Proteolytic Cleavage of the Linker Region of the Human P-glycoprotein Modulates Its ATPase Function*

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P-glycoprotein (Pgp), an anticancer drug-translocating ATPase, is responsible for multidrug resistance in cancer. We have previously shown (Nuti, S. L., Mehdi, A., and Rao, U. S. (2000) Biochemistry 39, 3424–3432) that tryptic cleavage of Pgp results in the activation of basal and drug-stimulated ATPase functions of Pgp. To understand this phenomenon, we determined the sites cleaved by trypsin and further examined whether the modulation of Pgp function is trypsin-specific or the result of the proteolysis in general. The effects of chymotrypsin and proteinase K on Pgp ATPase function were studied. The results show that proteolysis of Pgp irrespective of the protease employed resulted in the activation of basal ATPase activity. However, drug-stimulated ATPase activities were differentially modulated. Immunoblot analysis of proteolytic digests indicated that, irrespective of the protease employed, Pgp was predominantly cleaved in the middle of the molecule. N-terminal amino acid sequencing of Pgp tryptic and chymotryptic peptides indicated Arg<sup>680</sup> and Leu<sup>682</sup> as the sites of cleavage, respectively. These two cleavage sites are part of the predicted linker region that joins the two halves of Pgp. Together, these results suggest that the linker region in Pgp is primarily accessible to protease action and that cleavage of this region modulates Pgp ATPase function.

Multidrug resistance (MDR)<sup>1</sup> is a condition in which the tumor cells subjected to chemotherapy develop resistance not only to the anticancer drug used, but also to other structurally unrelated drugs (1–6). Emergence of MDR is often associated with overexpression of the MDR<sub>1</sub> gene product, Pgp, a plasma membrane-bound glycoprotein of ~170 kDa (4, 7, 8). It is now established that Pgp is a drug transporter that utilizes the energy released from ATP hydrolysis to pump the anticancer drugs out of cancer cells. The amino acid sequence deduced from the MDR<sub>1</sub> cDNA indicates that Pgp is a polypeptide of 1280 amino acids and shares considerable sequence and structural homology with members of the ABC superfamily of membrane transporters (1, 5, 9). The structure predicted by computer-assisted predictive algorithms indicates that Pgp consists of two homologous (N- and C-terminal) halves that share nearly 43% amino acid sequence similarity (6, 10). Each half contains six transmembrane segments and an ABD, which was further delineated into “homology A and homology B” consensus Walker sequences (11). Both halves are known to bind transport substrates and ATP (12–14). The cytoplasmically located region comprising amino acids 633–709, commonly called the “linker region,” is considered to link the N- and C-terminal halves of the molecule (6, 8, 10, 12). As this region harbors a number of phosphorylation sites and charged amino acids, it is thought to be analogous to the R domain of CFTR (cystic fibrosis transmembrane conductance regulator) (15–17), implying a regulatory function for the linker region of Pgp. Although phosphorylation/dephosphorylation of sites in the linker region have been shown to have no role in the establishment of the MDR phenotype (18), Sarkadi and co-workers (19) have shown that phosphorylation of certain sites in the linker region modulates the interaction of Pgp with certain drugs at low concentrations.

Human Pgp expressed in Sf9 insect cells is functionally similar to Pgp expressed in mammalian cells and has provided a wealth of information on the biochemical and mechanistic characteristics of this transporter (20–27). Although Pgp contains two ABDs, no significant basal ATPase activity was noticed in Pgp preparations made from Sf9 insect cells (25–28). However, basal ATPase activity was detected in Pgp preparations from mammalian cells (29–32). Although the reason for this discrepancy is unclear, it is conceivable that a mammalian plasma membrane component may be serving as a transport substrate, thus leading to the exhibition of apparent basal Pgp ATPase activity. In the presence of transport substrates, Pgp exhibits high ATPase activity, which is known as drug (transport substrate)-stimulated ATPase activity (20, 25–27, 30, 31, 33). Because of the ease with which the ATPase measurements can be carried out, drug-stimulated ATPase activity has been employed by several laboratories to monitor drug transport by Pgp (19, 22, 23, 25, 26, 30, 33–38).

