrane fraction was pelleted by centrifugation, separated by SDS/PAGE and transferred on to a PVDF membrane. Pgp and its fragments were detected by the mAb MD-7. Lane 1 is the control (undigested membranes). (B) Detection of Pgp fragments by three different antibodies. Digestion of MgATP-treated membrane proteins, SDS/PAGE and Western blot were performed as described in (A). Pgp and its fragments were detected by the mAb MD-7 (lane 1), C219 (lane 2) and C494 (lane 3). F, undigested full-length molecules; C, C-terminal-half molecules; N, N-terminal-half molecules; Y and Z, peptide fragments Y and Z.

mini-linker domain and amino acid residue 666. This was determined using the mAb MD-1, which has its epitope between amino acids 666–687 in the mini-linker domain [17,40]. Peptide Z represents a fragment including loop 8, linking TM8 and TM9 generated from the C-half fragment by trypsin digestion at site c (possibly Arg⁶⁸⁹ preceding TM7) and site b (possibly Lys⁸⁸⁵ following TM10) (see Figure 1 and ref. [17]). A minor peptide (indicated as Y) was also produced (Figure 2A, lane 2). This was thought to represent a product similar to peptide Z, but generated by trypsin cleavage at sites a and b because it reacts with mAb MD-1, which has the epitope in the mini-linker domain [17]. Small amounts of peptide C were also produced before trypsin was added owing to self-degradation, which occurred during the membrane-isolation process (Figure 2A, lane 1).

Effects of MgATP and MgADP on the trypsin-digestion profile of Pgp

To determine whether nucleotide binding causes any conformational change in Pgp, we used trypsin digestion of loop 8 as an indicator, using MD-7 on Western blots. If nucleotide binding causes a global conformational change in Pgp, generation of tryptic fragments, including loop 8, may be altered due to changes in exposure of the sensitive sites to trypsin. As shown in Figure 2(A), more peptide Y was detected relative to peptide Z in the presence of MgATP (lane 3) as compared with the control digestion (lane 2). The amount of peptide Z, on the other hand, was not changed. The increased amount of peptide Y is not due to the increased trypsin activity by MgATP (see Table 1 and the Discussion section).

To confirm the cleavage of Pgp into two half-molecules and smaller fragments in the presence of MgATP, we probed the digested products with two additional Pgp-specific mAbs, C219 and C494. C219 has two epitopes, one near each ATP-binding fold, whereas C494 has only one epitope near the C-terminal ATP-binding fold [41]. As shown in Figure 2(B), MD-7 detected the

Figure 3 Effects of various nucleotide ligands on the trypsin digestion of Pgp

Membrane proteins (30 μ g) were pretreated for 30 min at room temperature with 5 mM ATP in the absence of MgCl₂ (lane 1) or in the presence of 5 mM MgCl₂ (lane 2), 5 mM MgCl₂ with 5 mM of ATP (lane 3), ADP (lane 4), AMP (lane 5), ATP[S] (ATPγS) (lane 6), and AMP-PNP (lane 7). Trypsin was then added and the digestion was performed for 2 h at 37 °C. SDS/PAGE and Western blot were performed as described in the Experimental section. Pgp and its fragments were detected using the mAb MD-7. AMP-PNP, p[NH]ppA.

peptides C, Y and Z (Figure 2B, lane 1). C219 detected peptide C and an additional peptide N (N-half fragment) (Figure 2B, lane 2). C494 detected peptide C, but not peptide N (Figure 2B, lane 3). Neither C219 nor C494 reacted with peptides Y and Z. However, they detected an additional minor peptide (indicated by an arrow) which does not react with MD-7 (compare lane 1 with lanes 2 and 3, Figure 2B). This is likely to be a fragment generated from the C-terminal ATP-binding fold which does not have epitopes for MD-7. These observations suggest that Pgp is cleaved into two half-fragments in the presence of MgATP, and peptides Y and Z are unlikely to be from the ATP-binding domain containing C219 and C494 epitopes.

