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Mechanism of Action of Human P-glycoprotein ATPase Activity

PHOTOCHEMICAL CLEAVAGE DURING A CATALYTIC TRANSITION STATE USING ORTHOVANADATE REVEALS CROSS-TALK BETWEEN THE TWO ATP SITES*

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Human P-glycoprotein (P-gp), an ATP-dependent efflux pump responsible for cross-resistance of human cancers to a variety of lipophilic compounds, is composed of two homologous halves, each containing six transmembrane domains and an ATP-binding/utilization domain. To determine whether each site can hydrolyze ATP simultaneously, we used an orthovanadate (Vi)-induced ADP-trapping technique (P-gp·MgADP·Vi). In analogy with other ATPases, a photochemical peptide bond cleavage reaction occurs within the Walker A nucleotide binding domain consensus sequence ($GX_4GK(T/S)$) when the molecule is trapped with Vi in an inhibited catalytic transition state (P-gp·MgADP·Vi) and incubated in the presence of ultraviolet light. Upon reconstitution into proteoliposomes, histidine-tagged purified P-gp from baculovirus-infected insect cells had drug-stimulated ATPase activity. Reconstituted P-gp was incubated with either ATP or 8-azido-ATP in the presence or absence of Vi under ultraviolet (365 nm) light on ice for 60 min. The resultant products were separated by SDS-polyacrylamide gel electrophoresis and subjected to immunoblotting with seven different human P-gp-specific antibodies covering the entire length of the molecule. Little to no degradation of P-gp was observed in the absence of Vi. In the presence of Vi, products of approximately 28, 47, 94, and 110 kDa were obtained, consistent with predicted molecular weights from cleavage at either of the ATP sites but not both sites. An additional Vi-dependent cleavage site was detected at or near the trypsin site in the linker region of P-gp. These results suggest that both the amino- and carboxyl-terminal ATP sites can hydrolyze ATP. However, there is no evidence that ATP can be hydrolyzed simultaneously by both sites.

One of the main causes of broad-based cellular resistance to a wide variety of cytotoxic agents in cancer cells is expression of a 170-kDa plasma membrane polypeptide known as the multidrug transporter or P-glycoprotein (P-gp),[1] encoded by the *MDR*1 gene in humans (1, 2). This 1280-amino acid plasma membrane-associated glycoprotein is composed of two homologous halves, each containing six transmembrane domains and one ATP site. P-gp acts as an ATP-dependent efflux pump for chemotherapeutic agents and other drugs (3). The precise mechanism of action of P-gp, however, remains unknown. ATP binding and hydrolysis are essential for the proper functioning of P-gp. It has been previously demonstrated that each ATP site in P-gp can hydrolyze ATP and that both sites must be intact to retain activity of the transporter (4, 5). These data suggested a model of P-gp action in which the ATP sites alternate and do not hydrolyze ATP simultaneously (6, 7).

To determine whether both sites hydrolyze ATP simultaneously, we used orthovanadate (Vi), a phosphate analog that stabilizes the inhibited catalytic transition state of P-gp (P-gp·MgADP·Vi), mimicking the physiological state in which MgADP and phosphate are bound and subsequently released (8). Upon incubation with Vi and ATP, only one cycle of hydrolysis occurs as a result of the stabilization of the inhibitory complex (9). When this complex is irradiated with ultraviolet light, a photochemical reaction occurs modifying the amino acid in the third position within the Walker A nucleotide binding domain consensus sequence ($GX_4GK(T/S)$) (10) followed by cleavage of the peptide bond (11). This technique has been successfully used to study the mechanism of action of myosin-ATPase (12, 13), adenylate kinase (14), and most recently, the F_1-ATPase from rat liver mitochondria (15). The studies described here represent the first use of this technique in the study of an ATP-binding cassette (ABC) transporter. The results indicate that ATP hydrolysis occurs within one or the other ATP site but could not be detected in both simultaneously.