Utilizing human Pgp expressed in Sf9 insect cells, we have recently reported that incubation of Pgp with trypsin results in cleavage of the molecule in the middle region, as judged by the formation of N- and C-terminal halves (28). Pgp thus cleaved exhibits high basal ATPase and increased drug-stimulated ATPase activities. However, the identity of the sites that are cleaved was not determined. We report in this study the identity of a site cleaved by trypsin. The results presented also indicate that the linker region is a primary target of cleavage for proteases. We infer that the linker region plays a key role in coupling the ATPase and drug transport functions of Pgp.

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§ The abbreviations used are: MDR, multidrug resistance; Pgp, P-glycoprotein; ABD, ATP-binding domain; ENaC, amiloride-sensitive epithelial sodium channel.

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Experimental Procedures

Molecular Biological Methods—Standard molecular biological procedures (39) were utilized in the preparation of all DNA constructs. The Transformer Mutagenesis procedure (CLONTECH) was utilized to introduce the desired mutations into the MDR1 cDNA using mutagenic oligonucleotides synthesized at the DNA Sequencing Facility of the University of Nebraska Medical Center. The mutations were confirmed by sequencing the cDNAs at the above facility.

Preparation of Recombinant Baculoviruses—A cDNA sequence coding for peptide sequence HHHHHHM was introduced into the MDR1 cDNA such that the C terminus of the encoding Pgp ended with the above sequence. The protein is expressed as His6-Pgp, Lyn233 and Asp335 in the first ABD and Lyn776 and Asp1000 in the second ABD were mutated to Met233, Asn555, Met776, and Asn1200, respectively, and the corresponding mutant MDR1 cDNAs were prepared. Stop codons were introduced into the MDR1 cDNA to express Pgp proteins truncating at residues 1220, 1222, and 1264. All of these MDR1 cDNAs were subcloned into the pVL1393 baculoviral transfer vector under the transcriptional control of the polyhedrin promoter. The SF9 insect cells were cotransfected with these cDNAs and BaculoGold DNA (BD PharMingen) to prepare the corresponding recombinant baculoviruses according to the instructions provided by the manufacturer. Preparation of a baculovirus carrying the wild-type human Pgp cDNA was reported previously (25).

Infection of SF9 Insect Cells with Baculoviruses and Preparation of Total Membrane Fraction—The monolayers of SF9 insect cells growing in tissue culture flasks were infected with the above recombinant baculoviruses, and the total membrane fractions were prepared as described previously (25). The membrane fractions were resuspended in Tris/glycerol buffer (50 mM Tris, 50 mM mannitol, 2 mM EGTA, 2 mM 2-mercaptoethanol, and 50% [v/v] glycerol [pH adjusted to 7.0 with HCl]) and stored at –20°C for later use. A total membrane fraction obtained from SF9 insect cells infected with a baculovirus carrying α-ENaC (40) was used as the control.

Protease Digestion—Treatment of the membrane suspension with trypsin was carried out as described previously (28). Membranes were incubated with chymotrypsin or proteinase K (1 mg/ml of 1 mM HCl) at a membrane/proteinase ratio of 1000:1 (w/w) at room temperature. The final volume of protease in the mixture was always maintained at 1% (v/v). At the end of the incubation, proteolysis was arrested by the addition of excess soybean trypsin inhibitor or phenylmethylsulfonyl fluoride.

