Identification of the Cyclosporin-Binding Site in P-Glycoprotein†

Michel Demeule,‡ Alain Laplante,‡ Gérard F. Murphy,§ Roland M. Wenger,|| and Richard Béliveau*,‡

Laboratoire d'oncologie moléculaire, Université du Québec à Montréal et Hôpital Ste-Justine, P.O. Box 8888, Centre-Ville station, Montreal, Quebec H3C 3P8, Canada, Novartis Pharmaceutical Inc., Dorval, Quebec H9R 4P5, Canada, and Novartis Pharmaceutical Inc., CH-4002 Basel, Switzerland

Received August 18, 1998; Revised Manuscript Received October 22, 1998

ABSTRACT: The binding site of cyclosporin A to P-glycoprotein was characterized by using a multidrug-resistant Chinese hamster ovary cell line. P-glycoprotein photolabeled with diazirine−cyclosporin A analogue was purified by a two-step process involving continuous elution electrophoresis followed by wheat germ agglutinin−agarose precipitation. The cyclosporin A covalently bound to P-glycoprotein and to subsequent proteolytic fragments was detected by Western blot analysis using a monoclonal antibody against cyclosporin A. Proteolytic digestion of purified P-glycoprotein by V8 generated a major fragment of 15 kDa photolabeled by cyclosporin A, while proteolysis of P-glycoprotein photolabeled by [^{125}I]-iodoaryl azidoprazosin generated a major fragment of 7 kDa. Limited proteolysis of cyclosporin A-photolabeled P-glycoprotein with trypsin indicated that the major binding site for cyclosporin A was in the C-terminal half of the protein. This cyclosporin A binding site was further characterized with chemical agents (*N*-chlorosuccinimide, cyanogen bromide, and 2-nitro-5-thiocyanobenzoate). These three chemical agents established a proteolytic profile of P-glycoprotein for fragments photolabeled with cyclosporin A and for fragments that contained the C494 and C219 epitopes. The smallest fragments generated by these chemical agents include the transmembrane domains (TMs) 10, 11, and 12 of P-glycoprotein. When the fragments generated by these chemical agents are aligned, the region that binds cyclosporin A is reduced to the 953−1007 residues. These combined results suggest that the major binding site of cyclosporin A occurs between the end of TM 11 and the end of TM 12.

P-glycoprotein (P-gp)[1] is a plasma membrane protein (150−180 kDa) associated with the resistance to various anticancer agents that is often acquired during chemotherapy (*1*−*3*). A wide variety of agents, including vinca alkaloids, colchicine, antibiotics, and anthracyclines, have been shown to be transported by P-gp out of cells by an ATP-dependent mechanism (*4*). P-gp is a 1280 amino acid integral membrane glycoprotein with two homologous halves connected by a linker region. Hydropathy plots deduced from the amino acid sequence indicate that each of these two regions contains six transmembrane domains (TMs) and a consensus sequence for an ATP binding domain (*4, 5*). P-gp ATPase activity was shown to be essential for drug transport and to be sensitive to the presence of drug substrates in either isolated membranes or a reconstituted P-gp system. Furthermore, various reversal agents (also called chemosensitizers) such as verapamil, calcium antagonists, and cyclosporins have been shown to interact with P-gp (*3, 6*). These molecules inhibit P-gp from expelling anticancer drugs and reverse the multidrug resistant (MDR) phenotype in resistant cells in vitro (*4, 7*−*9*).

Previous studies have suggested that the immunosuppressive agent cyclosporin A (CsA) is also a transport substrate for P-gp (*3, 10*). Many CsA metabolites and analogues inhibit the in vitro activity of P-gp, and some analogues, such as PSC-833, possess more potent reversing properties than does the parent compound (*11*−*14*). Administration of CsA alone was also shown to cause increased P-gp expression in many rat tissues (*15*). Understanding the molecular mechanisms involved in the interaction between P-gp and CsA or CsA analogues would be invaluable in enabling the development of more effective anticancer chemotherapies. In contrast to the immunosuppressive activity of CsA, which involves cyclophilin A (CypA) and calcineurin (*16, 17*), the molecular mechanisms by which CsA reverses drug resistance remains unknown and the molecular events involved in the interaction between CsA (and its analogues) and P-gp remain to be established.

Combining proteolysis and mutagenesis with photoaffinity probes has allowed substantial progress in localizing binding

† This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada and from Novartis Pharmaceutical Canada Inc. A.L. received a scholarship from the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche and from the Fonds de Recherche en Santé du Québec.

* To whom correspondence should be addressed at the Département de Chimie-biochimie, Université du Québec à Montréal, P.O. Box 8888, Centre-Ville station, Montreal, Quebec H3C 3P8, Canada. Telephone (514) 987-3000, ext 8551; Fax (514) 987-0246; e-mail oncomol@er.uqam.ca.

‡ Département de Chimie-biochimie et Hôpital Ste-Justine, Université du Québec à Montréal.

§ Novartis Pharmaceutical Canada Inc.

|| Novartis Pharmaceutical Switzerland Inc.

[1] Abbreviations: P-gp, P-glycoprotein; CNBr, cyanogen bromide; CsA, cyclosporin A; dz, diazirine; ECL, enhanced chemiluminescence; IAAP, [^{125}I]iodoaryl azidoprazosin; mAb, monoclonal antibody; MDR, multidrug resistant; NCS, *N*-chlorosuccinimide; NTCB, 2-nitro-5-thiocyanobenzoate; pAb, polyclonal antibody; PVDF, poly(vinylidene difluoride); TBS-T, Tris-buffered saline with 0.3% or 0.1% Tween 20; TM, transmembrane domain; WGA, wheatgerm agglutinin.

10.1021/bi981992c CCC: $15.00
Published on Web 12/03/1998

site(s) of various drugs to P-gp. Incorporation of prazosin and forskolin analogues into P-gp followed by proteolysis suggested that there are two photoaffinity drug-binding domains in P-gp (*18, 19*). One of the domains is located in the N-terminal portion of the protein and includes residues within transmembrane domains 5 and 6, while the other site is within TMs 11 and 12. Furthermore, two nonidentical [^{125}I]-iodoaryl azidoprazosin (IAAP) binding sites in the human P-glycoprotein were identified (*20*), one in the N-terminal region that is insensitive to CsA and another in the C-terminal region that is inhibited by CsA in the presence of *cis*-flupentixol. P-glycoproteins produced from chimeras of human *MDR*1 and *MDR*3 also indicated that replacements limited to TM 12 impair the resistance to most of the anticancer drugs tested (*21*). The region containing the distal part of TM 4, the second cytoplasmic loop, and the proximal part of TM 5 was also identified as the binding site of iodomycin (*22*). A single serine residue within TM 11 of P-gps encoded by mouse *mdr1* (Ser^{941}) and *mdr3* (Ser^{939}) was shown to be critical for the recognition and transport of their specific substrates, including CsA (*23, 24*).

In this study, photoaffinity labeling of P-gp with diazirine−CsA analogue SDZ 212-122 was performed, followed by V8 proteolysis of the protein. CsA cross-linked to P-gp fragments was immunodetected with a monoclonal antibody (mAb) directed against CsA (*25*). The pattern of CsA-photolabeled fragments was compared to that obtained for fragments photolabeled with IAAP. CsA-photolabeled P-gp was cleaved with trypsin within its linker region and was also cleaved at cysteinyl, methionine, and tryptophan residues with 2-nitro-5-thiocyanobenzoate (NTCB), cyanogen bromide (CNBr), and *N*-chlorosuccinimide (NCS), respectively. This approach allowed us to characterize and compare fragments that had been photolabeled by CsA or that contained the C494, C219, and Ab-1 epitopes.