EXPERIMENTAL PROCEDURES

Expression and Purification of Wild-type P-gp Containing a C-terminal 6-Histidine Tag (P-gp·H_6)—Recombinant baculovirus encoding wild-type P-gp containing a six-histidine tag at the C terminus (BV-MDR1(H_6)) was used to infect *Trichoplusia ni* (High Five™) cells (Invitrogen, San Diego, CA) as described (16). P-gp·H_6 was purified by metal affinity chromatography as described (16). Protein concentration of the purified material was determined by the Amido Black 10B protein assay (17). Approximately 300 μg of purified protein was obtained from 20 mg of crude membrane protein prepared from 2×10^8 cells.

Preparation of Sodium Orthovanadate—Sodium orthovanadate was freshly prepared in water and heated at 100 °C for 3 min, vortexed, and cooled to room temperature. The concentration of the stock solution was determined spectrophotometrically at A_{268} (molar extinction coefficient,

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[1] The abbreviations used are: P-gp, P-glycoprotein; P-gp·H_6, human P-glycoprotein containing a 6-histidine tag at the C terminus of the protein; Vi, sodium orthovanadate; UV, ultraviolet light at 365 nm; PAGE, polyacrylamide gel electrophoresis; MOPS, 4-morpholinepropanesulfonic acid; aa, amino acid.

2925 M^{-1} cm^{-2}). Vi prepared in this manner may consist of varying amounts of monomeric vanadate and its oligomers, di-, tetra-, and pentavanadate.

Measurement of Vi-sensitive ATPase Activity in Proteoliposomes Reconstituted with Purified P-gp·H_6—Vi-sensitive ATPase activity in purified P-gp·H_6 preparations was performed as described (16, 18).

Photochemical Cleavage of Purified P-gp·H_6—Purified P-gp·H_6 (4 μl; ~1.4 μg) was first diluted 25-fold to a final volume of 100 μl to form proteoliposomes in 50 mM MOPS·KOH (pH 7.2), 125 mM KCl, 5 mM $MgCl_2$ in the presence and absence of 600 μM sodium orthovanadate in 12 × 75-mm glass test tubes and allowed to incubate at room temperature for 3 min. Verapamil (30 μM) was subsequently added to all samples from a 3 mM stock made in Me_2SO, and the tubes were allowed to stand at room temperature for an additional 3 min. Subsequently, 2.5 mM ATP was added, and the reaction mixture was immediately transferred to ice. In reactions where photoactivation was induced by UV light, the samples were transferred to a 96-well flat-bottom cluster and were placed under a 365-nm ultraviolet (UV) lamp (Black-Ray lamp from UVP, Upland, CA) on aluminum foil-covered ice under subdued light conditions. The samples were irradiated for 40 min covered with a glass plate at a distance of 1.3 cm and for an additional 20 min uncovered at a distance of 1 cm. For samples containing 8-azido-ATP, the reaction was pre-irradiated for 10 min on ice prior to addition of drug and Vi.

SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Immunoblot Analysis—Samples were prepared in 1× Laemmli sample buffer (19) and allowed to incubate at room temperature for 30 min prior to electrophoresis. SDS-PAGE was performed (19) using 8, 8–16, and 4–20% Tris/glycine gels (Novex, San Diego, CA) followed by immunoblotting as described (16). Immunoreactive bands were visualized by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

Antibodies—Monoclonal anti-P-gp antibody C219 (Centocor, Malvern, PA) (20) was used at a 1:2000 dilution. Human specific anti-P-gp polyclonal antibodies 4007 and 4077 (21) were used at dilutions of 1:1000 and 1:3000, respectively. Polyclonal human specific anti-P-gp antibodies PEPG-13, PEPG-2, and PEPG-7 were used at dilutions of 1:3000, and PEPG-12 was used at a dilution of 1:1000 (22).

Mild Trypsin Digestion of Purified P-gp·H_6—Purified P-gp·H_6 (1.4 μg) was diluted in a total volume of 100 μl in 50 mM Tris·HCl (pH 8.0). Modified trypsin (Promega) was added at a ratio of 30:1 protein:trypsin (0.046 μg). The reaction was carried out for 5 min at 37 °C. Subsequently, a 5-fold excess of trypsin inhibitor was added followed by Laemmli sample buffer.