Pgp ATPase Measurements—The ATPase activities were measured as reported previously (28). Briefly, membranes (5 μg) were incubated at 37°C for 10 min in 0.1 ml of the ATPase reaction mixture containing 50 mM Tris-HCl (pH adjusted to 6.8 with HCl), 2 mM dithiothreitol, 50 mM KCl, 2 mM EGTA, 5 mM NaCl, and 5 mM MgATP. The assays were stopped by the addition of 0.1 ml of 5% (w/v) SDS solution, and the liberated inorganic phosphate was measured colorimetrically as described by Sarkadi et al. (27). The ATPase activity in the Pgp-containing membranes after subtracting the ATPase activity in the control membrane fractions was measured using the above-mentioned ATPase reaction mixture that contained 1 μl of drug (verapamil/ vinblastine/calcineurin prepared in dimethyl sulfoxide), and the activity obtained was corrected for the basal ATPase activity. The concentrations of verapamil, vinblastine, and calcineurin in the ATPase assays were 100, 10, and 250 μM, respectively.

Isolation of Pgp Peptides—The His6-Pgp digests were incubated with octyl β-glucopyranoside (0.5%, w/v) in phosphate buffer (50 mM NaCl and 20% glycerol [pH 8.0]) for 1 h on ice with occasional mixing. The material was then centrifuged at 100,000 × g for 30 min; and the supernatant (soluble fraction), which contained the majority of the Pgp peptides, was collected and supplemented with Triton X-100 (0.05%, w/v). This was then subjected to Sepharose CL-6B gel filtration chromatography (1 × 100-cm column) in phosphate buffer containing Triton X-100 (0.05%, w/v). The fractions containing Pgp peptides were further enriched by reversed-phase–n-pentyltriacetic acid-agarose affinity chromatography (QIAGEN Inc.).

N-terminal Amino Acid Sequencing—The fractions enriched with the Pgp peptides were precipitated with trichloroacetic acid (6%, w/v) and subjected to SDS-PAGE, followed by electroblotting onto polyvinylidene difluoride membrane as described (28). The desired Pgp peptides identified by immunoblotting of a small portion of the membrane were subjected to N-terminal amino acid sequencing at the Protein Core Facility of the University of Nebraska Medical Center.

Results

Effect of Proteases on the Basal ATPase Activity of Pgp—To study the effect of protease treatment on Pgp, α-ENaC (control) and Pgp-containing membranes were incubated with either chymotrypsin or proteinase K at a membrane protein/protease ratio of 1000:1 (w/v); and at regular time intervals, the ATPase activity was measured in the ATPase reaction mixture lacking any Pgp transport substrate. The ATPase activity in the control membrane fractions was measured using the above-mentioned ATPase reaction mixture that contained 1 μg of drug (verapamil/vinblastine/calcineurin prepared in dimethyl sulfoxide), and the activity obtained was corrected for the basal ATPase activity. The concentrations of verapamil, vinblastine, and calcineurin in the ATPase assays were 100, 10, and 250 μM, respectively.

Materials—Verapamil, vinblastine, colchicine, soybean trypsin inhibitor, and Sepharose CL-6B-200 were obtained from Sigma. Chymotrypsin and proteinase K were obtained from Worthington. The C219 and C494 monoclonal antibodies were obtained from Dako Corp. (Carpinteria, CA), and Calbiochem. The SF9 insect cell culture media were obtained from Invitrogen. Prestained protein markers (broad range) were obtained from New England Biolabs Inc. The SDS-PAGE and immunoblotting reagents were obtained from Fisher and Bio-Rad.

Effect of Proteases on the Drug-stimulated ATPase Activity of Pgp—The protease digests obtained from the above experiment (Fig. 1) were assayed for verapamil-, vinblastine-, and colch-
icine-stimulated ATPase activities (28), and the results are shown in Fig. 2. The control membranes did not exhibit any drug-stimulated ATPase activity before or after incubation with proteases (data not shown). The verapamil-, vinblastine-, and colchicine-stimulated ATPase activities in the Pgp-containing membranes before the beginning of protease treatment were ~90, 30, and 15 nmol of Pi released per min/mg of membrane protein, respectively, which were considered to be 100%. The verapamil- and vinblastine-stimulated Pgp ATPase activities gradually decreased to ~75 and 60%, respectively, upon incubation with either chymotrypsin or proteinase K. Interestingly, the colchicine-stimulated ATPase activity gradually increased to a maximum of ~200% upon incubation with these proteases. To further establish that protease treatment increases the colchicine-stimulated ATPase activity, the initial rates of ATP hydrolysis by the chymotrypsin-treated and untreated Pgp membranes in the presence of colchicine were compared. The results indicated that the ATP hydrolysis by both the treated and untreated Pgp membranes was linear with time, with an increased initial rate of ATP hydrolysis by the chymotrypsin-treated Pgp membranes (data not shown). Together, these data suggest that the action of the two proteases on Pgp results in differential modulation of ATPase activity stimulated by different transport substrates.