Mg²⁺ is required for MgATP effects on the trypsin-digestion profile of Pgp

We then determined whether Mg²⁺ is required for MgATP to change the trypsin-digestion profile of Pgp. As shown in Figure 3, ATP in the absence of Mg²⁺ (lane 1) or with Mg²⁺ alone (lane 2) did not increase the amount of peptide Y. On the other hand, more peptide Y was detected in the presence of both ATP and Mg²⁺ (lane 3). It is interesting to note that more peptide Y was also detected in the presence of MgADP (lane 4), but not in the presence of MgAMP (lane 5). The effect of MgADP on trypsin digestion of Pgp also requires Mg²⁺ (results not shown). Therefore the complex of Mg²⁺ with either ATP or ADP is required to change the trypsin-digestion profile of Pgp. The poorly hydrolysable and non-hydrolysable ATP analogues MgATP[S] (lane 6) and Mg-p[NH]ppA (or AMP-PNP; lane 7) did not increase the amount of peptide Y. However, more peptide C was observed in the presence of MgATP[S] or Mg-p[NH]ppA. These results suggest that different trypsin-digestion profiles of Pgp were generated by the binding of nucleotide ligands, indicating that Pgp has several conformational states which can be stabilized by the binding of nucleotides with or without hydrolysis.

Table 1 Effects of ligands on trypsin activity

Trypsin activity was assayed using BAEE as the substrate, as described in the Experimental section, in the absence (control) or presence of various ligands. Trypsin activity (average of three experiments) in the presence of various ligands was normalized on the basis of the control reaction, which was set as 100%. The signs (+) and (—) are arbitrary: (+) indicates that the amount of tryptic fragments of Pgp was increased by the ligands, and (—) indicates no change.

Ligand	Remaining trypsin activity (%)	Digestion enhancement of Pgp
Control	100	—
5 mM ATP	46.7	—
5 mM MgATP	48.6	+++
5 mM MgADP	47.2	+++
5 mM Mg-p[NH]ppA	54.2	++++ ^a
5 mM MgAMP	18.1	—
5 mM MgITP	27.8	+++
5 mM MgGTP	10	++
5 mM MgCTP	76.4	+
5 mM MgUTP	68.1	+
10 μ M NEM	90	—
100 μ M Vanadate	79.2	—

^a The altered trypsin-digestion profile of Pgp in the presence of Mg-p[NH]ppA is different from that in the presence of MgATP or MgADP.

Figure 4 Concentration dependence of MgATP and MgADP effects

Membrane proteins (30 μ g) were pretreated for 30 min at room temperature with MgADP at concentrations of 5 mM (lane 2), 2.5 mM (lane 3), 1 mM (lane 4), 0.5 mM (lane 5), 0.05 mM (lane 6) or MgATP at 5 mM (lane 7), 2.5 mM (lane 8), 1 mM (lane 9), 0.5 mM (lane 10) and 0.05 mM (lane 11). Lane 1 is a control reaction without nucleotides. Trypsin digestion, SDS/PAGE and Western blot were performed as described in the Experimental section. Pgp and its fragments were detected using the mAb MD-7.

It should be noted that the amount of peptide C is normally reduced in the presence of 5 mM ATP without Mg²⁺ (compare lane 1 with lanes 2–7, Figure 3; see also Figure 7). This is presumably due to the fact that the trypsin activity was reduced by ATP (Table 1), and binding of ATP alone to Pgp does not change the conformation of Pgp.