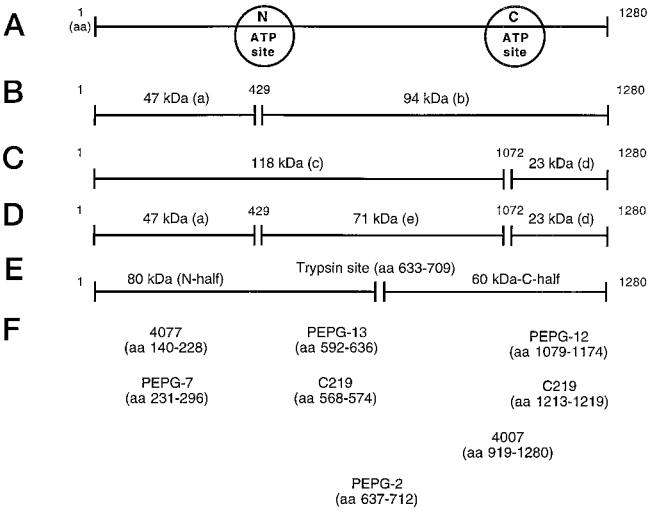

FIG. 1. **Schematic representation of human P-glycoprotein and the potential UV-induced vanadate-dependent polypeptide bond cleavage positions and products.** *A,* full-length P-gp shown with N- and C-terminal ATP sites as *circles*. *B,* peptide fragments generated if cleavage occurs in the N-terminal ATP site at serine 429. The predicted 47-kDa peptide is referred to as (*a*) and the predicted 94 kDa peptide as (*b*) in the text. *C,* peptide fragments generated if cleavage occurs in the C-terminal ATP site at serine 1072. The predicted 118-kDa peptide is referred to as (*c*) and the 23-kDa peptide as (*d*) in the text. *D,* peptide fragments generated if simultaneous peptide bond cleavage occurs in both ATP sites at serine residues 429 and 1072. The three peptides generated would be the 47 kDa (*a*), the 71 kDa (*e*), and the 23 kDa (*d*). These are referred by the letter designations in the text. *E,* peptide fragments generated after mild trypsinization of P-gp. An 80-kDa peptide representing the N-terminal half of P-gp (*N-half*) and a 60-kDa peptide representing the C-terminal half (*C-half*) result. *F,* antibodies used to detect the various fragments by immunoblot analysis. All antibodies are human P-gp-specific except for C219, which recognizes other species of P-gp with two epitopes as shown.

RESULTS AND DISCUSSION

Human P-gp is a 1280-amino acid protein with two homologous halves functionally connected by a flexible linker region. Each half contains six hydrophobic transmembrane regions implicated in the binding of substrates and inhibitors based on photoaffinity labeling studies and the behavior of mutant transporters and a highly conserved ATP binding/utilization domain (1). Through mutational analysis, it has been demonstrated that both sites are essential for function since disruption of either nucleotide binding domain results in an inactive protein (23–25). Biochemical analyses using P-gp from Chinese hamster ovary cells have revealed that each ATP site is capable of hydrolyzing ATP (4).

In this study, we sought to determine whether both ATP sites of human P-gp were acting independently and hydrolyzing ATP simultaneously or if cross-talk exists between the two sites that allows for only one hydrolysis event to occur at a time. This alternating catalytic site model of ATP hydrolysis was originally suggested by Senior and colleagues (6) in studies that demonstrated that 1 mol of Mg^{2+}-8-azido-ADP was bound per mol of hamster P-gp. This hypothesis has been supported by experiments involving chemical modification of one ATP site that prevented vanadate trapping at the other site (5).

