Activation of the Human P-Glycoprotein ATPase by Trypsin†

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ABSTRACT: The human *MDR1* gene product, P-glycoprotein (Pgp), a tandemly duplicated molecule containing two putative ATP- and perhaps two drug-binding sites, is responsible for multidrug resistance in tumors. In this report, we characterized the effects of trypsinization of Pgp on its ATPase function. Incubation of Pgp-containing membranes with trypsin at a ratio of 1000:1 (w/w) resulted in a gradual increase in the basal- and the drug-stimulated ATPase activities of Pgp in a time-dependent manner. The maximal basal-, verapamil-, and vinblastine-stimulated ATPase activities of the trypsinized Pgp were approximately 1.8-, 1.5-, and 1.75-fold higher than the activities of the native Pgp, respectively. Increased basal- and drug-stimulated ATPase activities of the Pgp were also observed when the ratio of membrane protein to trypsin in the incubation mixtures was raised to 10:1 (w/w). Immunoblotting analysis of Pgp tryptic digests using Pgp-specific $NH_2$11, C219, and C494 antibodies together revealed the degradation of full-length Pgp and formation of at least eight peptides migrating in the 36−60 kDa range. Immunoprecipitation reactions using $NH_2$11 and C494 antibodies have suggested that the peptides originating from the NH_2 half of Pgp are in strong association with the COOH half of the peptide. These findings suggest that while Pgp fragments together exhibit the ATPase functional characteristics, Pgp possesses a cleavage activation site or region, and its cleavage leads to the activation of basal ATPase function of Pgp.

Multidrug resistance is a condition in which the tumor cells become resistant not only to the anticancer drug used in the chemotherapy but also to a variety of other unrelated drugs (*1*−*3*). Often, the multidrug resistant human tumor cells overexpress a ∼170 kDa plasma membrane glycoprotein, referred to as Pgp[1] encoded by the *MDR1* gene (*4*, *5*). It is now well-established that Pgp acts as an energy-dependent transporter that extrudes from cells a spectrum of drugs and compounds with diverse structure. The deduced amino acid sequence indicates that the human Pgp is a 1280-amino acid polypeptide and shares considerable sequence and structural homology with the members of a superfamily of membrane transporters (*6*−*8*). Computer-assisted predictive algorithms indicate that Pgp consists of two homologous halves, each containing six membrane-embedded nonpolar peptide regions and "Homology A and Homology B" consensus sequences of Walker (*9*), characteristic of a nucleotide-binding domain. Pgp binds to photoaffinity analogues of ATP and exhibits high ATPase activity in the presence of a variety of compounds and chemotherapeutic agents (*10*−*12*). Direct interactions of Pgp with photoaffinity analogues of anticancer drugs and other compounds, including adriamycin, vinblastine, colchicine, and iodoaryl azidoprazosin, were also reported (*13*−*20*). Although it is fairly clear that drug transport and ATPase functions of Pgp are interrelated processes (*25*, *26*, *28*), the mechanism by which Pgp hydrolyzes ATP and couples the released energy to the drug transport remains largely unknown.

An understanding of the molecular mechanism of Pgp-mediated energy-dependent drug transport will greatly facilitate our ability to modulate its function in the drug resistant tumors. An approach most commonly used toward this end by several laboratories is the site-directed mutagenesis of amino acid residues in the putative transmembrane segments and in the drug- and ATP-binding domains followed by analyses using cell biological procedures (*21*, *22*). These studies have collectively provided a large body of information that relates to the key roles played by amino acids distributed throughout the Pgp molecule. However, a recent vanadate-induced inhibition procedure for Pgp pioneered by Senior and co-workers (*23*) and subsequently adapted by others (*20*, *26*, *29*, *37*) has reinvigorated the biochemical analyses, and provided a way of identifying selectively the drug- and ATP-binding sites in this transporter. These procedures have provided evidence that both of the ATP-binding sites present in the NH_2 and COOH halves of the Pgp molecule are catalytically active (*32*). Loo and Clarke (*24*) have observed that the recombinant NH_2 and COOH halves of Pgp when expressed alone in Sf9 insect cells do not catalyze the drug-stimulated ATPase function. However, noticeable drug-stimulated ATPase activity was

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[1] Abbreviations: Pgp, P-glycoprotein; MDR, multidrug resistance; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Sf9 insect cells, *Spodoptera frugiperda* ovarian insect cells; TPCK, L-1-tosylamide-2-phenylethyl chloromethyl ketone; PVDF, polyvinylidene difluoride; EGTA, ethylenebis(oxyethylenenitrilo)-tetraacetic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

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recovered with coexpression of these two Pgp halves. These observations suggest that interactions between the NH_2 and COOH halves of Pgp are essential for the display of functional characteristics. Studies from several laboratories aimed at identifying the ligand binding sites and conformational changes in Pgp have suggested that mild treatment with trypsin of Pgp results in the separation of the NH_2 and COOH halves (*16*, *30*, *31*). These observations probably suggest that Pgp is a relatively rigid molecule with very few accessible tryptic cleavage sites in the entire molecule. It was, however, unknown whether Pgp cleaved with trypsin exhibits any ATPase function. While pursuing the determination of a variety of biochemical aspects of Pgp, we noticed, as reported in this paper, that incubation of Pgp membranes with trypsin leads to the fragmentation of Pgp with concomitant activation of its basal- and drug-stimulated ATPase activities.

EXPERIMENTAL PROCEDURES

Preparation of Membrane-Containing Human Wild-Type Pgp. The Sf9 insect cells were infected with a recombinant baculovirus carrying the human wild-type *MDR1* cDNA, and the total membrane fraction was prepared as described previously (*25*). The membrane fraction was resuspended in glycerol buffer [50 mM Tris, 50 mM mannitol, 2 mM EGTA, 2 mM 2-mercaptoethanol, and 30% (w/v) glycerol at pH 7.0, which is adjusted with HCl] at a final concentration of 2 mg of membrane protein/mL and used immediately or stored at −20 °C for later use. Membranes prepared from Sf9 insect cells infected with recombinant baculoviruses carrying the cDNAs of the α-subunit of amiloride-sensitive sodium channel (α-ENaC membranes) and *Escherichia coli* β-galactosidase (β-gal membranes) were used as a control.