Concentration dependence of MgATP effects on the trypsin-digestion profile of Pgp

We also determined the concentration dependence of the MgATP effects on the trypsin-digestion profile of Pgp. As shown in Figure 4, 1 mM MgATP was sufficient to increase the amount of peptide Y (compare lanes 7–11 with lane 1). This is consistent

Figure 5 Effects on trypsin digestion of Pgp by NEM covalent modification

(A) The effects of Pgp trypsin digestion by MgATP are reversible. Membrane proteins (30 μ g) were pretreated for 30 min at room temperature with 5 mM MgATP (lanes 1 and 2), followed by centrifugation to separate the membrane fraction from free MgATP. The membrane pellet was then resuspended and digested with trypsin in the presence (lane 1) or absence (lane 2) of 5 mM MgATP. Re, removed. (B) Effects of NEM on MgATP-induced trypsin digestion of Pgp. Membrane proteins (30 μ g) were pretreated for 10 min with 10 μ M NEM (lane 2) or for 30 min with 5 mM MgATP (lane 3), followed by incubation with MgATP (lane 2) or NEM (lane 3) respectively, before being subjected to trypsin digestion. Samples in lanes 1 and 4 are controls: digestion without any treatment (lane 1) or treated with MgATP alone (lane 4). The numbers in the parentheses indicate the order of treatment.

with the estimated K_m (MgATP) of 0.6–1.4 mM [20–23]. However, concentrations > 1 mM of MgADP may be required to stimulate the increased accumulation of peptide Y (compare lanes 2–6 with lane 1, Figure 4).

Reversibility of the MgATP effects on the trypsin-digestion profile of Pgp

To determine whether the effects of MgATP on Pgp digestion are reversible, we first treated the membrane vesicles with 5 mM MgATP for 30 min at room temperature. The membrane was then separated from nucleotides by centrifugation, washed once and then subjected to trypsin digestion in the absence of MgATP. Since Pgp has low affinity for MgATP, Pgp should be free of MgATP molecules after their removal from the media. As shown in Figure 5(A), peptide Y was not detected if MgATP was removed before the trypsin digestion (lane 2). However, large amounts of peptide Y were produced when the digestion was performed after readdition of MgATP (lane 1). Similarly, effects by MgADP and Mg-p[NH]ppA were also eliminated by removing the nucleotides before performing the digestion (results not shown). These results suggest that the changes in the trypsin-digestion profile of Pgp induced by MgATP, MgADP or Mg-p[NH]ppA can be reversed by removing the nucleotides before trypsin digestion.

Inhibition of the MgATP-stimulated digestion of Pgp by covalent NEM modification

To determine whether covalent modification of Pgp with NEM affects the MgATP-induced change in the trypsin-digestion profile of Pgp, we first treated the membranes with 10 μ M NEM for 15 min at room temperature. The reaction was stopped by addition of dithiothreitol (DTT) (100 μ M) and the free NEM and DTT were removed by centrifugation. The membrane fraction was washed once and then digested with trypsin in the absence or presence of MgATP. As shown in Figure 5(B), the MgATP-stimulated increase in peptide Y accumulation was

Figure 6 Effects of MgUTP, MgCTP, MgGTP, and MgITP on trypsin digestion of Pgp

(A) Membrane proteins (30 μ g) were pretreated for 30 min at room temperature in the absence of MgATP (lane 1) or with equal concentrations (5 mM) of MgATP (lane 2), MgUTP (lane 3), MgCTP (lane 4), MgGTP (lane 5) or MgITP (lane 6). Trypsin digestion, SDS/PAGE and Western blot were performed as described in the Experimental section. Pgp and its fragments were detected using the mAb MD-7. (B) Schematic planar structures of ATP, UTP, CTP, GTP and ITP. R, ribose triphosphate.

inhibited by the pretreatment of Pgp with NEM (compare lanes 2 and 4, Figure 5B). However, if the membranes were pretreated with MgATP before treatment with NEM (Figure 5B, lane 3), the MgATP-stimulated peptide Y accumulation can be partially recovered (compare lanes 2–4, Figure 5B). Thus it is likely that the effect of MgATP on trypsin digestion of Pgp is due to the binding and hydrolysis of MgATP, which is inhibited by NEM

modification. Similar NEM effects were also observed to MgADP (results not shown).