Model for Photooxidative Peptide Bond Cleavage of Human P-gp—To assess directly whether ATP hydrolysis can occur at both sites simultaneously, we made further use of sodium Vi, a phosphate analog that is photochemically active. Irradiation with UV light at 365 nm results in specific oxidations of protein side chains within Vi-trapped species and in peptide bond cleavage. The mechanism of photocleavage for myosin, which involves a seryl radical intermediate, has been determined by Grammer *et al*. (11). ATPases form a MgADP·Vi·enzyme inhibitory complex, which upon irradiation results in peptide bond cleavage at the third position within the Walker A motif (GX_4GKT). This has been shown directly for myosin (serine) (12, 13), F_1-ATP synthase (alanine) (15), and adenylate kinase (proline) (14), and flagellar ATPase dynein (26). Human P-gp has a serine residue at the third position in both nucleotide binding domains.

In the case of the heavy chain of myosin, an additional nucleotide-independent vanadate cleavage site is observed upon incubation with Vi and UV light (12). This second site, termed V2, is a few amino acids away from the trypsin-sensitive site between the 50- and 20-kDa tryptic fragments of the myosin heavy chain, perhaps because of tetrameric vanadate binding to a series of positively charged lysine residues next to a potentially photosensitive residue (12). Interestingly, human P-gp also has a lysine/arginine-rich region in the linker region between the two halves of the protein, which contains a trypsin site that is sensitive to enzymatic cleavage (27).

Schematic Representation of Potential UV-induced Vanadate Cleavage Sites in Human P-gp—A schematic diagram of the potential vanadate cleavage sites in human P-gp is shown in Fig. 1. The cleavage products are identified using antibodies specific for P-gp. The epitopes for these various antibodies are shown in Fig. 1*F*. If simultaneous hydrolysis occurs at both ATP sites, three fragments would be generated (Fig. 1*D*) of approximately 47, 71, and 23 kDa. If hydrolysis occurs only at the N-terminal site (Fig. 1*B*), only 47- and 94-kDa fragments would be produced as a result of the UV-induced cleavage reaction in the presence of Vi. Conversely, if hydrolysis only

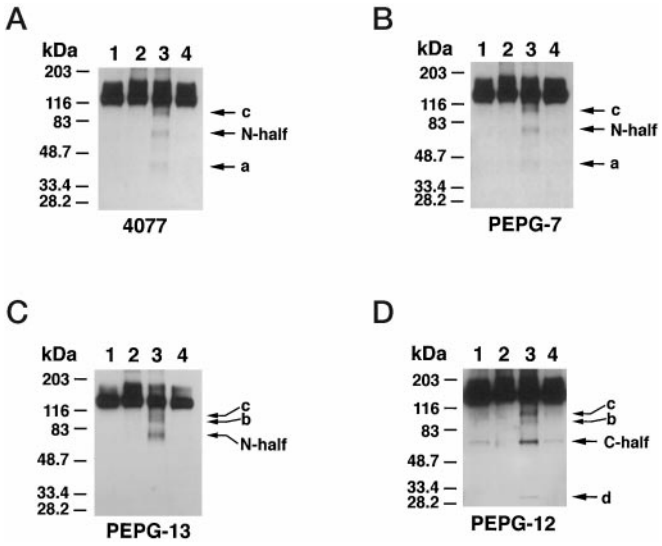

FIG. 2. **Immunoblot analysis of peptide fragments generated by UV-induced Vi-dependent photocleavage of a Mg^{2+}ADP·Vi·P-gp transition state complex.** Purified P-gp·H_6 (1.4 μg) was reconstituted by rapid dilution and subjected to photocleavage reaction conditions and UV irradiation as described under "Experimental Procedures." All samples contain 2.5 mM ATP. *Lane 1,* −Vi/−UV; *lane 2,* −Vi/+UV; *lane 3*, +Vi/+UV; *lane 4,* +Vi/−UV. Approximately 175 ng of protein from each reaction mixture was loaded per lane on 8–16% Tris/glycine polyacrylamide gels and subjected to SDS-PAGE and immunoblotting. Each *panel r*epresents a separate rabbit polyclonal anti-human P-gp antibody: *panel A,* 4077; *panel B,* PEPG-7; *panel C,* PEPG-13; *panel D,* PEPG-12. The specificities of these antibodies are shown in Fig. 1*F*. Peptide fragment assignments are denoted by *arrows* and *lowercase letters* to the *right* of the blots as defined in Fig. 1. N-half and C-half designations refer to the N-terminal and C-terminal halves of human P-gp generated by peptide bond cleavage at or near the trypsin site in the linker region of P-gp defined as being between amino acids 633 and 709.