Effect of Proteases on the Pgp Molecule—To determine whether Pgp was cleaved in the above protease incubations, the protease digests of the above experiments were analyzed by Western blotting using the NH211, C219, and C494 antibodies. (The locations of the epitopes of these antibodies are shown schematically in Fig. 5.) Fig. 3A shows that incubation of Pgp with chymotrypsin resulted in a gradual disappearance of full-length ~140-kDa Pgp and the concomitant appearance of a group of three closely migrating peptides in the ~80-kDa region of the SDS-polyacrylamide gels as detected with the NH211 antibody, indicating that these peptides originate from the N-terminal half of Pgp. Also, a group of three closely migrating peptides in the ~80-kDa region of the gels were reactive with the C219 antibody (Fig. 3B). Upon longer incubation with the protease, a minor population of three closely migrating faintly stained peptides were detected in the ~58–60-kDa region by the C219 antibody (Fig. 3B). Three closely migrating peptides (which appeared as a single band) in the ~58–60-kDa region of the gels were detected by the C494 antibody (Fig. 3C), suggesting that these peptides correspond to the C-terminal half of Pgp.

To determine the relationship between peptides identified by different Pgp-specific antibodies, the Pgp chymotryptic digest (1000:1 (w/w) ratio, 45 min) was loaded in a broader well of an SDS-polyacrylamide gel. Following electrophoresis and electrophoretic transfer onto polyvinylidene difluoride membrane, the membrane was cut into three portions, and each was developed with the indicated antibody (D).

Fig. 2. Effect of proteases on the drug-stimulated ATPase activity of Pgp. Pgp-containing membranes were incubated with either chymotrypsin (A) or proteinase K (B) at a ratio of 1000:1 (w/w). Aliquots were withdrawn at the indicated times and treated with excess soybean trypsin inhibitor or phenylmethylsulfonyl fluoride, and the ATPase activities were determined in the presence of 100 μM verapamil (○), 10 μM vinblastine (●), or 250 μM colchicine (▲) as described under “Experimental Procedures.”

Fig. 3. Western blot analysis of chymotryptic digests of Pgp. Pgp chymotryptic digests were subjected to Western blot analysis using the NH211 (A), C219 (B), and C494 (C) antibodies. The 140-kDa band represents full-length Pgp, and the Pgp peptides released are indicated by their approximate molecular masses. To determine the relationship between the peptides recognized by these antibodies, following electrophoresis of the proteins, the membrane was cut into three portions, and each was developed with the indicated antibody (D).
the mobilities of the three NH211 antibody-reactive ~80-kDa peptides and the three C219 antibody-reactive ~80-kDa peptides are similar (Fig. 3D), suggesting that these peptides contain both the NH211 and C219 antibody epitopes and therefore originate from the N terminus of Pgp, include the first C219 epitope (G688VQVALD574), and end somewhere after residue 574. The three C219 antibody-reactive ~58–60-kDa peptides did not react with the NH211 antibody. However, these three peptides comigrated with the three C494 antibody-reactive ~58–60-kDa peptides, suggesting that these peptides contain both the C219 (the second epitope, 1213VQALD1219) and C494 antibody epitopes and therefore correspond to the C-terminal half of Pgp.