Effects of other nucleotides on trypsin digestion of Pgp

To determine whether other nucleotides (MgUTP, MgCTP, MgGTP and MgITP) also affect the trypsin-digestion profile of Pgp, we performed trypsin digestion of membranes in the presence of these nucleotides. As shown in Figure 6(A), all nucleotides enhanced the accumulation of peptide Y, but less than that evoked by MgATP. The effectiveness of these nucleotides on the amount of peptide Y accumulated was in the following order: MgATP > MgITP > MgGTP > MgUTP $\approx$ MgCTP. This correlates well with the degree of structural similarity between MgATP and the other nucleotides (Figure 6B).

Mg²⁺ requirement for the effects of Mg-p[NH]ppA

The effects of MgATP on the trypsin-digestion profile of Pgp suggest that a change in Pgp conformation may be induced by MgATP. However, due to the high basal ATPase activity, the effect of MgATP on the trypsin-digestion profile of Pgp may not be due only to the binding of MgATP, but also to its hydrolysis product MgADP. To investigate further the MgATP-bound state, we conducted experiments using the non-hydrolysable ATP analogue, Mg-p[NH]ppA. As shown in Figure 7(A), less peptide C was detected in the presence of p[NH]ppA without Mg²⁺ than the control reaction (compare lanes 1 with 2). This decrease is likely to be due to the fact that p[NH]ppA reduced the trypsin activity (Table 1) and also that binding of p[NH]ppA to Pgp in the absence of Mg²⁺ does not change the Pgp conformation. However, when Mg²⁺ was added, more peptide C was observed in the presence of p[NH]ppA (Figure 7A, lane 3). Apparently, the effect of p[NH]ppA on the trypsin-digestion profile of Pgp requires Mg²⁺. This is consistent with the observation of ATP (see Figure 3).

Since more peptide Y was detected in the presence of MgATP, whereas more peptide C was detected in the presence of Mg-p[NH]ppA, we conclude that the binding of Mg-p[NH]ppA results in a trypsin-sensitive state of Pgp which is different from

Figure 7 Effects of Mg-p[NH]ppA on trypsin digestion of Pgp

(A) Mg²⁺ dependence of AMP-PNP (p[NH]ppA) effects. Membrane proteins (30 μ g) were pretreated for 30 min at room temperature with 5 mM p[NH]ppA in the absence (lane 1) or presence (lane 3) of 5 mM MgCl₂, followed by trypsin digestion. Pgp and its fragments were detected using the mAb MD-7. Lane 2 is a control reaction in the absence of nucleotide ligands. (B) Competition of MgATP effects by Mg-p[NH]ppA. Membrane proteins (30 μ g) were pretreated for 30 min at room temperature with MgATP (lanes 2 and 4) or Mg-p[NH]ppA (lanes 3 and 5), followed by Mg-p[NH]ppA (lane 2) or MgATP (lane 3), respectively. The membrane was then used for trypsin digestion. Lane 1 represents the control digestion without nucleotide treatment. (C) Concentration dependence of the Mg-p[NH]ppA competition. Membrane proteins (30 μ g) were pretreated for 30 min at room temperature with a mixture of 5 mM MgATP (lanes 3–7) and Mg-p[NH]ppA at 0 mM (lane 3), 0.1 mM (lane 4), 1 mM (lane 5), 3 mM (lane 6) or 5 mM (lane 7). The membrane was then used for trypsin digestion. Lanes 1 and 2 are control reactions in the absence of nucleotide treatment (lane 1) or pretreated with 5 mM Mg-p[NH]ppA only (lane 2). The increased production of C-half molecules in the presence of Mg-p[NH]ppA was partially due to the generation of a peptide slightly smaller than the peptide C (indicated by arrows). SDS/PAGE and Western blot were performed as described in the Experimental section. Pgp and its fragments were detected using the mAb MD-7.