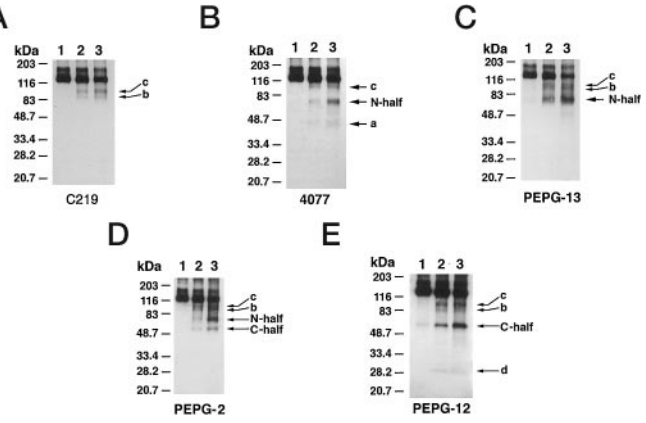

FIG. 3. **Both ATP and 8-azido-ATP can facilitate UV-induced Vi-dependent photocleavage, and an additional polypeptide cleavage occurs near the trypsin-sensitive site in the linker region of P-gp.** Purified P-gp·H_6 (1.4 μg) was reconstituted by rapid dilution and subjected to photocleavage reaction conditions as described under "Experimental Procedures." All samples were subjected to UV irradiation. *Lane 1*, +ATP/−Vi; *lane 2,* +ATP/+Vi; *lane 3,* +8-azido-ATP/+Vi. Approximately 175 ng of each reaction mixture was loaded per lane on 4–20% Tris/glycine polyacrylamide gels and subjected to SDS-PAGE and immunoblotting. Each *panel r*epresents an anti-P-gp antibody: *panel A,* C219; *panel B,* 4077; *panel C,* PEPG-13; *panel D,* PEPG-2; *panel E,* PEPG-12. Fragment assignments are as described in the legend to Fig. 2.

occurs at the C-terminal ATP site (Fig. 1*C*), only fragments of predicted molecular masses of 118 and 23 kDa would be generated. If both sites were active but not in the same molecule, 47-, 94-, 118-, and 23-kDa fragments would be predicted. If nucleotide-independent cleavage at or near the trypsin site of P-gp occurs, the molecule would also be cleaved into two peptides of 80 and 60 kDa representing the N-terminal and C-terminal halves of the protein, respectively (Fig. 1*E*).

Purified P-gp Retains Vi-sensitive Drug-stimulated ATPase Activity—To facilitate identification of the UV-induced vanadate cleavage products and to eliminate other interfering ATPases, we used wild-type P-gp containing a six-histidine tag at the C terminus (P-gp·H_6) purified from insect cells using metal affinity chromatography as described under "Experimental Procedures" (16). Using a rapid dilution method to reconstitute the protein into proteoliposomes, Vi-sensitive drug-stimulated ATPase activity of P-gp·H_6 was confirmed (16). The reconstituted protein demonstrated high specific activity (5.8 μmol/min/mg of protein) in the presence of 30 μM verapamil. This method reconstitutes approximately 20% of the starting material, 50% of which has ATPase activity (16). This yield of functional P-gp was used to calculate the specific activity of the protein.

UV-induced Vi Cleavage of the Human P-gp·H_6 Polypeptide Chain—We performed the UV-induced vanadate cleavage reactions under the same optimal conditions as in the ATPase activity assay described above, except that MOPS buffer was substituted for Tris because it is known that Tris buffers result in less efficient photocleavage because of the formation of stable Tris·Vi complexes (28). P-gp ATPase activity is comparable in either Tris or MOPS buffer (data not shown). Purified P-gp·H_6 was reconstituted into proteoliposomes by rapid dilution either in the presence or absence of Vi. To start the formation of the MgADP·Vi·P-gp complex, 2.5 mM ATP was added, and the samples were immediately irradiated on ice for a total of 60 min and subjected to SDS-PAGE and immunoblot analysis as described under "Experimental Procedures."