Protease Treatment of Pgp-Containing Membranes. A TPCK-treated trypsin solution (1−10 mg in 1 mL of 1 mM HCl, freshly prepared) was mixed with a membrane suspension (1 mg of membrane protein/mL of glycerol buffer) at different ratios as indicated in each individual experiment, and the mixture was incubated at room temperature. At regular time intervals, aliquots were taken out, mixed with a 1000-fold excess (w/w) of soybean trypsin inhibitor prepared in glycerol buffer, and then used in the ATPase activity and SDS−PAGE analyses. In some experiments, potassium chloride was added to the tryptic digests to a final concentration of 250 mM and centrifuged at 170000*g* for 30 min. The supernatants and the pelleted membrane fractions resuspended in glycerol buffer were used in the ATPase and immunoblotting analyses.

To determine the initial rates of ATPase activity, membrane fractions were digested with trypsin at a membrane protein to trypsin (prepared in 1 mM HCl) ratio of 1000:1 (w/w) at room temperature for 45 min. Trypsinization was stopped by the addition of a 1000-fold excess (w/w) of soybean trypsin inhibitor. The α-ENaC membranes similarly treated with trypsin were used as a control. The basal- and drug-stimulated ATPase activities in these membrane preparations were measured as described below.

Pgp ATPase Activity Determination. The Pgp ATPase activity in the membranes was determined as described previously (*25*, *26*). Briefly, 5 μg of membrane protein was incubated with 0.1 mL of the ATPase reaction mixture at 37 °C for 10 min. To determine the basal ATPase activity, membranes were included in a reaction mixture containing 50 mM Tris (pH adjusted to 6.8 with HCl), 2 mM DTT, 50 mM KCl, 2 mM EGTA, 5 mM NaN_3, and 5 mM MgATP and incubated at 37 °C for 10 min. To determine the drug-stimulated ATPase activity, the total ATPase activity in the reaction mixture described above was measured in the presence of 1 μL of drug (verapamil or vinblastine prepared in dimethyl sulfoxide) and corrected for the basal ATPase activity. The concentrations of verapamil and vinblastine in the ATPase assays were 100 and 10 μM, respectively. The assays were stopped by the addition of 0.1 mL of a 5% SDS solution, and the amount of liberated P_i was measured colorimetrically as described by Sarkadi et al. (*33*). Each experiment described in this paper was carried out at least six times using different membrane preparations with essentially identical results.

Antibodies. To recognize the NH_2-terminal half of Pgp, a 24-mer peptide corresponding to residues 11−34 of Pgp (AKKKNFFKLNNKSEKDKKEKKPTV) was selected as the antigen. Synthesis of this peptide and generation of antibodies in rabbits were carried out at Animal Pharm, CA. An immunoblotting procedure was used to screen for the presence of polyclonal antibodies in the test bleed sera. Serum collected from rabbit 3030 exhibited strong and specific binding to Pgp. No proteins in the control membranes were reactive with respect to these sera. Bleeds collected from this rabbit were used in the future experiments, with the antibody being termed the $NH_2$11 antibody. Monoclonal antibodies, C219 which recognizes two epitopes, 568-VQVALD-573 and 1213-VQVELD-1218 (*39*), and C494, which recognizes epitope 1028-PNTLEGN-1034 (*39*), in the Pgp molecule were also utilized in these studies.

Immunoprecipitation. Pgp-containing membranes were digested with trypsin at a ratio of 1000:1 (w/w) for 45 min, and the resulting digests were centrifuged at 50000*g* for 30 min and the supernatants removed. The pelleted material obtained from 1 mg of membrane protein was solubilized in 1 mL of buffer [20 mM Tris, 137 mM NaCl, and 2 mM EDTA (pH 8.0)] containing either 1% (w/v) octyl β-D-glucopyranoside or 1% CHAPS (w/v). The soluble peptide solution was precleared with rabbit preimmune serum and then incubated overnight at 4 °C with either 3.3 μL of NH_2-11 or C494 antibody and 66 μL of protein A/G−agarose beads. The protein A/G−agarose beads were pelleted and washed twice in a buffer containing 10 mM Tris and 0.1% (v/v) NP-40. The peptides bound to the beads were extracted with 300 μL of SDS−PAGE disaggregating buffer and heated at 60 °C for 10 min, and 10 μL of these samples was run on SDS−PAGE gels followed by electroblotting onto PVDF membranes as described below. The Pgp peptides were detected using Pgp-specific antibodies as described in each individual experiment.

SDS−PAGE, Immunoblotting, and Quantitation of Pgp. The proteins in the proteolytic digests were first precipitated with 6% (w/v) trichloroacetic acid and then dissolved in SDS−PAGE disaggregating buffer. The proteins were separated on 7.5% acrylamide minigels and electroblotted onto polyvinyldene difluoride (PVDF) membranes as described previously (*25*). At the end of electrotransfer, the gels were stained with silver to ascertain the transfer of Pgp and its

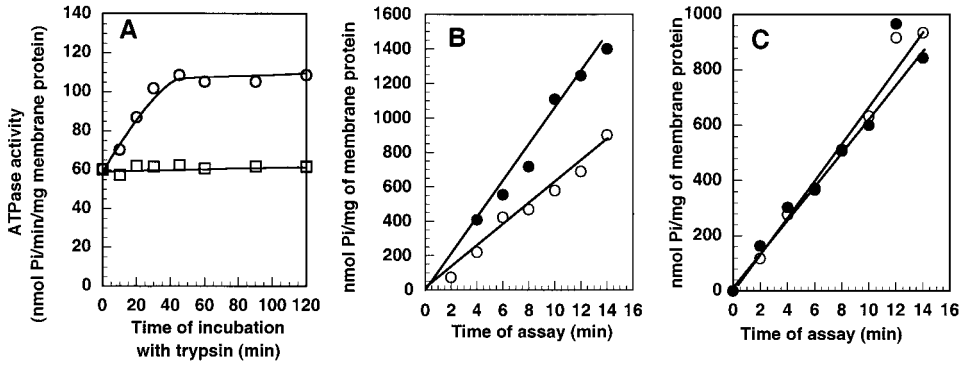

FIGURE 1: Effect of trypsin incubation on the basal ATPase activity. (A) Membranes containing wild-type Pgp (E) and αENaC (G) were incubated with trypsin [1000:1 (w/w)]. Aliquots were withdrawn at the indicated times and treated with excess soybean trypsin inhibitor, and the basal ATPase activity was measured as described in Experimental Procedures. (B) To determine the initial rates of ATP hydrolysis, the trypsin-treated [1000:1 (w/w) for 1 h] (●) and untreated (○) Pgp membranes were incubated in the ATPase assay mixtures for the indicated periods of time. (C) Initial rates of ATP hydrolysis in control membranes (α-ENaC) that were similarly untreated (○) and treated (●) with trypsin.

peptides onto the PVDF membranes. The results clearly indicated that the transfer of Pgp and its peptides onto the PVDF membranes was complete (data not shown). The membranes were probed with $NH_2$11, C219, and C494 antibodies, and the immunoblots were developed by using the enhanced chemiluminiscence method according to the manufacturer's instructions (ECL kit of Amersham Corp.). The staining intensities of the proteins on the fluorograms were quantitated by volume integration analysis using ImageQuant software of Molecular Dynamics (Molecular Dynamics), according to the manufacturer's instructions.