EXPERIMENTAL PROCEDURES

Materials. The minimum essential medium Alpha and fetal bovine serum were from Gibco (Gaithersburg, MD). Penicillin and streptomycin were from Flow Laboratories (Mississauga, ON). The Mini-Protean II apparatus for electrophoresis and electrophoresis reagents were purchased from Bio-Rad (Mississauga, ON). Poly(vinylidene difluoride) (PVDF) membranes and Milliblot-Graphite electroblotter I were from Millipore (Mississauga, ON). mAbs C219 and C494 were purchased from ID Labs (London, ON). The pAb Ab-1 directed against P-gp was from Oncogene Science Inc. (Uniondale, NY). Anti-mouse and anti-rabbit IgG horseradish peroxidase-linked whole antibody and enhanced chemiluminescence reagents were purchased from Amersham (Oakville, ON). CsA analogues, the drug parent, and the mAb against CsA were furnished by Novartis Pharmaceutical (Basel, Switzerland), while IAAP was from Dupont NEN (Boston, MA). *N*-Chlorosuccinimide (NCS) and cyanogen bromide (CNBr) were from Aldrich Chemical Co. (Milwaukee, WI). 2-Nitro-5-thiocyanobenzoate (NTCB) was from Sigma Chemical Co. (Oakville, ON).

Cell Culture and Crude Membrane Preparations. Cells of the pleiotropic drug-resistant CH^{R}C5 cell line, selected for resistance to colchicine (*2*), were grown in monolayers on 175 cm^2 plastic tissue culture flasks at 37 °C under 5% CO_2 in minimum essential medium Alpha supplemented with 10% fetal bovine serum and 1% penicillin (50 units/mL)−streptomycin (50 μg/mL). Cells were grown to confluence and incubated for 24 h at 37 °C with fresh culture medium. They were then harvested with 15 mM sodium citrate in phosphate-buffered saline (PBS, which contains 138 mM NaCl, 5.4 mM KCl, and 5 mM $Na_2HPO_4-KH_2PO_4$, pH 7.4), washed by centrifugation, and resuspended in PBS. Cells were homogenized in a buffer (10 mL/g wet weight of cells) composed of 50 mM mannitol and 5 mM Hepes/Tris, pH 7.5, with a Polytron tissue homogenizer (Brinkmann Instruments, Rexdale, ON) at position 5.5 for 1 min and were then centrifuged at 600*g* for 10 min at 4 °C. The supernatants were centrifuged at 33000*g* for 30 min at 4 °C. The pellets were resuspended in the same buffer, stored at −80 °C, and used within 2 weeks. Protein was estimated by the Bradford assay (*26*).

Western Blots. P-gp and P-gp fragments were detected by Western blot analysis. Sodium dodecyl sulfate (SDS)−polyacrylamide gel electrophoresis (PAGE) was performed according to the method of Laemmli (*27*). The samples were resuspended in sample buffer and loaded on 6% or 15% acrylamide−bisacrylamide (29.2:0.8) gels without heating. The proteins were transferred electrophoretically onto a 0.45 μm pore-size PVDF membrane. Detections with the antibodies (mAbs C219, C494, and anti-CsA and pAb Ab-1) were performed as described previously (*15, 25*). Briefly, for detections with mAbs C219 and C494 the blots were blocked overnight at 4 °C with 5% (w/v) nonfat milk in 50 mM Tris, 150 mM NaCl, and 0.3% (w/v) Tween 20 (pH 7.0; TBS-T buffer). For detections with mAb anti-CsA the blots were blocked overnight at 4 °C in TBS only, while the blots for pAb Ab-1 were blocked in TBS and 0.1% Tween 20. PVDF membranes were washed three times with TBS-T and were incubated with antibodies (C219, C494, anti-CsA, or Ab-1) for 2 h at 25 °C. Anti-mouse and anti-rabbit horseradish peroxidase-conjugated IgGs were used as secondary antibodies. Detections were made with ECL reagents according to the manufacturer's instructions. The blots were exposed to preflashed Fuji films.

S. aureus V8 Proteolysis of P-gp. Membrane proteins were incubated with diazirine−CsA analogue SDZ 212-122 (0.5 μM) or IAAP (20 nM) for 60 min at 25 °C and irradiated on ice at 254 nm. P-gp was then precipitated with WGA−agarose as described (*28*). The final pellet was resuspended in Laemmli's sample buffer and membrane proteins were electrophoretically separated on 6.25% acrylamide gels as described by Laemmli (*27*). Regions from SDS gels that corresponded to P-gp were cut out and exposed to V8 proteolysis for 30 min as described by Cleveland using a second acrylamide gel of 15%. CsA-labeled fragments were detected by Western blot as described previously (*25*). IAAP-labeled polypeptides were revealed by exposing dried gels to Fuji film for 1 week.

Limited Proteolysis with Trypsin. Membrane proteins (50 μg) were incubated with diazirine−CsA analogue SDZ 212-122 (100 nM) for 60 min at 25 °C and irradiated on ice at 254 nm for 10 min. Unbound SDZ 212-122 was removed by centrifugation at 50000*g* for 30 min. The pellet was resuspended in 50 mM Tris-HCl, pH 7.5. Trypsin digestion

was performed as previously described (*22*) with 0.5 μg of trypsin at 37 °C for 2.5 h. Proteins were separated by SDS−PAGE on a 10% acrylamide/bisacrylamide gel. Immunodetection of photolabeled fragments and of fragments containing the C219, C494, and Ab-1 epitopes was performed by Western blot using a horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence as previously described.

Purification of Photolabeled P-gp by Diazirine−CsA Analogue. Proteins (60 mg) from CH^{R}C5 membranes were incubated with 0.5 μM diazirine−CsA analogue SDZ 212-122 in 10 mM Tris-HCl (pH 7.0) and protease inhibitors for 60 min at 25 °C and irradiated on ice at 254 nm for 10 min. Laemmli sample buffer without β-mercaptoethanol was added to the suspensions and proteins were separated by continuous elution electrophoresis on 4.5% acrylamide/bisacrylamide gels with a Model 491 Prep Cell (Bio-Rad). Electrophoresis was performed at 75 mA and the fraction collector was settled at 0.75 mL/min. Proteins exiting the gel were collected and the presence of photolabeled P-gp in these fractions was determined by Western blot with the pAb Ab-1 directed against P-gp or the mAb directed against CsA. The fractions containing P-gp were pooled and P-gp was then precipitated with wheat germ agglutinnin cross-linked to agarose, as previously described (*28*). Laemmli sample buffer was added to the WGA suspension and proteins were then separated once more by continuous elution electrophoresis on 4.0% acrylamide/bisacrylamide gels. The fractions that contained photolabeled P-gp were determined by Western blots, as previously mentioned, pooled, and concentrated with Centricon 30 filtration devices (Amicon, Danvers, MA). This material was separated into aliquots and kept at −80 °C.

Cleavage of Purified Photolabeled P-gp by N-Chlorosuccinimide. Cleavage of CsA-photolabeled P-gp at tryptophan residues was performed with 50 mM NCS in 50 mM citrate buffer, pH 3, for 16 h at 25 °C. The fragments were then precipitated by a chloroform/methanol/water method (*29*) and separated by SDS−PAGE on a 12.5% acrylamide/bisacrylamide gel. Western blot analysis was used to detect CsA labeled fragments or fragments containing the epitopes recognized by C219, C494, and Ab-1.