Photooxidative Peptide Bond Cleavage at the ATP Sites of Human P-gp Is Vi- and UV-dependent—Peptide bond cleavage occurs only in the presence of Vi and UV irradiation (Fig. 2, *lanes 3*). UV irradiation alone generated little or no cleavage products (Fig. 2, *lanes 2*). Additionally, little or no cleavage was observed in the absence of UV light either in the presence or absence of Vi (Fig. 2, *lanes 1* and *4*). Because both the amount of functional P-gp·H_6 in the reaction (200 ng) and the efficiency of the cleavage reaction were extremely low, we could not visualize the bands by Coomassie Brilliant Blue or silver stain nor could we generate enough of each fragment for N-terminal sequencing. However, using a variety of human P-gp-specific antibodies that recognize different regions of the molecule, we were able to clearly identify the cleavage products (*a*), (*b*), (*c*), and (*d*) but not (*e*) (Fig. 1*F*). Because of the hydrophobic nature of the peptides generated, there was a slight disparity between the predicted molecular weights of the products (Fig. 1) and the apparent molecular weights as determined by SDS-PAGE. The crucial results are, however, that we detect the higher molecular weight fragments (*b* and *c*) but not fragment (*e*) (Fig. 1), arguing against simultaneous hydrolysis and cleavage at both ATP sites. The absence of this fragment does not prove unequivocally that it is not being formed in amounts below the level of detectability. We do not believe that it is present but migrating anomalously because we could not detect this fragment in several gel systems with different antibodies.

Human P-gp-specific polyclonal antibodies 4077 (21) (Fig. 2*A*) and PEPG-7 (22) (Fig. 2*B*) directed against peptides prior to the N-terminal ATP site recognize fragments (*a*) and (*c*). Polyclonal antibody PEPG13 (22) directed against a peptide between the two ATP sites recognizes fragments (*b*) and (*c*) (Fig. 2*C*). Polyclonal antibody PEPG-12 directed against a peptide after the C-terminal ATP site recognizes fragments (*b*), (*c*), and (*d*) (Fig. 2*D*). PEPG-12 was made against the entire loop

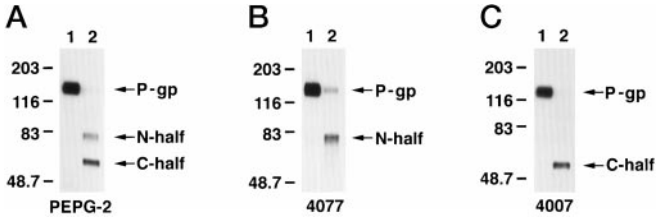

FIG. 4. **Mild trypsin digestion of purified P-gp·H_6.** Purified P-gp·H_6 (1.4 μg) was subjected to mild trypsin digestion as described under "Experimental Procedures" followed by SDS-PAGE on 8% Tris/glycine polyacrylamide gels (~100 ng/lane) and immunoblot analysis with human anti-P-gp-specific antibodies PEPG-2 (*panel A*), 4077 (*panel B*), and 4007 (*panel C*). The migration positions of the full-length P-gp (*P-gp*) and the N-terminal (*N-half*) and C-terminal (*C-half*) halves of the protein are shown with *arrows*.

between the Walker A and the linker dodecapeptide in the ATP-binding domain (22). This region is 50% identical and 70% similar to the homologous region in the N-terminal half, which may explain the cross-reactivity and the recognition of fragment (*c*). Polyclonal antibody 4007, which spans both sides of the C-terminal ATP site and is expected to react with all three bands, gave the same pattern as PEPG-12, which is also suggestive of cross-reactivity (data not shown). Fragment (*e*), the expected product if simultaneous cleavage occurs, was not detected using PEPG-13, C219, PEPG-2, and 4007.