Protein Estimation. Protein in the membrane suspensions was quantitated with Coomassie Protein Assay Reagent (Pierce) according to the manufacturer's instructions, using bovine serum albumin as a standard.

Materials. TPCK-treated trypsin, vinblastine, and verapamil were obtained from Sigma. The SDS−PAGE and immunoblotting reagents were obtained from Fisher and Bio-Rad. The monoclonal antibodies C219 and C494 were obtained from Dako Corp. (Carpinteria, CA). The monoclonal JSB-1 antibody was obtained from Signet Corp. (Boston, MA). The protein A/G−agarose beads were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The Sf9 insect cell culture media were obtained from Gibco-BRL.

RESULTS

Effect of Trypsin on the Basal ATPase Activity. Membranes containing Pgp and α-ENaC (control) were incubated with trypsin at a membrane protein:trypsin ratio of 1000:1 (w/w), and aliquots were withdrawn from the incubation mixtures at regular time intervals and assayed for the ATPase activities in the absence of added drug as described in Experimental Procedures. Figure 1A shows the results of this experiment. The basal ATPase activity in the Pgp-containing membranes before the beginning of incubation was ∼60 nmol min^{-1} mg^{-1}. Upon incubation with trypsin, the ATPase activity increased linearly, and reached a maximum of ∼108 nmol min^{-1} mg^{-1} within 45 min which persisted throughout the period of incubation. In contrast, the basal ATPase activity in the α-ENaC membranes (∼60 nmol min^{-1} mg^{-1}) did not increase at all with trypsin treatment. Membranes prepared from Sf9 insect cells infected with a baculovirus carrying the *E. coli* β-galactosidase cDNA also did not exhibit any increase in the basal ATPase activity when similarly incubated with trypsin (results not shown). Because the increase in the basal ATPase activity was observed only with the Pgp-containing membranes, these results suggest that the increase is a reflection of the trypsin effect on Pgp.

To further establish that trypsin treatment increases the basal ATPase activity of the Pgp-containing membranes, initial rates of ATP hydrolysis of the untreated and trypsin-treated Pgp membranes were compared and the results are shown in Figure 1B. The extent of ATP hydrolysis by both membranes increased linearly with time. However, the initial rate of ATP hydrolysis by the trypsin-treated Pgp membranes was ∼116 nmol of P_i released min^{-1} mg^{-1}, which was twice the value of the untreated Pgp-containing membranes (∼58 nmol of P_i released min^{-1} mg^{-1}). On the other hand, the initial rates of ATP hydrolysis obtained with trypsin-treated and untreated control (α-ENaC) membranes were essentially unchanged (Figure 1C). These results provide strong evidence that trypsin treatment results in the stimulation of basal ATPase activity of the Pgp-containing membranes.

Effect of Trypsin on Drug-Stimulated Pgp ATPase Activity. Pgp has been shown to exhibit ATPase activity in the presence of a variety of anticancer drugs and chemosensitizers (*11*, *12*). To characterize the effects of trypsinization on the drug-stimulated Pgp ATPase activity, Pgp-containing membranes were incubated with trypsin (1000:1 ratio), aliquots withdrawn at regular time intervals were assayed for the verapamil- and vinblastine-stimulated activities, and the results are shown in Figure 2. Before the beginning of the incubation, the verapamil- and vinblastine-stimulated Pgp ATPase activities were approximately 140 and 47 nmol of P_i released min^{-1} (mg of membrane protein)$^{-1}$, respectively. Incubation with trypsin resulted in the activation of verapamil (panel A)- and vinblastine-stimulated (panel B) ATPase activities in a time-dependent manner with maxima of ∼210 and 85 nmol of P_i released min^{-1} (mg of membrane protein)$^{-1}$, respectively. The α-ENaC or β-gal membranes did not have any verapamil- or vinblastine-stimulated ATPase activity prior to or after treatment with trypsin (data not shown). These data suggest that incubation of Pgp-containing membranes with trypsin greatly stimulates the drug-dependent ATPase function of Pgp.

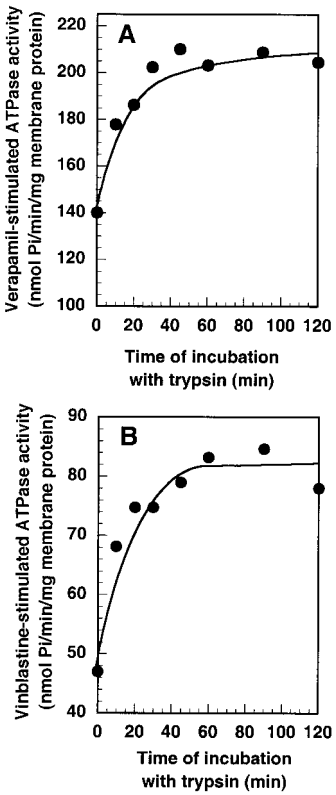

FIGURE 2: Effect of trypsin incubation on the drug-stimulated ATPase activity. Pgp-containing membranes were incubated with trypsin [1000:1 (w/w)]; aliquots were withdrawn at the indicated times and treated with excess soybean trypsin inhibitor, and the ATPase activities were measured as described in Experimental Procedures in the presence of 100 μM verapamil (A) and 10 μM vinblastine (B).