Cleavage of Purified Photolabeled P-gp by 2-Nitro-5-thiocyanobenzoate. Cleavage of CsA-photolabeled P-gp at cysteinyl residues was performed with NTCB. P-gp was incubated with 100 mM NTCB in 0.2 M Tris-acetate buffer, pH 8, for 1 h at 37 °C. The pH was then adjusted to 9 with NaOH and the reaction proceeded for a further 16 h. Fragments were precipitated by a chloroform/methanol/water method (*29*) and separated on a 15% acrylamide/bisacrylamide gel. Western blot analysis was used to detect CsA-labeled fragments or fragments containing the epitopes recognized by C219, C494, and Ab-1, as previously mentioned.

Cleavage of Purified Photolabeled P-gp by Cyanogen Bromide. Cleavage of CsA-photolabeled P-gp at methionine residues was performed with CNBr, as previously described (*30*) with slight modifications. Instead of drying under vacuum, the fragments were precipitated by a chloroform/methanol/water method (*29*) and separated on a 15% acrylamide/bisacrylamide gel. Western blot analysis was used to detect CsA-labeled fragments or fragments containing the epitopes recognized by C219, C494, and Ab-1, as previously mentioned.

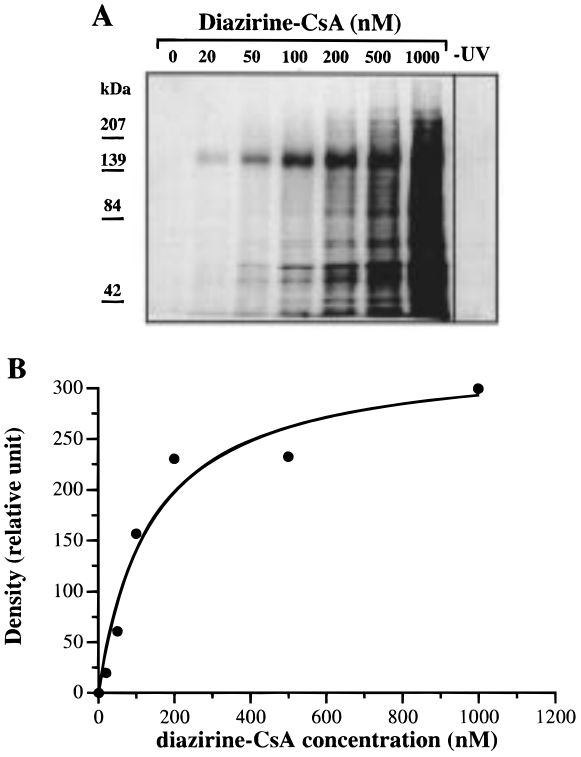

FIGURE 1: Photoaffinity labeling of P-gp by diazirine−CsA analogue SDZ 212-122. Membrane proteins (40 μg) from CH^{R}C5 cells were incubated for 60 min at 25 °C with increasing concentrations of diazirine−CsA analogue SDZ 212-122 (0−1000 nM) and irradiated on ice for 10 min, as described under Experimental Procedures. (A) CsA covalently bound to P-gp was immunodetected with the anti-CsA mAb. A control with SDZ 212-122 (50 nM) but without irradiation (−UV) was also performed. (B) The anti-CsA autoradiogram was scanned and the band density corresponding to P-gp was expressed as a function of SDZ 212-122 concentration.

RESULTS

CH^{R}C5 membranes were incubated with increasing concentrations of diazirine−CsA analogue SDZ 212-122 and irradiated with UV light (Figure 1). CsA-labeled proteins were detected by Western blot analysis with a mAb directed against CsA. The photolabeling of CH^{R}C5 proteins increased with SDZ 212-122 concentration (Figure 1A). A control was also performed with CH^{R}C5 proteins incubated with 50 nM diazirine−CsA without irradiation (−UV). Under these conditions, no labeled protein was detected when blots were probed with anti-CsA mAb. The region of 150−180 kDa, corresponding to P-gp, was scanned by laser densitometry and the measured band volumes were analyzed as a function of SDZ 212-122 concentration (Figure 1B). The photolabeling of P-gp by SDZ 212-122 was saturable and the approximate concentration needed to achieve 50% photolabeling was 135 nM.

The CsA binding domain of P-gp was first characterized by V8 proteolysis. P-gp photolabeled with diazirine−CsA analogue SDZ 212-122 or IAAP was precipitated with WGA−agarose, separated on a first gel, and then digested with V8 protease on a second polyacrylamide gel according to the Cleveland method. The fragments labeled by CsA were

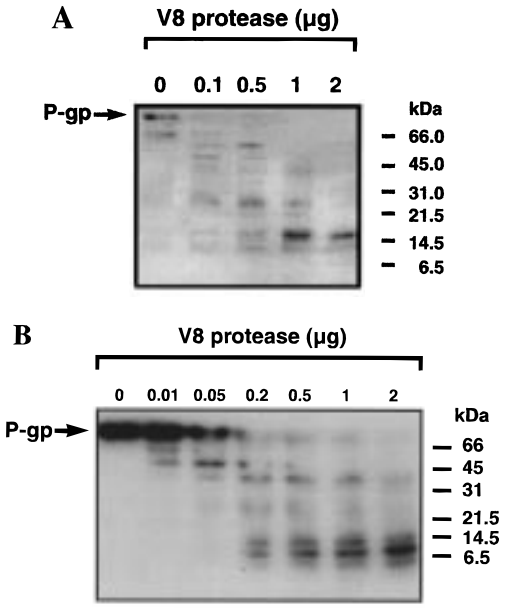

FIGURE 2: P-gp fragments obtained from V8 proteolysis after photolabeling with [^{125}I]iodoaryl azidoprazosin (IAAP) and CsA. P-gp was first photolabeled with diazirine−CsA analogue SDZ 212-122 or IAAP and precipitated with WGA as described under Experimental Procedures. A slice of the preparative gel (10%) was removed from the 150−180 kDa area and loaded in the wells of a second gel (15%). V8 was added to the wells in the indicated amount and proteolysis was performed according to the Cleveland method. (A) The molecular masses of P-gp proteolytic fragments photolabeled with CsA were estimated by Western blot analysis. After the second electrophoresis, the gel was blotted and the fragments photolabeled with CsA were detected with a mAb directed against CsA as indicated under Experimental Procedures. (B) The molecular masses of P-gp proteolytic fragments photolabeled with IAAP were estimated by autoradiography. After the second electrophoresis, the gel was dried and exposed to Fuji film.

Table 1: Molecular Masses of the Fragments Photolabeled by IAAP and Diazirine−CsA (SDZ 212-122) Obtained in Figure 2 by V8 Proteolysis Estimated by Laser Densitometry

fragments from IAAP (kDa)	fragments from SDZ 212-122 (kDa)
60	61
53	52
34	42
25	26
22	**15**[a]
11	13
7[a]	
5	

[a] Major fragments.

immunodetected with anti-CsA mAb (Figure 2A), while the fragments labeled with IAAP were revealed by autoradiography (Figure 2B). The fragments photolabeled with SDZ 212-122 or IAAP were analyzed by laser densitometry to determine their molecular masses (Table 1). The major proteolytic fragment photolabeled with SDZ 212-122 was 15 kDa, while the major fragment photolabeled with IAAP was 7 kDa. The difference between the molecular masses of the two major fragments (8 kDa) cannot be solely due to the difference in molecular mass between these two photoaffinity probes.