The data shown in Fig. 3 provide further evidence for the generation of fragments (*a*), (*b*), (*c*), and (*d*) but not (*e*). These experiments were done in the presence and absence of Vi using both ATP (*lanes 1* and *2*) and 8-azido-ATP (*lanes 3*) as nucleotides. We initially used 8-azido-ATP, which is also hydrolyzable by P-gp (6), to determine whether ATP cross-linked to P-gp would block UV-induced vanadate cleavage and found that it does not. As can be seen in *panel A*, using the P-gp-specific monoclonal antibody C219, both nucleotides can support the cleavage reaction and the antibody recognizes the (*b*) and (*c*) products; however, under the blotting conditions used, (*d*) fragment is not recognized. Both PEPG-2 (*panel D*) and PEPG-13 (*panel C*) recognize the (*b*) and (*c*) cleavage products. 4077 (*panel B*) recognizes products (*a*) and (*c*) and PEPG-12 (*panel E*), and 4007 (data not shown) recognizes (*b*), (*c*), and (*d*).

Vi-induced Cleavage of Human P-gp Near the Trypsin-sensitive Site in the Linker Region—In our experiments, two additional bands migrating at approximately 80 and 60 kDa are apparent (Figs. 2 and 3). The 80-kDa band cross-reacts with 4077 (Figs. 2*A* and 3*B*), PEPG-13 (Figs. 2*C* and 3*C*), and PEPG-7 (Fig. 2*B*), and the 60-kDa band is preferentially recognized by PEPG-12 (Figs. 2*D* and 3*E*) and 4007 (data not shown). Both bands were recognized by PEPG-2 (Fig. 3*D*). Because the 80-kDa peptide is recognized well by 4077, PEPG-7, and PEPG-9 directed against amino acids 348–419 (data not shown), it is unlikely that this fragment represents fragment (*e*) (Fig. 1), the product of UV-induced Vi peptide bond cleavage at both ATP sites in the same P-gp molecule. Because both bands were recognized by PEPG-2, the cleavage site must necessarily reside between amino acids 637 and 712. Conversely, PEPG-12 and 4007 preferentially recognize the C-terminal half although PEPG-12 can under certain conditions weakly recognize some fragments containing the N-terminal half. Under the conditions used in this experiment, C219 does not detect these fragments (Fig. 3*A*). Importantly, the linker region of human P-gp, defined as the peptide segment between amino acids 633 and 709, is known to be sensitive to cleavage by mild trypsin digestion (27). Taken together, these results suggest that the two bands most likely represent the N- and C-terminal halves of P-gp produced by vanadate cleavage at or near this lysine/arginine-rich trypsin-sensitive region of P-gp, as has been previously observed for myosin (12). Additionally, upon overexposure of these immunoblots (data not shown), the natural degradation products generated during manipulation of the untreated samples for electrophoretic analysis are apparent and migrate in the same positions as the Vi-induced 80- and 60-kDa products, lending credence to the argument that these bands represent the two halves of the protein.

To further confirm the identity of these fragments representing the N- and C-terminal halves of P-gp·H_6, the purified protein was treated mildly with trypsin, followed by SDS-PAGE, immunoblotting, and probing with human P-gp-specific antibodies (Fig. 4). PEPG-2 (Fig. 4*A*) recognizes both halves of the protein whereas 4077 (Fig. 4*B*) recognizes the N-terminal half and 4007 preferentially recognizes the C-terminal half (Fig. 4*C*). Migration positions are similar to those observed in the cleavage reactions shown in Figs. 2 and 3.

Mechanism of ATP Hydrolysis of Human P-gp—In this study, we have demonstrated that both ATP sites of human P-glycoprotein are capable of hydrolyzing ATP because we generate peptide products accounting for cleavage at both active sites. We have no evidence, however, for any detectable amount of the 71 kDa (*e*) double cleavage fragment under the reaction conditions tested and with any of the antibodies used. This fragment along with the (*a*) and (*d*) fragments would have been generated if simultaneous hydrolysis was occurring at both of the ATP sites. Our data suggest that cleavage can occur only at one site at a time and that the two sites are functionally interdependent. These data do not demonstrate, however, that the catalytic sites alternate with equal efficiency but only that they are unlikely to act simultaneously.

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