Effect of Increasing Concentrations of Trypsin on Pgp. It was somewhat surprising to find that trypsinized Pgp exhibits increased basal- and drug-stimulated ATPase functions, and we therefore sought to determine if increased concentrations of trypsin would further activate Pgp ATPase function. The Pgp-containing membranes were incubated with trypsin at membrane protein:trypsin ratios of 100:1 and 10:1 (w/w), and aliquots withdrawn at regular time intervals were assayed for basal- and verapamil-stimulated ATPase activities as described in Experimental Procedures. Figure 3A shows that Pgp treated with trypsin at a ratio of 100:1 (w/w) exhibited a maximum basal ATPase activity of ~180% within 10 min, which decreased slightly after incubation for 45 min. These tryptic digests also exhibited verapamil-stimulated ATPase activity, which increased to nearly 150% within 10 min. The activation persisted throughout the period of incubation [Figure 3B (○)].

The basal ATPase activity of Pgp-containing membranes treated with trypsin at a ratio of 10:1 (w/w) increased to a maximum of ~150% after incubation for 20 min, which decreased slowly with time to the untreated Pgp ATPase activity level [Figure 3A (●)]. Although verapamil-stimulated ATPase activity in these digests increased initially, longer incubation time resulted in slight decreases in activity [Figure 3B (●)]. These results collectively suggest that incubation of Pgp with larger amounts of trypsin also results in the stimulation of basal- and the drug-stimulated ATPase activities of Pgp.

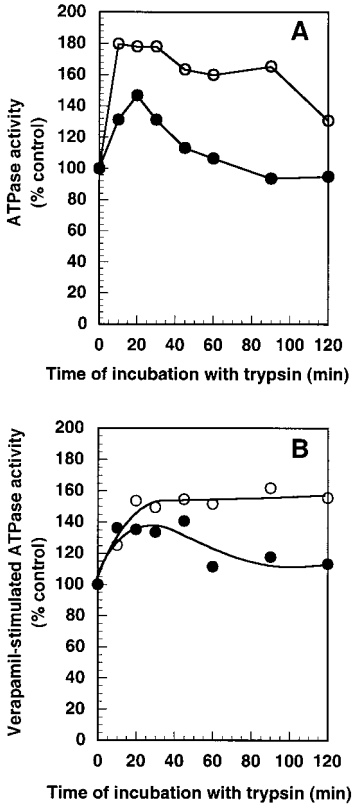

FIGURE 3: Effect of high concentrations of trypsin on Pgp ATPase activity. Pgp-containing membranes were incubated with trypsin at ratios (w/w) of 100:1 (○) and 10:1 (●), and at regular time intervals, aliquots were removed and the basal (A) and the verapamil-stimulated ATPase activities (B) measured. All the activities were expressed as a percentage of the activity in the untreated membranes.

Analysis of the ATPase Function in the Soluble and Membrane-Bound Fractions of the Pgp Tryptic Digest. Examination of the deduced amino acid sequence of the human wild-type Pgp indicates the presence of 136 tryptic sites in the putative cytoplasmic side of the molecule. It is therefore possible that Pgp peptides released by cleavage of some of these cytoplasmically located tryptic sites are probably held together by weak ionic interactions with the regions of Pgp that are membrane-anchoring, leaving the trypsinized Pgp in a functional conformation. To test this possibility, the Pgp-containing membranes were digested with trypsin at a 1000:1 ratio (w/w) for 45 min at room temperature and mixed with a high concentration of KCl, and the soluble and pelleted membrane-bound Pgp peptide fractions were separated by centrifugation, as described in Experimental Procedures. Figure 4A shows that the soluble peptide fraction did not contain any ATPase activity. The verapamil-stimulated ATPase activity was, however, recovered completely in the membrane-bound Pgp peptide fraction. These results suggest that cytoplasmic peptides that are released, if any, are not necessary for the Pgp ATPase function.

Kinetic Characterization of the Trypsin-Activated Pgp ATPase Function. Activation of drug-stimulated Pgp ATPase function by trypsin suggested that the affinities of ATP and/or drug binding to their respective binding sites might be altered. To test this possibility, Pgp-containing membranes were digested with trypsin at ratios of 1000:1 and 10:1 (w/

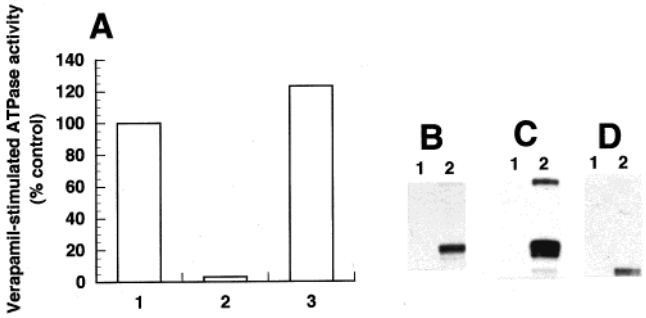

FIGURE 4: Analysis of soluble and membrane-bound Pgp fragments. Pgp-containing membranes were digested with trypsin [1000:1 (w/w)] for 45 min, and KCl was added to a final concentration of 250 mM. The digest was centrifuged, and the supernatant (soluble) and pelleted (membrane-bound) peptide fractions were separated. (A) The verapamil-stimulated ATPase activities in the untreated Pgp (lane 1), soluble (lane 2), and membrane-bound (lane 3) peptide fractions were determined as described in Experimental Procedures. The soluble (lane 1) and membrane-bound (lane 2) peptide fractions were analyzed by immunoblotting using the $NH_2$11 antibody (B), C219 antibody (C), and C494 antibody (D) as described in Experimental Procedures.

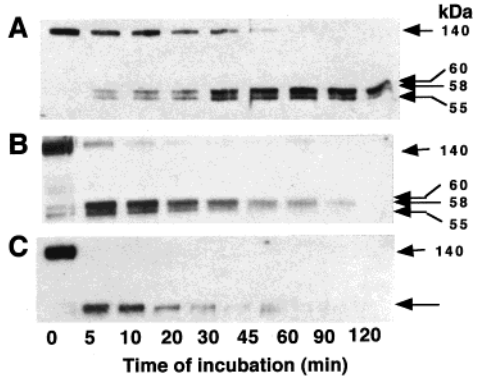

FIGURE 5: Immunoblot analysis of Pgp digests with the $NH_2$11 antibody. The Pgp-containing membranes were incubated with trypsin at ratios of 1000:1 (w/w) (A), 100:1 (B), and 10:1 (C), and at regular time intervals, aliquots were withdrawn and analyzed with immunoblotting procedures as described in Experimental Procedures using the $NH_2$11 antibody. The 140 kDa band in these blots corresponds to the full-length Pgp. The 60−55 kDa peptide mixture in panel C is denoted with an arrow.