Figure 8 Effects of vanadate and ouabain on trypsin digestion of Pgp

(A) Effects of vanadate on trypsin digestion of Pgp. Membrane proteins (30 μ g) were pretreated for 30 min at room temperature with 5 mM MgATP (lanes 2 and 4) or MgAMP-PNP (Mg-p[NH]ppA) (lanes 3 and 5) in the absence (lanes 2 and 3) or presence (lanes 4 and 5) of 100 μ M vanadate. Lane 1 is the control digestion in the absence of MgATP, Mg-p[NH]ppA and vanadate. (B) Effects of ouabain on trypsin digestion of Pgp. Membrane proteins (30 μ g) were pretreated for 30 min at room temperature with 5 mM MgATP (lanes 1 and 3) in the absence (lane 1) or presence (lane 3) of 1 mM ouabain. Lanes 2 and 4 are control reactions without pretreatment (lane 1) or pretreated with 1 mM ouabain only (lane 2). SDS/PAGE and Western blot were performed as described in the Experimental section. Pgp and its fragments were detected using the mAb MD-7.

that induced by MgATP. It is likely that MgATP is hydrolysed to generate product MgADP, resulting in the MgADP-bound state of Pgp which has a different trypsin sensitivity from the Mg-p[NH]ppA-bound state (see Figure 1). This is consistent with the observation that the same proteolysis profile was generated by MgATP and MgADP (see Figure 3). It should be noted that the increase in the amount of C-half molecules in the presence of Mg-p[NH]ppA was partly due to the generation of a peptide fragment which is slightly smaller than peptide C. The two peptides run as a doublet on SDS/PAGE (see the peptide indicated by an arrow in Figure 7B).

Competition of MgATP-stimulated trypsin digestion of Pgp by Mg-p[NH]ppA

To investigate the possible existence of different trypsin-sensitive states of Pgp, we performed a competition study using Mg-p[NH]ppA and MgATP. As shown in Figure 7(B), pretreatment of Pgp with Mg-p[NH]ppA prevents the increased accumulation of peptide Y induced by MgATP (compare lanes 3 and 4, Figure 7B). Treatment of Pgp with Mg-p[NH]ppA following the pretreatment with MgATP (compare lanes 2 and 4, Figure 7B) also prevented the increase in the amount of peptide Y. Furthermore, more peptide C was detected in all cases with added Mg-p[NH]ppA (Figure 7B, lanes 2, 3 and 5), indicating the existence of an alternative trypsin-sensitive state of Pgp bound by Mg-p[NH]ppA. Similar results were also observed using MgADP (results not shown).

To determine the concentration dependence of Mg-p[NH]ppA on inhibition of the MgATP-induced change in the trypsin-digestion profile of Pgp, we performed a trypsin digestion with increasing concentrations of Mg-p[NH]ppA in the presence of 5 mM MgATP. As shown in Figure 7(C), a decrease in the amount of peptide Y began in the presence of 0.1 mM Mg-p[NH]ppA. More than 80% of the MgATP-induced accumulation of peptide Y was eliminated in the presence of 1 mM Mg-

p[NH]ppA, and MgATP-induced peptide Y was not observed in the presence of 5 mM Mg-p[NH]ppA. With the elevated concentration of Mg-p[NH]ppA, the amount of peptide Y was decreased and that of peptide C was increased. These observations further suggest that more than one trypsin-sensitive state of Pgp exists, indicating that Mg-p[NH]ppA can lock Pgp into one of the states.

Effects of vanadate on MgATP-stimulated trypsin digestion of Pgp

It has been shown previously that vanadate is a potent inhibitor of Pgp ATPase [25]. To determine whether vanadate affects the MgATP-induced or the MgADP-induced change in trypsin sensitivity of Pgp, we performed trypsin digestion of Pgp in the presence of vanadate (100 μ M) and MgATP (5 mM). As shown in Figure 8(A), MgATP increased the amount of peptide Y (lane 2), whereas vanadate alone had no effect (results not shown). However, vanadate decreased the amount of peptide Y detected in the presence of MgATP (lane 4). It is interesting to note that the decreased amount of peptide Y was accompanied by the increased amount of peptide C in the presence of vanadate and MgATP (Figure 8A, lane 4). The increased accumulation of peptide C is normally associated with binding to the non-hydrolysable analogue Mg-p[NH]ppA (see Figure 7), whereas that of peptide Y is associated with the hydrolysed product MgADP. We therefore believe that the digestion profile generated in the presence of MgATP and vanadate implicates the existence of an intermediate state between the MgATP-bound and MgADP-bound molecules.