To determine whether the binding site for CsA is located in the N-terminal or in the C-terminal half of the protein, limited proteolysis with trypsin was performed on CsA-photolabeled CH^{R}C5 membranes (Figure 3). Fragments generated by trypsin were analyzed by Western blot with the mAbs anti-CsA, C494, C219, and pAb Ab-1. MAb C219 recognizes two related epitopes, one located in the N-terminal half of P-gp and the other in the C-terminal portion of the protein, and it detected two major fragments of 60 and 120 kDa. The band at 170 kDa represents a population of P-gp that has not been cleaved by trypsin. MAb C494, which recognizes an epitope in the C-terminal half of P-gp, detected only the 60 kDa fragment. This fragment was also detected by the pAb Ab-1, which is directed against an epitope in the C-terminal region of P-gp. These results indicate that the 120 kDa fragment generated by trypsin corresponds to the N-terminal portion of P-gp while the 60 kDa fragment corresponds to the C-terminal portion of P-gp. The mAb directed against CsA detected the 60 kDa fragment, suggesting that the C-terminal half of P-gp contains the major binding site for CsA.

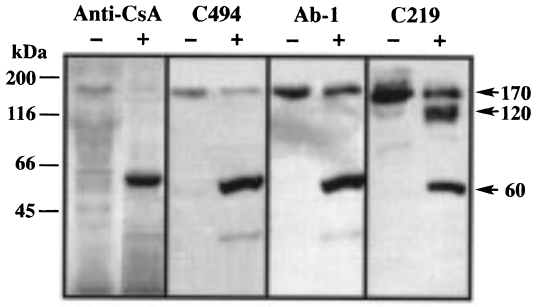

FIGURE 3: CsA-photolabeled membranes from CH^{R}C5 digested by trypsin. Crude membranes from CH^{R}C5 were photolabeled with diazirine−CsA analogue SDZ 212-122 as described under Experimental Procedures. Photolabeled P-gp was then digested with trypsin (0.25 μg) at 37 °C for 2.5 h. Proteins, either cleaved (+) or uncleaved (−), were resolved on SDS−PAGE. P-gp fragments were analyzed by Western blots with mAb C494 and C219 and pAb Ab-1. Photolabeled fragments were detected by mAb anti-CsA.

To further characterize this CsA-binding site in the C-terminal portion of P-gp, large amounts of CH^{R}C5 membrane proteins (60 mg) were photolabeled and separated by continuous elution gel electrophoresis (Figure 4). Aliquots from the eluted fractions were separated on SDS−PAGE and the fractions that contained P-gp photolabeled with CsA were determined by Western blotting with a mAb directed against CsA (Figure 4A) and a pAb directed against P-gp (Figure 4B). These two antibodies revealed that photolabeled P-gp was present in fractions 25−43. To increase P-gp purification, these fractions were pooled and P-gp was precipitated by WGA immobilized on cross-linked agarose beads. The precipitated P-gp was separated once more by continuous gel electrophoresis to eliminate other proteins that might have bound to the WGA−agarose beads. The fractions that contained P-gp labeled with CsA were once again determined by Western blotting analysis (Figure 5). Antibodies directed against CsA or P-gp revealed the presence of photolabeled P-gp in fractions 13−25. The difference in the fraction range in which P-gp is eluted between the two columns may be related to the concentration of acrylamide used (4.5% for the first column and 4.0% for the second one). These fractions (13−25) were pooled and P-gp was concentrated by ultrafiltration. By this procedure, approximately 30−40% of the photolabeled P-gp initially present in CH^{R}C5 membranes was recovered.

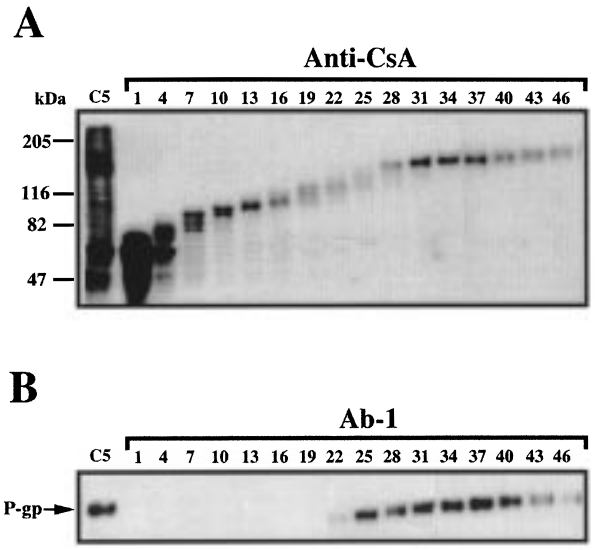

FIGURE 4: Separation of photolabeled CH^RC5 proteins by continuous elution electrophoresis column. CH^RC5 proteins (60 mg) were photolabeled with diazirine−CsA analogue SDZ 212-122 and separated on a continuous elution electrophoresis column, as described under Experimental Procedures. The collected fractions were screened by Western blot to determine which fractions contained CsA-photolabeled P-gp. (A) CsA bound to the proteins in various fractions was immunodetected as previously mentioned. (B) P-gp in these fractions was also immunodetected with a pAb (Ab-1) directed against P-gp. Photolabeled CH^RC5 proteins (C5) were used as positive controls for both detections.

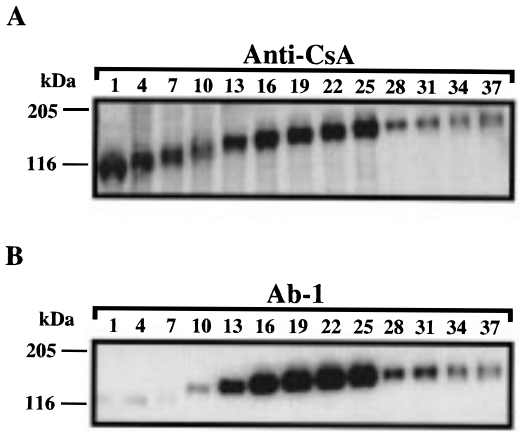

FIGURE 5: Separation of CsA-photolabeled proteins after WGA precipitation. P-gp was resolved on a second continuous elution electrophoresis column as described under Experimental Procedures. The fractions obtained were screened as described for the first continuous elution electrophoresis column by Western blotting analysis using (A) the anti-CsA mAb and (B) the anti-P-gp pAb.