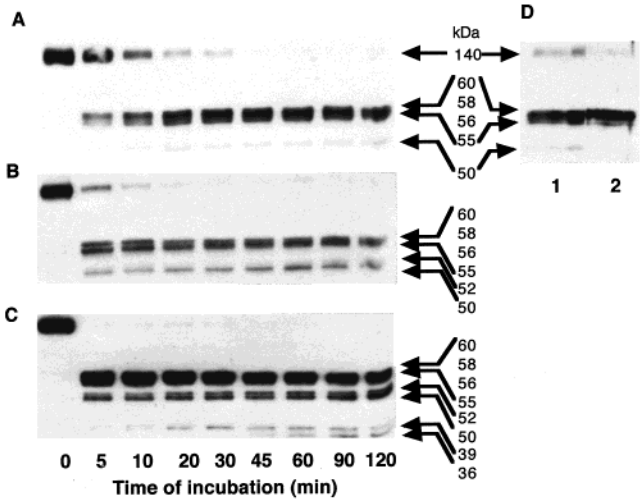

FIGURE 6: Immunoblot analysis of Pgp digests with the C219 antibody. The Pgp-containing membranes were incubated with trypsin at ratios of 1000:1 (w/w) (A), 100:1 (B), and 10:1 (C), and at regular time intervals, aliquots were withdrawn and analyzed by immunoblotting procedures as described in Experimental Procedures using the C219 antibody. The 140 kDa band in these blots corresponds to the full-length Pgp. The aliquot obtained at 60 min in the experiment whose results are depicted in panel A was run on SDS−PAGE, electroblotted onto a PVDF membrane, and cut into two portions. Portion 1 was probed with C219 and portion 2 with the $NH_2$11 antibody (D).

w) for 1 h at room temperature, and the membrane-bound peptide fraction was prepared as described above and used to determine the K_m values of ATP, verapamil, and vinblastine. The results indicated that the K_m values of ATP, verapamil, and vinblastine obtained with trypsinized Pgp were 0.5 mM, 1.0 μM, and 1.0 μM, respectively, which were similar to the K_m values obtained with the native Pgp (data not shown). These results suggest that both the native and trypsinzed Pgp forms bind ATP and drugs with equal affinity.

A variety of natural and synthetic peptides are known to interact with Pgp and stimulate the Pgp ATPase function (*41*). We first assumed that the increased basal- and drug-stimulated ATPase activity observed in membranes treated with trypsin is due to the stimulation of Pgp ATPase function by peptides released from Sf9 cell membrane proteins. To test this possibility, the tryptic digests of α-ENaC membranes were included in the Pgp ATPase assays. The results of this experiment indicated the absence of any stimulation of Pgp ATPase activity (data not shown). These observations establish that the increase in the basal- and drug-stimulated ATPase activities in the Pgp-containing membranes is a direct effect of trypsin on the Pgp molecule.

Immunoblot Analysis of Trypsin-Treated Pgp. The changes in the Pgp molecule during trypsin treatment were investigated with immunoblotting procedures using $NH_2$11, C219, and C494 antibodies as described in Experimental Procedures. For reasons of space economy, only the regions in the immunoblots that contained Pgp peptides are shown. Figure 5 shows the immunoblots of tryptic digests developed using the $NH_2$11 antibody. Digestion of membranes with low concentrations trypsin (1000:1 ratio) resulted in the gradual disappearance of the full-length ∼140 kDa Pgp, with concomitant appearance of three peptides, migrating in the ∼55−60 kDa region of SDS−PAGE. For clarity of presentation, these peptides were termed Nt60, Nt58, and Nt55 peptides (panel A). Quantitation of the staining intensity by densitometry indicated that the total amount of these peptides was similar to the amount of full-length Pgp that disappeared at each time point (data not shown). When Pgp-containing membranes were incubated with 10-fold higher amounts of trypsin (100:1 ratio), the staining intensities of these peptides gradually decreased with time (panel B). When Pgp-containing membranes were digested with trypsin at a ratio of 10:1 (w/w), the staining intensity of these peptides was rapidly lost (panel C), presumably due to the loss of the NH_2-11 epitope.

To further characterize the tryptic degradation of Pgp, the tryptic digests obtained above were analyzed by immunoblotting using the C219 antibody and the results are shown in Figure 6. In the presence of mild amounts of trypsin (1000:1 ratio), the full-length Pgp was degraded gradually, with concomitant appearance and accumulation of four peptides in the ∼55−60 kDa region of the gel (panel A). To determine whether any of these peptides are related to

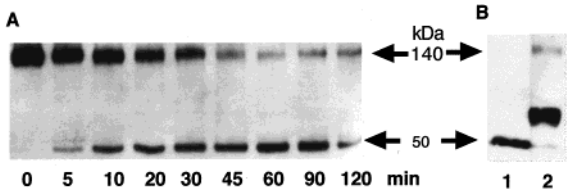

FIGURE 7: Immunoblot analysis of Pgp digests with C494 antibody. The Pgp-containing membranes were incubated with trypsin at a ratio of 1000:1 (w/w) (A), and at regular time intervals, aliquots were withdrawn and analyzed with immunoblotting procedures as described in Experimental Procedures using the C494 antibody. The 140 kDa band is the full-length Pgp. The aliquot obtained at 120 min in the experiment whose results are depicted in panel A was run on SDS−PAGE, electroblotted onto a PVDF membrane, and cut into two portions. Portion 1 was probed with the C494 antibody and portion 2 with the C219 antibody (B).