Fig. 4 shows the results from Western blot analyses of the proteinase K digests of Pgp-containing membranes. The blots developed with three different antibodies indicate the gradual disappearance of full-length 140-kDa Pgp upon incubation with proteinase K (Fig. 4, A–C). Three peptides migrating in the region of ~80 kDa and reactive with the NH211 antibody and thus originating from the N terminus of Pgp were generated with time of digestion with proteinase K (Fig. 4A). On the other hand, at least 10 peptides in the ~80–54-kDa range were reactive with the C219 antibody (Fig. 4B). Five of these peptides (marked with braces) migrated closely in the ~80-kDa region and together appeared as a single peptide. This was due to overexposure of the blot to the x-ray film, which allowed the identification of the remaining minor peptides (60–54-kDa range) present in the digests. Finally, the blot developed with the C494 antibody indicated the presence of at least four peptides in the ~58–60-kDa range (Fig. 4C), indicating that these peptides correspond to the C-terminal half of Pgp. To determine the relationship between all of these peptides, Western blots of a Pgp digest of proteinase K (1000:1 w/w ratio, 45 min) were developed with the NH211, C219, and C494 antibodies. Two bands were aligned as described above and indicated that the five peptides in the ~80-kDa group reacted with both the NH211 and C219 antibodies (Fig. 4D). This suggests that these peptides contain epitopes of both the NH211 and C219 antibodies and thus represent the N-terminal half of Pgp. The four C219 antibody-reactive peptides in the ~58–60-kDa region also reacted with the C494 antibody, but not the NH211 antibody, suggesting that these peptides likely contain the second C219 epitope (1213VQALD1219) and correspond to the C-terminal half of Pgp. However, the two C219 antibody-reactive minor peptides that migrated in the 56–54-kDa range did not react either with the NH211 or C494 antibody, suggesting further cleavage of some of these above peptides.

**Effects of Mutations in ABDs**—To test the hypothesis that the N- and C-terminal halves generated by proteolytic cleavage of Pgp exhibit ATPase activity independently, a mutation was introduced in either of the ABDs in the molecule. It has been previously demonstrated that mutations in the consensus sequences of Walker A (Lys433 → Met and Lys1076 → Met) and Walker B (Asp355 → Asn and Asp1200 → Asn) of either the first or second ABD result in a surface-expressed yet inactive form of Pgp (12, 38). Therefore, to test the above hypothesis, four Pgp forms with the above indicated single mutations were prepared. These Pgp mutants did not exhibit any ATPase activity, corroborating the observations of others (12, 38). Incubation of these Pgp mutants with different proteases did not result in any increase in basal or drug-stimulated ATPase activity. These results suggest that both halves of Pgp with functionally competent ATP-binding sites are essential for the protease-mediated modulation of Pgp function.

**Identification of Cleavage Sites in the Pgp Molecule**—To identify the sites that are cleaved by proteases, the ~60-kDa C-terminal peptide generated by trypsin2 and the ~58–60-kDa peptides generated by chymotrypsin were partially purified by the method of Groffen (28) and aligned as described above and indicated that the five peptides in the ~80-kDa group reacted with both the NH211 and C219 antibodies (Fig. 4D). This suggests that these peptides contain epitopes of both the NH211 and C219 antibodies and thus represent the N-terminal half of Pgp. The four C219 antibody-reactive peptides in the ~58–60-kDa region also reacted with the C494 antibody, but not the NH211 antibody, suggesting that these peptides likely contain the second C219 epitope (1213VQALD1219) and correspond to the C-terminal half of Pgp. However, the two C219 antibody-reactive minor peptides that migrated in the 56–54-kDa range did not react either with the NH211 or C494 antibody, suggesting further cleavage of some of these above peptides.