To determine whether vanadate has any effect on the Mg-p[NH]ppA-induced proteolysis of Pgp, we performed a trypsin digestion of Pgp in the presence of vanadate and Mg-p[NH]ppA. As shown in Figure 8(A), vanadate apparently did not change the amount of peptide C generated in the presence of Mg-p[NH]ppA (compare lanes 3 and 5, Figure 8A). It should be noted that a small amount of peptide Y was detected in the presence of MgATP and vanadate. However, no peptide Y was found in the presence of Mg-p[NH]ppA (compare lanes 3 and 4, Figure 8A). Thus the proteolysis profile in the presence of MgATP/vanadate is different from that in the presence of Mg-p[NH]ppA. We conclude that the effect of vanadate on the MgATP-induced proteolysis of Pgp is exerted at the step after ATP hydrolysis and this locks Pgp into an intermediate conformational state.

We also determined whether ouabain affects the MgATP-induced change in the trypsin-digestion profile of Pgp. As shown in Figure 8(B), 1 mM ouabain did not alter the MgATP-induced change in trypsin digestion of Pgp. This observation is consistent with the previous studies that ouabain does not inhibit the ATPase activity of Pgp [27,28].

DISCUSSION

ABC transporters accomplish their functions at the expense of ATP [3]. ATP-binding domains are thought to be important in supplying the energy required for substrate translocation. The binding and hydrolysis of nucleotides is likely to change the protein conformation. Pgp has a high basal ATPase activity and thus the hydrolysis of MgATP by Pgp is not necessarily coupled to drug transport. This provides a convenient way to investigate conformational changes in Pgp induced by nucleotide ligands, without a drug-induced conformational change acting as a complicating factor [30], although membrane lipids may be potential substrates [42–44]. In this study, we used limited proteolysis of Pgp in inside-out membrane vesicles, in com-

bination with Western blotting, to investigate the conformational change of Pgp during its catalytic cycle. We observed that nucleotides in the presence of Mg^{2+} changed the trypsin-digestion profile of Pgp, suggesting that Pgp conformation is changed by the binding of nucleotide ligands.

The nucleotide-enhanced accumulation of peptide fragments from Pgp was not due to the increased trypsin activity in the presence of nucleotides. In fact, this enhancement in the presence of MgATP, MgADP or Mg-p[NH]ppA was accomplished by only 46–54% of the remaining trypsin activity. There is no correlation between the change in trypsin activity and the change in proteolysis profile of Pgp (see Table 1). It appears that all the nucleotides tested reduced the trypsin activity, but to differing degrees. However, there is no direct relationship between the reduction of trypsin activity and the change in trypsin-digestion profile of Pgp. For example, 5 mM ATP, MgATP and Mg-p[NH]ppA all reduced approx. 50% of the trypsin activity, yet the trypsin-digestion profile of Pgp in the presence of these nucleotides is different.

Limited trypsin digestion has been used previously to investigate conformational changes in non-membrane-bound ATP-binding subunits of bacterial MalK transporter [33], F_1 -ATPase [34,35] and the ArsA anion pump [36]. In all these cases, it was found that the trypsin sensitivity of the ATP-binding domain was decreased by MgATP, which could have been explained by the decreased trypsin activity in the presence of MgATP. The most important and novel observation of the current study is that the trypsin sensitivity of a domain away from the nucleotide-binding fold, including the linking region between the N- and C-half domains and the loop linking TM10 and TM11, was altered in the presence of MgATP or MgADP. This change causes the increased accumulation of peptide Y (see discussion below on Figure 1). The requirement of Mg^{2+} for the effects of ATP, ADP and Mg-p[NH]ppA on the trypsin-digestion profile of Pgp suggests that Mg^{2+} is necessary for the effects of these nucleotides on the conformational changes in Pgp. Previously, it has been shown that the ATPase activity of Pgp requires Mg^{2+} [21,22] and that the inhibition of Pgp ATPase activity by vanadate-induced trapping of nucleotides also requires Mg^{2+} [25]. MgAMP has no effect on trypsin digestion of Pgp, suggesting that it does not bind to Pgp, consistent with the study by Al-Shawi and Senior [21].