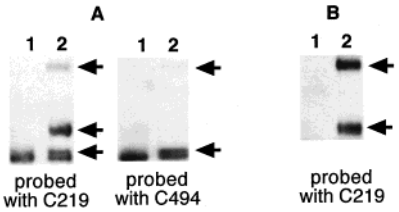

FIGURE 8: Coimmunoprecipitation of Pgp peptides. The tryptic digests of control (lanes 1) and Pgp (lanes 2) membranes were solubilized in 1% (w/v) octyl β-D-glucopyranoside and immunoprecipitated with $NH_2$11 (A) and C494 (B) antibodies. The blots of immunoprecipitates were probed with antibodies as indicated.

the Nt60, Nt58, and Nt55 peptides observed in the immunoblots developed with the $NH_2$11 antibody (Figure 5A), a membrane blot of the tryptic digests was cut into two portions, and one portion was probed with the $NH_2$11 antibody and the other with the C219 antibody. Alignment of these two portions clearly suggested that the upper two peptides and the lowest peptide of the four peptides observed in the C219 antibody immunoblot aligned with the Nt60, Nt58, and Nt55 peptides (panel D). The third peptide which was stained with the C219 antibody, but not with the NH_2-11 antibody, is termed the Nt56 peptide. These observations suggest that while the Nt60, Nt58, and Nt55 peptides contain both of the $NH_2$11 and C219 epitopes, the Nt56 peptide contained only the C219 antibody epitope. In addition, a faintly stained peptide with a molecular mass of ~50 kDa, termed the 50 kDa peptide, that was reactive with the C219 antibody was also detected in these tryptic digests.

Immunoblot analysis using the C219 antibody of the Pgp digests obtained after incubation with trypsin (100:1 ratio) indicated the conversion of the four peptides observed above (panel A) into two discrete peptides at the beginning of the incubation with trypsin (Figure 6B). However, longer incubations resulted in the loss of resolution of these peptides, presumably due to further degradation. An additional peptide termed the ~52 kDa peptide, migrating just above the ~50 kDa peptide, was formed near the end of the incubation. The staining intensity of the ~50 kDa peptide has remained constant throughout the incubation.

With high concentrations of trypsin in the incubation mixture (10:1 ratio), two additional C219 antibody-reactive Pgp peptides with molecular masses of ~39 and 36 kDa were identified along with the Nt60, Nt58, Nt56, Nt55, Nt52, and Nt50 peptides (Figure 6C).

To identify additional Pgp peptides, the tryptic digests obtained from the experiments described above were also analyzed by immunoblotting procedures using the C494 antibody, and the results are shown in Figure 7. A single peptide with a molecular mass of ~50 kDa was found in the digests obtained from incubations of Pgp membranes with small amounts of trypsin (1000:1 ratio). To determine whether this peptide is related to the ~50 kDa peptide observed in the immunoblots developed with the C219 antibody (Figure 6), a blot of tryptic digest was cut into two portions, each of which was probed with the C219 or C494 antibody. The ~50 kDa peptide reacted with both of these antibodies and aligned nicely, suggesting that this peptide contains C219 and C494 antibody epitopes (Figure 7B). The staining intensity of the ~50 kDa peptide was reduced slightly in the high tryptic digests without any additional peptide formation (data not shown).

As shown, the membrane-bound Pgp tryptic peptide fraction exhibits ATPase function (Figure 4). To determine which of the Pgp peptides recognized in the immunoblots described above are partitioned into the membrane-bound fraction, the soluble and membrane-bound peptide fractions were prepared and analyzed by immunoblotting using NH_2-11, C219, and C494 antibodies as described in Experimental Procedures. The results (Figure 4B−D) clearly indicate that only the membrane-bound peptide fraction contained all of the $NH_2$11, C219, and C494 antibody-reactive peptides, which probably carry out the Pgp ATPase function together.

Coimmunoprecipitation. To assess the possible interactions between the Pgp peptides identified above, the Pgp tryptic digests were solubilized in buffers containing 1% (w/v) octyl β-D-glucopyranoside. The soluble materials were then immunoprecipitated using $NH_2$11 and C494 antibodies as described in Experimental Procedures. The immunoprecipitates were separated on SDS−PAGE gels followed by electrotransfer onto the PVDF membranes. The blots were then probed with various Pgp-specific antibodies, and the results are shown in Figure 8. Probing the $NH_2$11 antibody immunoprecipitates with the C219 antibody revealed the presence of full-length Pgp and a mixture of ~55−60 kDa peptides (Figure 8A). The presence of C219 antibody-reactive ~55−60 kDa peptides further strengthened the notion that these peptides contained both of the epitopes of $NH_2$11 and C219 antibodies. Interestingly, these immunoprecipitates also contained the ~50 kDa peptide, although it does not have the $NH_2$11 antibody epitope (see Figure 5).

The $NH_2$11 antibody immunoprecipitates were also probed with the C494 antibody, and the results are shown in Figure 8A. The C494 antibody reacted with full-length Pgp and a ~50 kDa peptide. Thus, the presence of the ~50 kDa peptide in the $NH_2$11 antibody immunoprecipitates further supports the observation that this peptide is held to the mixture of ~55−60 kDa peptides via protein−protein interactions. To further corroborate this observation, we have carried out the immunoprecipitations with the C494 antibody and then analyzed the mixtures for the presence of ~55−60 kDa peptides using the C219 antibody, and the results are shown in Figure 8B. The results clearly indicated the presence of full-length Pgp and a mixture of ~55−60 kDa peptides in the immunoprecipitates. The presence of the C494 antibody-reactive ~50 kDa peptide in these immunoprecipitates could

not be ascertained due to the interference by the antibody heavy chain, which also migrates at this region of the gel and reacts with the HRP-linked secondary antibody. Identical results were obtained when the whole immunoprecipitation procedure was carried out using 1% CHAPS in place of octyl β-D-glucopyranoside in the buffer system described above (data not shown). Thus, identification of an $NH_2$11 and C219 antibody-reactive ~55−60 kDa peptide mixture and the C219 and C494 antibody-reactive ~50 kDa peptide in these immunoprecipitates together suggests that all these peptides are likely held together by protein−protein interactions.