**Effect of Deletions of C-terminal Regions on Pgp ATPase Activity**—To determine whether the removal of the C terminus of Pgp by proteases that could not be detected in the Western blot analyses was responsible for the observed modulation of its ATPase activities, three Pgp forms were prepared by introducing stop codons just after the putative tryptic sites Lys1220, Arg1222, and Lys1264. None of these mutants exhibited any basal or drug-stimulated ATPase activities in the presence of verapamil, vinblastine, or colchicine before or after protease digestions (data not shown). Because the longest Pgp among these mutants is Pgp-(1–1264) and because the only available tryptic site after Lys1264 is Lys1275, which is the penultimate residue in the Pgp molecule, these results suggest that the C terminus is essential in Pgp ATPase function. Thus, it is unlikely that the C terminus of Pgp was cleaved by proteases in the incubations.

*Fig. 4. Western blot analysis of proteinase K digests of Pgp. Proteinase K digests of Pgp were subjected to Western blot analysis using the NH211 (A), C219 (B), and C494 (C) antibodies. The 140-kDa band represents full-length Pgp, and the Pgp peptides released are indicated by their approximate molecular masses. To determine the relationship between the peptides recognized by these antibodies, following electrotransfer of the proteins, the membrane was cut into three portions, and each was developed with the indicated antibody (D).*

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2 The molecular masses of Pgp tryptic peptides reported previously (28) were based on the relative mobilities of prestained marker proteins purchased from Bio-Rad. Due to inconsistencies in the molecular masses of these prestained marker proteins, we recalculated the sizes of these trypic peptides using prestained marker proteins purchased from New England Biolabs Inc. and found that the ~50-kDa trypic peptide reported previously is ~60 kDa.
DISCUSSION

We have recently reported that trypsin cleaves the Pgp molecule predominantly in the middle region, resulting in the formation of N- and C-terminal halves, and that such cleaved Pgp exhibits increased basal and drug-stimulated ATPase activities (28). However, the sites cleaved by trypsin were not determined. Also, it was unclear whether the activation of Pgp ATPase function is a trypsin-specific effect or a general effect of proteolysis. Thus, the goal of this work was to determine the effects of proteolysis in general on Pgp ATPase function and to identify the sites in the Pgp molecule that are accessible and modulated by proteolysis.

Together, these results suggest that, in general, proteolysis of Pgp leads to the activation of basal and colchicine-stimulated ATPase activities and differential modulation of ATPase activity stimulated by other transport substrates studied.

Western blot analyses of chymotryptic and proteinase K digests indicated that the Pgp molecule, although cleaved at several sites, appears to be predominantly cleaved within a small region in the middle of the molecule. Trypsin exhibits high specificity and cleaves at very few sites, again in the middle region, leading to the appearance of a few Pgp peptides belonging to the N-terminal half and a single ~60-kDa peptide from the C-terminal half of the molecule (28). Therefore, the identity of the N-terminal amino acid of this ~60-kDa C-terminal peptide would indicate the site of tryptic cleavage of Pgp. We directed our attempts to first purify and sequence the single ~60-kDa C-terminal peptide released by trypsin to determine the Pgp tryptic cleavage site. As the Pgp peptides were only partially soluble in most commonly used detergents, a series of experiments was carried out, and the procedure described under “Experimental Procedures” was found to yield nearly total solubilization of Pgp peptides from the membranes. Both the N- and C-terminal peptides were always tightly associated and eluted together in Sepharose CL-6B gel filtration and DEAE ion-exchange chromatographic procedures (data not shown).

Although this observation supported our previous data of co-immunoprecipitation, which suggested that the N- and C-terminal halves of trypsinized Pgp are held together by protein-protein interactions (28), the Pgp peptide-containing fractions were not found to be suitable for N-terminal amino acid sequencing due to the presence of other contaminating proteins. These problems were further compounded by the fact that the Pgp halves transferred onto the polyvinylidene difluoride membranes could not be stained by Coomassie Blue, a procedure commonly used to identify the peptide of interest so that it can be cut out and subjected to N-terminal amino acid sequencing. To circumvent these problems, a six-histidine tag was added at the C terminus of Pgp (His6-Pgp) to isolate the C-terminal halves by nickel affinity column chromatography. The C-terminal peptides by nickel affinity chromatography. His6-Pgp was identical to wild-type Pgp with respect to its ATPase activity before and after proteolysis (data not shown), suggesting that the proteolytic sites identified using His6-Pgp will be useful in understanding the mechanism of proteolysis-mediated Pgp modulation. Utilizing His6-Pgp, the tryptic cleavage site Arg680 and the chymotryptic cleavage site Leu682 were identified. Attempts were made to obtain additional sequences from the procedures described under “Experimental Procedures.”}