Since Pgp has high basal ATPase activity [19–22] and the assay was followed for 2.5 h, the observed effects of MgATP on Pgp may well be due to its hydrolysed product MgADP. This is supported by several observations. First, both MgATP and MgADP increased the amount of peptide Y. Secondly, the effects of both MgATP and MgADP on the trypsin-digestion profile of Pgp differ from those of the non-hydrolysable analogues Mg-p[NH]ppA or MgATP[S]. Thirdly, both pre- and post-treatment with Mg-p[NH]ppA inhibit the effects of MgATP on trypsin digestion of Pgp. This inhibition may be due to the binding of Mg-p[NH]ppA after the release of hydrolysed product MgADP (see Figure 1). Fourthly, vanadate inhibits the effects of MgATP on trypsin digestion of Pgp, but not those of Mg-p[NH]ppA, indicating that MgATP is hydrolysed.

The increase in trypsin digestion of Pgp was observed when 0.5–1 mM MgATP was present. This is consistent with the estimated K_m (MgATP) value of 0.6–1.4 mM for Pgp [20–23]. Although the K_m (Mg-p[NH]ppA) for Pgp is not known, Al-Shawi and Senior [21] have estimated that the K_i of Mg-p[NH]ppA for Pgp ATPase activity is 0.44 mM. This is consistent with our observation that the inhibition of Mg-p[NH]ppA on the MgATP-stimulated accumulation of peptide Y was initiated at 0.1 mM of Mg-p[NH]ppA and more than 80% of peptide Y was converted into peptide C with 1 mM Mg-p[NH]ppA.

MgITP, MgGTP, MgCTP and MgUTP also changed the trypsin-digestion profile of Pgp. This is consistent with previous studies showing that Pgp can hydrolyse both MgITP and MgGTP [21]. Moreover, both MgUTP and MgCTP can support vanadate-induced inhibition of Pgp ATPase activity [25]. We also observed that the effectiveness of these nucleotides on trypsin sensitivity of Pgp varies in the order of $MgATP > MgITP > MgGTP > MgCTP \approx MgUTP$. This order of action is consistent with their order of preference for hydrolysis by Pgp [21], and also with their structural similarity to ATP. However, considering that the trypsin activity was inhibited two-fold more by MgITP and four-fold more by MgGTP than that achieved by MgATP (Table 1), the enhanced trypsin digestion of Pgp by these three nucleotides may be very closely matched. On the other hand, MgUTP and MgCTP inhibited trypsin activity less than MgATP, their effects on trypsin digestion of Pgp would be less than those observed had their inhibition been the same as MgATP. In all cases, nucleotides were not found to increase trypsin activity (Table 1). These observations suggest that the effects on trypsin cleavage of Pgp by all nucleotides is likely to be due to their direct binding and hydrolysis by Pgp.

On the basis of the results of this study, we propose that the different trypsin-sensitive states of Pgp represent different conformational states in its catalytic cycle. We speculate that Pgp has four conformational states, which were trapped by the binding of nucleotides and inhibitors. These four states are shown schematically in Figure 1. It is not known whether the catalytic cycle is different in the presence of drug substrates. Since Pgp has high basal ATPase activity and the ATPase activity is not tightly coupled with drug transport, Pgp can pass through the cycle in the absence of drug substrates. However, it has been shown that Pgp can catalyse the transport of lipids [42–44]. Thus one can speculate that the ATP hydrolysis catalysed by Pgp in the absence of drug substrates is coupled to lipid flipping in the membrane.