DISCUSSION

The results presented in this paper indicate that cleavage of full-length Pgp leads to the activation of its ATPase function. Prior to the treatment with trypsin, both of the control and Pgp-containing membranes exhibit nearly identical basal ATPase activities of ~60 nmol of P_i released min^{-1} (mg of membrane protein)$^{-1}$, suggesting that Pgp as prepared in these studies does not exhibit any basal ATPase activity. On the basis of the mechanisms of related transporters such as Ca^{2+}- and Na^+/K^+-ATPases (*43*−*45*), it is speculated that the Pgp-mediated ATP hydrolysis in the presence of transport substrate (anticancer drug, for example) is likely a reflection of the utilization of the energy released from ATP hydrolysis for the transport of the transport substrate (*25*, *26*). This interpretation points out that these two reactions, ATP hydrolysis and drug transport, are coupled processes. Because Pgp did not exhibit any ATPase activity in the absence of the transport substrate (drug) in our assays, and ATPase activity was observed in the presence of anticancer drugs and chemosensitizers known to be transported by Pgp, these observations provide support to the aforementioned notion. It may be important to mention that whenever membranes were prepared in the absence of protease inhibitors, some amount of degraded Pgp was observed (unpublished observations). The pattern of degradation was similar to the action of trypsin. Such membranes exhibited high basal ATPase activity. On the basis of the observations made in this paper, we conclude that whenever high basal ATPase activity was observed in the Pgp membranes, it is due to the activity of the degraded Pgp fragments. Incubation of Pgp with small amounts of trypsin resulted in an increase in basal ATPase activity with concomitant cleavage of Pgp as indicated by the identification of several well-defined Pgp-specific peptides. The time course of formation of these peptides correlated well with the rise in the basal ATPase activity, indicating that fragmentation of Pgp leads to the activation of Pgp basal ATPase function. As described in the introductory section, the native Pgp exhibits a unique high-capacity ATPase activity in the presence of a wide variety of anticancer drugs and chemosensitizers, termed drug-stimulated ATPase activity, a fundamental property which is now being utilized as a tool in the biochemical investigations of Pgp (*38*). The data presented in Figures 2 and 3 demonstrate that cleavage of Pgp also activates the drug-stimulated Pgp ATPase activity. These observations are the first to provide evidence that degradation of Pgp leads to the activation of its basal- and drug-stimulated ATPase functions.

Detection of increased basal- and drug-stimulated ATPase activities in membranes lacking full-length Pgp but containing several Pgp-specific peptides raises an important issue regarding the significance of full-length Pgp in elucidating its functional characteristics. Kinetic characterization of the interactions of drugs and ATP with the normal and trypsinized Pgps showed no evidence for changes in the affinities with which these ligands interact with their binding sites. It thus seems apparent that although Pgp is degraded, the regions of the molecule that are involved in ATP and drug binding are unaffected by proteolysis.

As a next logical step in assigning the location of the peptides released by trypsinization in the linear polypeptide sequence, we have employed several Pgp-specific antibodies to recognize various regions in the Pgp molecule, which are schematically shown in Figure 9. Although the possible sites at which trypsin is cleaved are indicated by the letter X solely in the cytoplasmically located region of the molecule, some of these sites might as well be on the extracellular side. The $NH_2$11 antibody that recognizes amino acids 11−34 in the Pgp sequence identified Nt60, Nt58, and Nt55 peptides in the mild tryptic digests, establishing that these peptides originate from the NH_2-terminal end of the Pgp molecule. The COOH-terminal end of these peptides is fairly clear from the results of immunoblots developed with the C219 antibody. The C219 antibody recognized four peptides with molecular masses ranging from ~55 to 60 kDa, suggesting that these peptides must contain the C219 antibody epitope, 568-VQVALD-574, and end somewhere after amino acid 574. This interpretation also suggests that these peptides must contain the consensus Walker A and Walker B motif of the first ATP binding site and are thus capable of hydrolyzing ATP. Because the Nt56 peptide did not stain with the NH_2-11 or C494 antibody, it is likely that this peptide also originates from the NH_2 terminus of Pgp but lost the $NH_2$11 antibody epitope. Interestingly, amino acid 185 in the polypeptide, which has been shown to play an important role in the interactions of Pgp with drugs, including colchicine, verapamil, and cyclosporin A (*25*), is also present in the Nt55−Nt60 peptides, suggesting that these peptides interact with drugs. Previous studies from other laboratories have in fact shown that trypsinization of human and Chinese hamster Pgps expressed in mammalian cells leads to the formation of a ~95 kDa fragment originating from the NH_2 terminus of Pgp, which is similar to the Nt55−Nt60 peptide mixture observed in the study presented here. The apparent discrepancy of the molecular masses of these peptides is due to the fact that the ~95 kDa peptide is glycosylated (*30*, *31*) and the Nt55−Nt60 peptides are not. In addition, photoaffinity labeling experiments have provided evidence that the 95 kDa fragment contains the drug- and ATP-binding sites (*30*, *31*). Taken together, these observations suggest that although Pgp is degraded, the Nt55−Nt60 peptides originating from the NH_2-terminal end of Pgp contain the functional drug- and ATP-binding sites, and are capable of eliciting the Pgp ATPase function.

The facts that the staining intensity of Nt55−Nt60 peptides decreased gradually in the presence of large amounts trypsin and no other peptides were detected by the $NH_2$11 antibody suggest that the $NH_2$11 antibody epitope from these peptides was lost. However, small amounts of three additional peptides with molecular masses of ~42, 39, and 36 kDa were recognized by the C219 antibody in the high tryptic digests, suggesting that trypsin was able to degrade the Nt55−Nt60 peptides from the NH_2-terminal end, without affecting the