The purified CsA-photolabeled P-gp was then cleaved by three chemical agents (NCS, NTCB, and CNBr). The fragment profiles obtained with these reagents were more complex than anticipated due to partial cleavages of P-gp by these reagents, yielding more fragments. To facilitate their analysis, we focused on the C-terminal portion of P-gp since we showed that CsA preferentially binds to this part of the protein. First, P-gp was cleaved at tryptophan residues by NCS. Because the peptide sequence of P-gp contains only 11 cleavage sites for NCS compared to 130 for V8 protease, the fragments obtained with NCS are more easily analyzed than are the fragments derived from enzymatic digestion (Figure 6A). A profile of the fragments photolabeled with CsA was established by immunodetection with a mAb

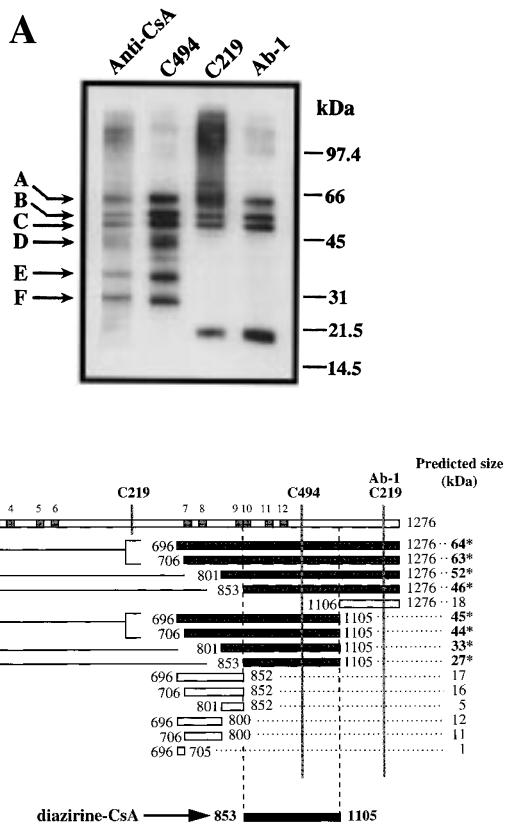

FIGURE 6: CsA-photolabeled P-gp cleavage at tryptophan residues by NCS. (A) Purified CsA-photolabeled P-gp was digested by NCS and the fragments were resolved on SDS−PAGE as described under Experimental Procedures. The sizes of the fragments generated by NCS were determined by Western blot using antibodies directed against P-gp (C494, C219, and Ab-1) and a mAb directed against CsA. (B) A schematic model of the P-gp molecule is presented. The solid boxes in the linear representation of P-gp correspond to the 12 putative TMs. The C219, C494, and Ab-1 epitopes are indicated by vertical lines, and the photolabeled fragments are represented by dark bars and asterisks. The molecular masses measured for the fragments generated by NCS that contained the C219, C494, and Ab-1 epitopes or were photolabeled with dz−CsA were compared to predicted fragments indicated by light bars.

directed against CsA. The fragments that contained the C494, C219, and Ab-1 epitopes were also identified. The molecular masses of the fragments from these four profiles are summarized and compared with the molecular masses of the fragments predicted from the C-terminal portion of P-gp (Figure 6B). Alignment of the digestion profiles shows that the CsA-photolabeled fragments (A−F), indicated by dark bars, are very similar to those that contain the C494 epitope. In addition, the smallest fragment (18 kDa) detected by mAb C219 and pAb Ab-1, which corresponds to amino acids 1106−1276 of P-gp, was not photolabeled. These results suggest that the smallest CsA-photolabeled fragment (F) includes the C494 epitope and corresponds to residues 853−1105 of P-gp.

CsA-photolabeled P-gp was also digested at cysteinyl residues by NTCB (Figure 7). The primary structure of P-gp contains seven cleavage sites for this reagent. In Figure 7A, the detections were performed on two different gels for mAbs C219 and C494. This explains the small variations in the migration of the molecular mass standards. The CsA-photolabeled fragments (A−D) were compared to the fragments that contained the C494 and C219 epitopes. Once

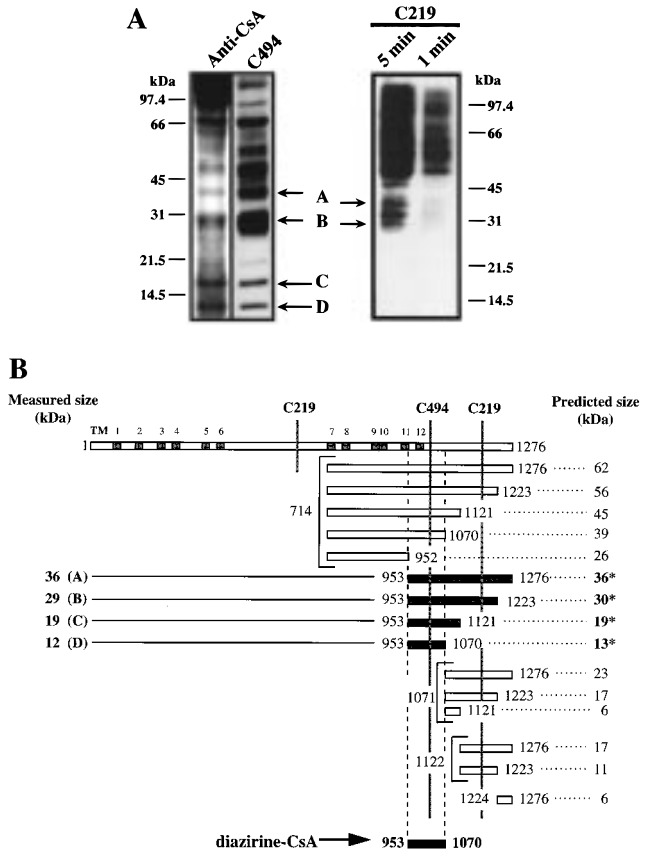

FIGURE 7: CsA-photolabeled P-gp cleavage at cysteinyl residues by NTCB. (A) Purified photolabeled P-gp was digested by NTCB and the fragments were resolved on SDS−PAGE as described under Experimental Procedures. The sizes of the fragments were determined by Western blot, with mAb directed against P-gp (C494 and C219) and a mAb directed against CsA. Different times of detection (1 and 5 min) are shown for mAb C219. (B) A schematic model of the P-gp molecule is presented. The solid boxes in the linear representation of P-gp correspond to the 12 putative TMs. The C219 and C494 epitopes are indicated by vertical lines, and the photolabeled fragments are represented by dark bars and asterisks. The molecular masses measured for the fragments generated by NTCB that contained the C219 and C494 eptiopes or photolabeled with dz−CsA were compared to predicted fragments indicated by light bars.

again, the digestion profile of the CsA-photolabeled fragments was similar to that obtained for fragments that contained the C494 epitope while there was a strong difference from the C219 blot. Since both of the mAbs (C219 and C494) recognize two different epitopes that are located in different region of P-gp, these results are not surprising. The cleavage profiles were compared to the molecular masses of fragments predicted from the C-terminal amino acid sequence of P-gp (Figure 7B). The fragments labeled with CsA, indicated by dark bars, show that the 36 and 29 kDa photolabeled fragments could be detected by mAbs C494 and C219, while the 19 kDa and 12 kDa photolabeled fragments were only detected by mAb C494. For the C219 blot, other fragments that are not recognized by C494 were detected, suggesting that these fragments probably originated from the N-terminal portion of P-gp, which contains the second C219 epitope. Thus, the smallest CsA-photolabeled fragment (12 kDa) obtained, indicated by hatched lines (D), corresponds to amino acid residues 953−1070 and includes TM 12 and the end of TM 11.