These peptides were subjected to N-terminal amino acid sequencing by standard Edman degradation. Ten cycles of amino acid sequence for each peptide were derived and matched with the deduced amino acid sequence for human Pgp. The amino acid sequence obtained from the ~60-kDa tryptic peptide was KL/T/TKEALDE..., which could easily be matched with the Pgp sequence..., 677AQDRKLSTKEALDE...after the arrow. Thus, trypsin cleaves Pgp at Arg680. The amino acid sequence obtained from the ~58–60-kDa chymotryptic peptide mixture was XXKEALXESI... , which matches the Pgp sequence..., 677AQDRKLSTKEALDE...after the arrow. Thus, Leu682 is a site of chymotryptic cleavage. The amino acids in the sequence indicated with X could not be identified unequivocally due to the presence of contaminating peptides. Chymotrypsin is known to catalyze the hydrolysis of bonds of leucyl, methionyl, asparaginyl, and glutamyl residues in addition to bonds involving aromatic amino acids (41, 42), which supports the identity of the above chymotryptic cleavage site. The peptides generated by proteinase K were not sequenced, as its effect on Pgp ATPase is similar to that of chymotrypsin action.

**FIG. 5. Model of Pgp.** A model of Pgp with 12 transmembrane segments is shown. The epitopes recognized by the NH211, C219, and C494 antibodies are shown as filled boxes. The putative ABDs (ABD1 and ABD2) are represented with circles. The linker region is highlighted with a thick line. The positions of the cleavage sites Arg680 and Leu682 are indicated with an arrow.
the chymotryptic digests without any success. Because all these chymotryptic peptides migrated very closely upon SDS-PAGE, it is likely that the additional cleavage sites are located close to the identified site. The calculated molecular mass of these identified (~60 kDa) peptides was ~66 kDa, which is close to the sizes estimated by SDS-PAGE. Pgp lacking the last 16 amino acids (Pgp-(1–1264)) was nonfunctional and did not exhibit any ATPase activity before or after proteolysis, suggesting that the C terminus was not lost during proteolysis. However, the possibility that the C-terminal region is cleaved upon proteolysis but still held to the molecule by protein-protein interactions cannot be ruled out with the existing data. Also, the evidence suggests that the N-terminal end probably was not cleaved by the proteases because the NH2-terminal antibody, whose epitope spans amino acids 11–34 in the linear sequence, was able to recognize quantitatively the N-terminal peptides in the Western blots. Together, these observations suggest that cleavage in the middle of the molecule is responsible for the observed increase in the basal ATPase activity of Pgp.

The predictive algorithms suggest that the identified Leu692 and Arg680 sites are located in the intracellular side, just before the beginning of the putative seventh transmembrane segment of the Pgp polypeptide (Fig. 5). The region (amino acids 633–709) encompassing these sites, commonly known as the linker region, was the subject of extensive study by other investigators (20, 18, 19). Because Pgp is thought to be a tandemly duplicated molecule, Loo and Clarke (50) investigated whether or not the N- and C-terminal halves would function independently if separated in the linker region. These investigators estimated that the N-terminal half (amino acids 1–682) and the C-terminal half (amino acids 683–1280) by molecular biological procedures, incidentally identical to the proteolytic action on Pgp observed in our studies. Although this study suggested that both halves are necessary for function, it is not possible to draw any conclusion as to whether ATPase function is modulated by this incision. Julien and Gros (44) and Zhang and co-workers (45) independently studied the accessibility of the amino acid sequencing data. We thank Rajgopal Govindarajan for help in the construction of the C-terminal deletion mutants.

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