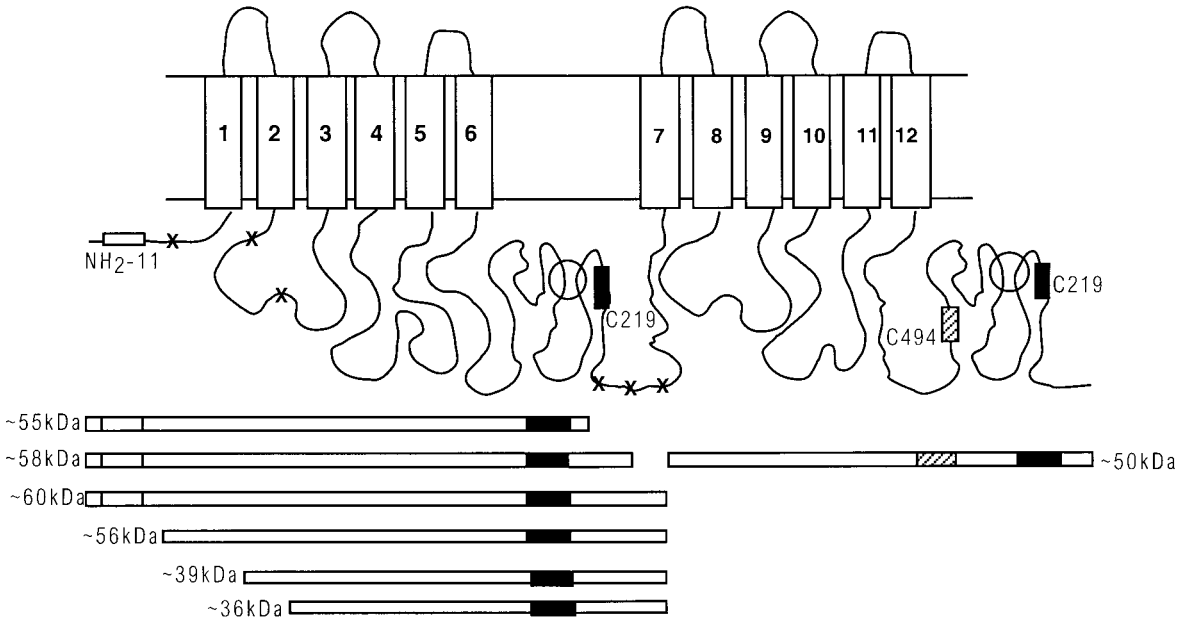

FIGURE 9: Model of Pgp with possible sites accessible to trypsin. The regions of Pgp recognized by antibodies $NH_2$11, C219, and C494 used in this study are represented with open, filled, and hatched boxes. The ATP-binding domains are represented with circles. The possible tryptic cleavage sites are represented with X, and the corresponding peptides that are released are schematically shown in the lower panel.

C219 antibody epitope, 568-VQVALD-574, at the COOH-terminal end of these peptides. Because the C219 antibody-reactive Nt55−Nt60 peptides are the predominant species, and the amounts of the ~52, 39, and 36 kDa peptides are small, it is possible that the NH_2 half of Pgp is relatively resistant to further degradation by trypsin.

We have utilized the C494 antibody which is known to recognize residues 1028−1034 (PNTLEGN) in the Pgp polypeptide (*39*) to account for the peptides originating from the COOH-terminal half of Pgp. The C494 antibody reacted with the ~50 kDa peptide in the tryptic digests, which, as expected, was detected along with the Nt55−Nt60 peptides. This peptide also reacted with the JSB-1 antibody (data not shown), another monoclonal antibody whose epitope was predicted to overlap the C494 antibody-recognizing sequence (*40*), further establishing the COOH-terminal origin of this peptide. The staining intensity of this peptide in immunoblots developed with C494 and JSB-1 antibodies was identical to that of full-length Pgp, indicating the quantitative recovery of this peptide. This peptide was not further degraded even in the presence of large amounts of trypsin. The exact NH_2- and COOH-terminal ends of this peptide are unclear at present. As mentioned above, the ~50 kDa peptide also reacted with the C219 antibody, albeit weakly, suggesting that this peptide contains the second C219 antibody epitope, 1213-VQEALD-1219. Although the C219 antibody has been shown recognize amino acid sequences VQVALD (residues 568−574) and VQEALD (residues 1213−1219) in the Pgp polypeptide, its interaction with the latter could be weaker, and may provide an explanation for the poor staining intensity of the ~50 kDa peptide on immunoblots developed with the C219 antibody. Our initial attempts to sequence the ~50 kDa peptide did not yield any meaningful information. We speculate that the NH_2 terminus of this peptide may be just after the end of the Nt60 peptide, and end somewhere after the second C219 eiptope, 1213-VQEALD-1219. Thus, the ~50 kDa peptide, as in the case of Nt55−Nt60 peptides, will have six putative transmembrane segments (7−12) and the second ATP-binding motif. Interestingly, previous studies (*30*) showed that a ~50 kDa tryptic peptide released from human Pgp expressed in mammalian cell lines similar to the peptide observed in the study presented here was also shown by a variety of epitope-specific antibody reactions to represent the COOH-terminal half of the Pgp molecule and to contain an ATP-binding site. Furthermore, Bruggemann et al. (*16*) and Yoshimura et al. (*31*) have demonstrated that trypsinization of azidopine-labeled Pgp results in the generation of an approximately 55 kDa labeled fragment, which reacts with antibodies raised against regions in the COOH-terminal half of Pgp. Recently, Gottesman and co-workers have demonstrated that putative transmembrane segement 12 is involved in drug binding (*36*). These observations together provide strong evidence that the ~50 kDa peptide possesses ATP- and drug-binding sites.

As mentioned in the introductory section, earlier works by Loo and Clarke (*24*) suggested that the interactions of the NH_2- and COOH-terminal halves of Pgp are necessary for the functionality of Pgp. As shown in this study, the presence of the ~50 kDa COOH-terminal peptide in the immunoprecipitates of the $NH_2$11 antibody and the presence of Nt55−Nt60 peptides originating from the NH_2 terminus in the immunoprecipitates of the C494 antibody provide strong evidence that the NH_2- and COOH-terminal halves of Pgp are probably held together by protein−protein interactions. Because such interactions between the two halves of Pgp were unaffected by trypsinization, it is possible that these halves are able to exhibit Pgp-specific ATPase function.

Although the mechanism by which trypsinization activates Pgp ATPase function is unclear at present, the following considerations are noteworthy. Utilizing the vanadate-induced inhibition procedures, Senior and his associates have previously demonstrated that both ATP-binding sites of Chinese hamster Pgp are capable of hydrolyzing ATP, and proposed that these two ATP-binding sites may alternate in the ATP hydrolysis reaction (*35*). This suggests that full-length Pgp

normally functions at half of its maximum efficiency. Since there was no change in the affinities with which ATP and drug bind to the trypsinized Pgp as determined in the study presented here, the activation can be attributed to an increased level of catalysis. Although the cleavage of the exact proteolytic site(s) that leads to activation of basal- and drug-stimulated ATPase activities is unclear, we speculate that this site(s) is likely present in a region termed the cleavage activation region, between the NH_2- and COOH-terminal halves of Pgp. Such a region may be the linker region of Pgp, which is predicted to include residues 653−683 (*42*), and contains an unusually high number of tryptic cleavage sites. It is also possible that such a cleavage activation region might as well be present elsewhere in the Pgp molecule. Because proteolysis of Pgp leads to a maximum ∼1.8-fold increase in the Pgp ATPase activity, we speculate that the separated NH_2- and COOH-terminal halves are now catalytically independent, and function at their maximum efficiency. The future efforts of identifying the exact cleavage site and the role of the cleavage activation region in the ATPase function will be of considerable value in understanding the mechanism of this clinically important drug transporter.

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