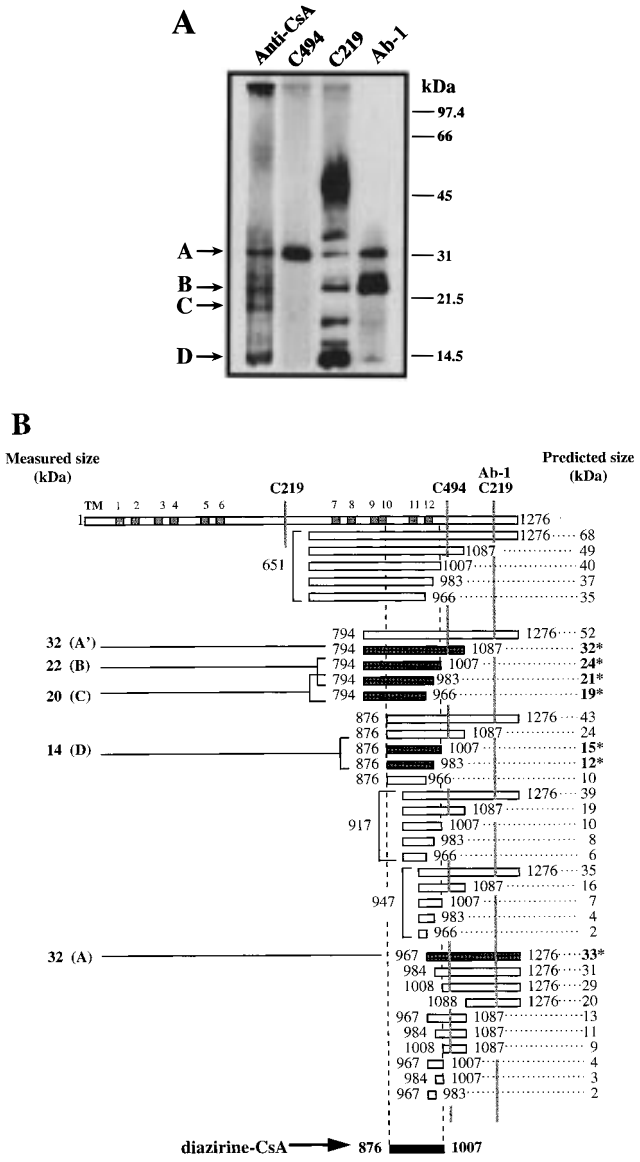

FIGURE 8: CsA-photolabeled P-gp cleavage at methionine residues by CNBr. (A) Purified photolabeled P-gp was digested by CNBr and the fragments were resolved on SDS−PAGE as described under Experimental Procedures. The sizes of the fragments were determined by Western blot, with antibodies directed against P-gp (C494, C219, and Ab-1) and a mAb directed against CsA. (B) A schematic model of the P-gp molecule is presented. The solid boxes in the linear representation of P-gp correspond to the 12 putative TMs. The C219, C494, and Ab-1 epitopes are indicated by vertical lines, and the photolabeled fragments are represented by dark bars and asterisks. The molecular masses measured for the fragments generated by CNBr that contained the C219, C494, and Ab-1 epitopes or photolabeled with dz−CsA were compared to predicted fragments indicated by light bars.

To confirm the results obtained with NCS and NTCB, cleavage of the CsA-photolabeled P-gp was also performed at methionine residues by CNBr. As shown in Figure 8A, a 32 kDa fragment (A) was detected with mAbs anti-CsA, C494, and C219 and with the pAb Ab-1. Surprisingly, this 32 kDa fragment was the only one detected by C494. The cleavage profiles obtained by CNBr cleavage were compared to the molecular masses of fragments predicted from the C-terminal amino acid sequence of P-gp (Figure 8B). The CsA-photolabeled fragments are indicated by dark bars. Two fragments (A and A′) of 32 kDa could be generated by CNBr

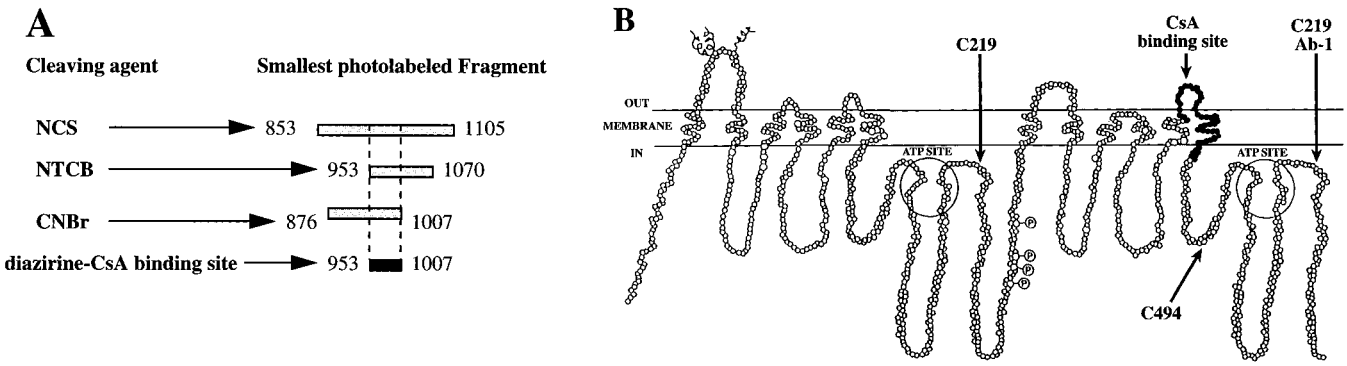

FIGURE 9: Alignment of the smallest CsA-photolabeled fragments generated by the three chemicals. (A) The smallest CsA-photolabeled fragments obtained from the tryptophan, cysteine, or methionine cleavage were aligned according to the primary amino sequence predicted for P-gp. The common region of the three fragments, indicated between hatched bars, corresponds to residues 953−1007. (B) A schematic drawing of the P-gp molecule is presented. The epitopes for mAb C219 and C494 and for pAb Ab-1 are indicated by black arrows. Solid circles represent the residues involved in the binding site of CsA, including the end of TM 11, TM 12, and the extracellular loop between these two TMs.

treatment of the published amino acid sequence of P-gp. Fragment A (residues 967−1276) includes the C494, C219, and Ab-1 epitopes, while fragment A′ (residues 794−1087) includes only the C494 epitope. Cleavage at sites inside both of these fragments (A and A′) could explain the detection of smaller photolabeled fragments (B, C, and D). These latter fragments were not detected by C494, suggesting that the 32 kDa fragments (A and/or A′) were cleaved at their C-terminal end, resulting in the loss of the C494 epitope. However, for both 32 kDa fragments (A and A′) the smallest photolabeled fragment (D, 14 kDa), undetected by C494, may correspond to two predicted fragments. One of these fragments (15 kDa) corresponds to amino acid residues 876−1007 and the other (12 kDa) includes the residues 876−983. Fragments of 22−25 kDa are detected by both C219 and Ab-1. These must originate from the 32 kDa fragments that have been cleaved between the C494 and C219 epitopes. Other fragments, between 14 and 18 kDa, were only detected with mAb C219, suggesting that these fragments originated from the N-terminal half of P-gp. These results with CNBr-mediated cleavage suggest that the binding site of CsA in P-gp corresponds to amino acid residues 876−1007 and includes TMs 10, 11, and 12.

The difference in the fragments generated by the three chemical agents and detected by the antibodies (C219, C494, Ab-1, and anti-CsA) helped us to determine the CsA-binding site in P-gp. The results obtained with mAbs C494 and anti-CsA were certainly the ones that gave us the most important information. The CsA-photolabeled fragments identified with the three chemical proteolytic agents were aligned (Figure 9A). All three fragments are consistent with a major binding site for CsA in the C-terminal portion of P-gp. Our results showed that the CsA-photolabeled fragments obtained with NCS, NTCB, and CNBr correspond to the amino acid residues 853−1105, 953−1070, and 876−1007, respectively. When these fragments are aligned, the common region (represented by a black bar) where CsA must bind includes the residues 953−1007, which are located within TM 11 and TM 12 (Figure 9B).