In this study, state II of Pgp was stabilized by using the binding of non-hydrolysable ATP analogues, Mg-p[NH]ppA or MgATP[S]. When Pgp molecules have reached this state, more peptide C is produced, presumably because the linking domain between the two half-molecules is further exposed. State IV of Pgp was identified by the binding of MgADP. Trypsin digestion of the stabilized state IV molecules enhanced the accumulation of peptide Y. In the presence of MgATP, the trypsin-digestion profile of Pgp is the same as that in the presence of MgADP. This is likely to be due to the hydrolysis of MgATP, resulting in the MgADP-bound form (state IV). The state III molecule is presumably present as a short-lived, transient and unstable intermediary state. The conformational state of Pgp generated in the presence of vanadate and MgATP may represent this state. The fact that the trypsin-digestion profile generated in the presence of both MgATP and vanadate is different from that generated by MgADP (state IV) or by Mg-p[NH]ppA alone (state II) suggests that it is likely to represent the intermediate state (see Figure 8). It has been shown previously that MgATP was hydrolysed in the vanadate-bound species of Pgp [25]. We therefore propose that the vanadate-inhibited species does not contain MgATP, but contains MgADP and vanadate (Figure 1). This is supported by the observation that vanadate did not affect the Mg-p[NH]ppA-induced digestion of Pgp. Future work is being directed to determine whether the changes between each state are reversible, and how the putative conformational-change cycle driven by the binding and hydrolysis of MgATP couples with the drug transport.

Using IR spectroscopy, Sonveaux et al. [29] detected an overall conformational change of Pgp in the presence of MgATP and

verapamil. MgATP alone induced an increased accessibility to the solvent of a population of amino acids of Pgp. MgATP-verapamil resulted in a subtraction of a part of the protein from access to the aqueous solvent. However, the nature and the site of the change were not discussed. In this study using trypsin digestion, we not only observed the change in Pgp conformation, but also detected the four states of Pgp in its catalytic cycle and localized the change of conformation in the region close to the loop 8. It should be noted that Sonveaux et al. [29] detected a smaller change of Pgp conformation in the presence of MgADP in comparison with that in the presence of MgATP. This is different from our observation that MgADP had similar effects to MgATP. The reason for this difference is currently unknown. However, the property of Pgp may have been altered by solubilization, purification and reconstitution procedures, since the ATPase activity of Pgp depends on lipid composition and is affected by detergent [27,30]. Alternatively, the specific change detected using MD-7 in our study may be compensated by changes in other regions of the molecule which cannot be distinguished by IR spectroscopy.

Previously, different topologies of Pgp in MDR cells were observed [17] (see also Figure 1). Colicin Ia, a voltage-sensitive channel, has been shown to involve a massive change in membrane topology between the opening and closed states [45]. At least 31 amino acids of colicin Ia located in the *cis*-side of the membrane appear to be translocated to the *trans*-side of the membrane when the channel changes from a closed to an open state. It therefore cannot be ignored that Pgp may also involve a dramatic topological change. It is interesting to note that more of peptide Y, including loop 8, was generated from Pgp by MgATP or MgADP. This observation is in fact consistent with the generation of more model B topology, as shown in Figure 1. Although the evidence for the existence of alternative topologies of Pgp is still controversial [16,18], we cannot rule out the possibility that the different states of Pgp detected in this study represent the different topological orientations of Pgp. This hypothesis is also supported by the observation that more amino acids were exposed to aqueous solvent (presumably movement of residues from inside the membrane to outside) induced by the binding of MgATP [29]. More detailed work is clearly needed to map the precise topology and the possible topological changes of Pgp during the drug-transport process.

We thank Drs. Victor Ling and Adam Shapiro for the mAbs MD-7, C219, C494 and their valuable suggestions. We also thank our colleagues at University of Texas Medical Branch for their helpful discussion over the course of this study. Critical comments on this manuscript from Drs. Guillermo Altenberg, Steve King and Malcolm Brodwick are appreciated. This work was supported by a NIH grant CA64539 and by a grant from the U.S. Army Medical Research and Development Command.

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