DISCUSSION

The molecular mechanism involved in the binding and transport of drugs by P-gp has been of great interest over the years. Photoaffinity labeling experiments with radioactive drug analogues were performed to determine the binding properties of P-gp (*18*−*20*, *22*, *31*). In the present study, enzymatic proteolysis and chemical cleavage of P-gp photolabeled with a nonradioactive CsA derivative was performed to localize the binding site of this chemosensitizer in hamster P-gp. The fragments photolabeled by CsA were detected with a mAb directed against CsA and compared to the fragments containing the C219, C494, and Ab-1 epitopes.

Previous studies have shown multiple binding domains on P-gp for various drugs. Photoaffinity probes for the α1-adrenergic receptor (IAAP) and for the calcium channel (azidopine) were shown to bind to a common domain within 6 kDa of the P-gp encoded by mouse *mdr* 1b gene (*30*). Two photoaffinity drug domains have been proposed, one for each half of the protein (*18*, *19*) The domain in the C-terminal half of the protein includes residues close to or within TM 11−12 (*18*), and the domain in the N-terminal half of the protein includes residues close to or within TM 5−6 (*19*). To achieve a functionally significant interaction with its substrate, the two halves of P-gp must be associated in a particular manner (*32*). It is speculated that the two photolabeling sites, located in the two halves of the protein, come close together to form a drug translocating pore with two distinguishable substrate interaction pockets within the N- and C-terminal portions (*20*). In the present study, the major P-gp fragment labeled with IAAP after V8 digestion was 7 kDa. This fragment is very similar to the P-gp binding domain for IAAP, estimated at 6 kDa after V8 proteolysis in J7.V1-1 cells (*31*). Proteolysis was also performed on P-gp photolabeled with diazirine−CsA analogue SDZ 212-122, according to the Cleveland method. The major fragment obtained after CsA labeling was 15 kDa. The difference in the molecular mass of these two major fragments, labeled either with IAAP or with CsA, could not be explained by the difference between the molecular mass of these two probes (around 1 kDa). Particularly, since there is no evidence for significantly aberrant migration of photolabeled fragments in the chemical cleavage experiments with NCS, NTCB, or CNBr, our results with V8 proteolysis suggest that these two affinity probes bind to different sites on P-gp. However, these two sites may be closely located since there

are approximately 130 sites for V8 proteolysis, many of which are present in TM 11 and TM 12.

Limited proteolysis with trypsin was previously used to cleave P-gp in its linker region and to show that both halves of P-gp were photolabeled by an iodinated analogue of forskolin and by IAAP (*19, 20*). In the present study, the two halves of diazirine−CsA-photolabeled P-gp generated by trypsin were immunodetected by C219 while the C-terminal half of P-gp was immunodetected by C494 and Ab-1. Although photolabeling experiments can be difficult to interpret quantitatively (*19*), our results indicate that the C-terminal half of P-gp is the major binding site for CsA since the mAb directed against CsA mainly recognizes the 60 kDa fragments detected by C494 and Ab-1. This is in contrast to a trypsin digestion performed on P-gp that had been photolabeled by an analogue of iodomycin, which indicated that the binding site of this drug was mainly in the N-terminal half of P-gp (*22*).

To further characterize the CsA binding site, continuous elution gel electrophoresis and wheatgerm agglutinin precipitation were used to obtain sufficient amounts of highly enriched CsA-photolabeled P-gp. This procedure, coupled with Western blotting analysis using a mAb directed against CsA, allowed us to optimize the chemical cleavage of P-gp by NCS, NTCB, and CNBr. These fragments were also compared with the fragments that contained the C494, C219, and Ab-1 epitopes. The mAb C219 binds to an amino acid sequence a short distance from the ATP binding sites in the N- and C-terminal portions of P-gp (Figure 9B), while C494 recognizes an amino acid sequence on the other side of the C-terminal ATP-binding domain (*3*). The pAb Ab-1 was generated against an amino acid sequence that overlaps the C-terminal epitope of C219. The profiles of CsA-photolabeled fragments generated by NCS and NTCB were very similar to those obtained for the C494 epitopes. In addition, when the smallest fragments generated by these three chemical agents were aligned, one common region in the C-terminal portion of P-gp could be determined. Using these approaches we were unable to detect any CsA-photolabeled fragments generated from the N-terminal portion of P-gp. These results, combined with those obtained with trypsin, suggest that CsA interacts mainly with one site in the C-terminal portion of P-gp.

Mutations of Phe^{335} within TM 6 of P-gp encoded by human *mdr1* gene (*33*) or Ala ^{339} of hamster P-gp1 (*34*) diminished the sensitivity of P-gp to CsA. In addition, P-gp mutated at Phe^{335} was less sensitive to PSC-833, a non-immunosuppressive analogue of CsA (*33*). These studies suggested that TM 6 is involved in the interaction of P-gp with CsA. However, two nonidentical binding sites for IAAP were identified, one within the N-terminal half of P-gp (TM 5−6) that was insensitive to CsA and the other within its C-terminal half (TM 11−12) that was sensitive to CsA (*20*). This study is in agreement with our results, suggesting that CsA interacts mainly with the C-terminal portion of P-gp. The loss of sensitivity toward reversal agents observed with previous TM 6 mutants (*33, 34*) could be the result of a conformational modification in the protein that would make the C-terminal site unable to recognize CsA or PSC-833.

The iodomycin binding site in hamster P-gp was localized to the region that includes amino acid residues 230−312 (*22*). This binding site for iodomycin was also sensitive to the presence of vinblastine and CsA. According to our results, the sensitivity of the iodomycin binding site toward CsA could occur as the result of a conformational modification of the protein following CsA interaction with P-gp, rather than by direct interaction of CsA with the region involved in the binding of iodomycin.

Mutagenesis and enzymatic proteolysis studies indicated that the region limited by the ends of TM 11 and TM 12 of the predicted topology (Figure 9B) seems to possess very important component(s) and structure(s) that allow the interaction of drugs with P-gp. More particularly, mutagenesis studies (*24, 35*) have shown that a serine residue within the predicted TM 11 of P-gps encoded by mouse *mdr1* (Ser^{941}) and *mdr3* (Ser^{939}) plays an important role in the substrate specificity of P-gp. This $Ser^{939/941}$ was also shown to be an important determinant in the recognition of P-gp modulators such as CsA (24). The region (953−1007) identified in the present paper as the binding site of CsA is not far from this important $Ser^{939/941}$. This may be explained by the fact that the SDZ 212-122 is a relatively large undecapeptide with a bulky substitution (diazirine) on position 8 that may, under UV light, cross-link to an adjacent region close to this $Ser^{939/941}$. Chimeric constructs of P-gp indicated that changes to TM 12 markedly impaired resistance to actinomycin D, vincristine, and doxorubicin, but not to colchicine (*21*). In addition, replacement of the loop between TM 11 and TM 12 created a more efficient pump for actinomycin D, colchicine, and doxorubicin but not for vincristine. Thus changes in the TM 11−12 region were associated with fundamental differences in the P-gp phenotype, reiterating the importance of this region for the drug-binding pocket. Studies using *cis*-(*Z*)-flupentixol, a modulator of P-gp function, indicated that this drug binds to a different site than does IAAP and that it preferentially increased the affinity for IAAP of the C-terminal half of the protein through an allosteric site on P-gp (*20*). Directed mutagenesis of the less conserved amino acid residues of TM 12 in human MDR1 showed that TM 12 contains this allosteric site and that the phenylalanine at position 983 is crucial for its interaction with *cis*-(*Z*)-flupentixol (*36, 37*). These results once again suggest that TM 12 possesses an important site-(s) for drug interactions with P-gp.

In the present study the cleavage of CsA-photolabeled P-gp in its linker region, and also at its tryptophan, cysteinyl, and methionine residues, revealed that this chemosensitizer binds predominantly to a region that includes amino acids 953−1007. According to the topology predicted for P-gp from hydropathy plots (*5, 38, 39*), this region corresponds to the ends of TM 11 and TM 12 as well as the extracellular loop between the TMs (Figure 9B). For the first time, we have demonstrated that this region of P-gp is directly involved in the binding of CsA to P-gp. Furthermore, PSC-833, one of the most potent reversal agents (*11−14*), might interact at the same site as does CsA, since it competes with a higher affinity than does CsA for the photolabeling of P-gp by diazirine−CsA analogue SDZ 212-122 (25). Because PCS-833 and CsA are among the drugs that have the strongest interaction with P-gp, our results suggest that TM 11 and TM 12 play a crucial role in the P-gp binding of these two chemosensitizers.

ACKNOWLEDGMENT

We thank Julie Poirier for her technical support and Dr. Richard Desrosiers and Dr. Michael Coady for their critical reading of the manuscript.

REFERENCES

1. Ling, V., and Thompson, L. H. (1974) *J. Cell. Physiol. 83*, 103−116.
2. Ling, V., Kartner, N., Sudo, T., Siminovitch, L., and Riordan, J. R. (1983) *Cancer Treat. Rep. 67*, 869−874.
3. Georges, E., Sharom, F. J., and Ling, V. (1990) *Adv. Pharmacol. 21*, 185−220.
4. Gottesman, M. M., and Pastan, I. (1993) *Annu. Rev. Biochem. 62*, 385−427.
5. Chen, C. J., Chin, J. E., Ueda, K., Clark, D. P., Pastan, I., Gottesman, M. M., and Roninson, I. B. (1986). *Cell 47*, 381−389.
6. Goldberg, H., Ling, V., Wong, P. Y., and Skorecki, K. (1988) *Biochem. Biophys. Res. Commun. 152*, 552−558.
7. Didier, A., Wenger, J., and Loor, F. (1995) *Anticancer Drugs 6*, 669−680.
8. Boesch, D., Gavériaux, C., Jachez, B., Pourtier-Manzanedo, A., Bollinger, P., and Loor, F. (1991) *Cancer Res. 51*, 4226−4233.
9. Boesch, D., and Loor, F. (1994) *Anticancer Drugs 5*, 229−238.
10. Tanaka, K., Hirai, M., Tanigawara, Y., Yasuhara, M., Hori, R., Ueda, K., and Inui, K. (1996) *Pharm. Res. 13*, 1073−1077.
11. Gavériaux, C., Boesch, D., Boelsterli, J. J., Bollinger, P., Eberle, M. K., Hiestand, P., Payne, T., Traber, R., Wenger, R. M., and Loor, F. (1989) *Br. J. Cancer 60*, 867−871.
12. Gavériaux, C., Boesch, C., Jachez, B., Bollinger, P., Payne, T., and Loor F. (1991) *J. Cell Pharmacol. 2*, 225−234.
13. Twentyman, P. R., and Bleehen, N. M. (1991) *Eur. J. Cancer 27*, 1639−1642.
14. Twentyman, P. R. (1992) *Biochem. Pharmacol. 43*, 109−117.
15. Jetté, L., Beaulieu, É., Leclerc, J. M., and Béliveau, R. (1996) *Am. J. Physiol. 270*, F756−F765.
16. Quesniaux, V. F., Schreir, M. H., Wenger, R. M., Hiestand, P. C., Harding, M. W. and Van Regenmortel, M. H. (1987) *Eur. J. Immunol. 17*, 1359−1365.
17. Liu, J., Farmer, J. D. Jr, Lane, W. S., Friedman, J., Weissman, I., and Screiber, S. L. (1991) *Cell 66*, 807−815.
18. Greenberger, L. M., Lisanti, C. J., Silva, J. T., and Horwitz, S. B. (1991) *J. Biol. Chem. 266*, 20744−20751.
19. Morris, D. I., Greenberger, L. M., Bruggeman, E. P., Cardarelli, C., Gottesman, M. M., Pastan, I., and Seamon, K. B. (1994) *Mol. Pharmacol. 46*, 329−337.
20. Dey, S., Ramachandra, M., Pastan, I., Gottesman, M. M. and Ambudkar, S. V. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 10594−10599.
21. Zhang, X., Collins, K. I., and Greenberger, L. M. (1995) *J. Biol. Chem. 270*, 5441−5448.
22. Demmer, A., Thole, H., Kubesch, P., Brandt, T., Raida, M., Fislage, R., and Tümmler, B. (1997) *J. Biol. Chem. 272*, 20913−20919.
23. Gros, P., Dhir, R., Croop, J., and Talbot, F. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 7289−7293.
24. Kajiji, S., Dreslin, J. A., Grizzuti, K., and Gros, P. (1994) *Biochemistry 33*, 5041−5048.
25. Demeule, M., Wenger, R. M., and Béliveau, R. (1997) *J. Biol. Chem. 272*, 6647−6652.
26. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
27. Laemmli, U.K. (1970) *Nature 227*, 680−685.
28. Demeule, M., Vachon, V., Delisle, M. C., Beaulieu, É, Averill-Bates, D., Murphy, G. F., and Béliveau, R. (1995) *Anal. Biochem. 230*, 239−247.
29. Wessel, D., and Flügge, U. I. (1984) *Anal. Biochem. 138*, 141−143.
30. Matsudaira, P. (1990) in *Guide to Protein Purifcation* (Deutscher, M. P., Ed.) pp 610−611, Academic Press Inc., San Diego, CA.
31. Greenberger, L. M., Yang, C. P., Gindin, E., and Horwitz, S. B. (1990) *J. Biol. Chem. 265*, 4394−4401.
32. Loo, T. W., and Clarke, D. M. (1994) *J. Biol. Chem. 269*, 7750−7755.
33. Chen, G., Duran, G. E., Steger, K. A., Lacayo, N. J., Jaffrézou, J.-P., Dumontet, C., and Sikic, B. I. (1997) *J. Biol. Chem. 272*, 5974−5982.
34. Ma, J. F., Grant, G., and Melera, P. W. (1997) *Mol. Pharmacol. 51*, 922−930.
35. Dhir, R., Grizzuti, K., Kajiji, S., and Gros, P. (1993) *Biochemistry 32*, 9492−9499.
36. Dey, S., Ramachandra, M., Pastan, I., Gottesman, M. M., and Ambudkar, S. V. (1998) *Proceedings of the AACR Meeting 39*, abstract 2470, p 362.
37. Hafkemeyer, P., Dey, S., Ambudkar, S. V., Hrycyna, C. A., Pastan, I., and Gottesman, M. M. (1998) *Proceedings of the AACR Meeting 39*, abstract 2469, p 362.
38. Gros, P., Croop, J., Roninson, I., Varshavsky, A., and Housman, D. E. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 337−341.
39. Gros, P., Croop, J., and Housman, D. (1986) *Cell 47*, 371−380.

BI981992C