Evidence for two nonidentical drug-interaction sites in the human P-glycoprotein

(multidrug transporter/cis(Z)-flupeptixol/photoaffinity labeling/[125I]iodoarylazidoprazosin/ATP hydrolysis)

SAIBAL DEY*, MURALIDHARA RAMACHANDRA†‡, IRA PASTAN†, MICHAEL M. GOTTESMAN*, AND SURESH V. AMBUDKAR*§

*Laboratory of Cell Biology and 1Laboratory of Molecular Biology, Division of Basic Sciences, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

Contributed by Ira Pastan, July 31, 1997

ABSTRACT Human P-glycoprotein (Pgp) confers multidrug resistance to cancer cells by ATP-dependent extrusion of a great many structurally dissimilar hydrophobic compounds. The manner in which Pgp recognizes these different substrates is unknown. The protein shows internal homology between its N- and C-terminal halves, each comprised of six putative transmembrane helices and a consensus ATP binding/utilization site. Photoactive derivatives of certain Pgp substrates specifically label two regions, one on each half of the protein. In this study, using [125I]iodoarylazidoprazosin ([125I]IAAP), a photoactive analog of prazosin, we have demonstrated the presence of two nonidentical drug-interaction sites within Pgp. Taking advantage of a highly susceptible trypsin cleavage site in the linker region of Pgp, we characterized the [125I]IAAP binding to the N- and C-terminal halves. cis(Z)-Flupentixol, a modulator of Pgp function, preferentially increased the affinity of [125I]IAAP for the C-terminal half of the protein (C-site) by reducing the Kd from 20 to 6 nM without changing the labeling or affinity (Kd = 42–46 nM) of the N-terminal half (N-site). Also, the concentration of vinblastine (Pgp substrate) and cyclosporin A (Pgp modulator) required for 50% inhibition of [125I]IAAP binding to the C-site was increased 5- to 6-fold by cis(Z)-Flupentixol without any effect on the N-site. In addition, [125I]IAAP binding to the N-site was less susceptible than to C-site to inhibition by vanadate which blocks ATP hydrolysis and drug transport. These data demonstrate the presence of at least two nonidentical substrate interaction sites in Pgp.

Human P-glycoprotein (Pgp) confers resistance to various structurally unrelated chemotherapeutic and cytotoxic agents (1, 2). Resistance results from the ability of Pgp to function as an ATP-dependent efflux pump for drug molecules (3, 4). Structurally, Pgp is a 1,280-amino acid integral membrane phospho-glycoprotein with two homologous halves connected by a short stretch of linker region (5). Each half consists of six putative transmembrane (TM) domains followed by a consensus nucleotide binding domain (5). Both of the ATP binding sites are catalytically active (6) and are required for the protein to function as a transporter (7). Pgp in isolated membranes (8) as well as in purified and reconstituted form (9) exhibits intrinsic ATPase activity that is stimulated upon interaction with the drug substrates. Besides substrate molecules, Pgp is also known to interact with a battery of chemical compounds, collectively known as reversing agents, that inhibit its ability to extrude anticaner drugs (1). These reversing agents, some of which are believed to be transport substrates by themselves, are also diverse in their chemical composition.

The mechanism by which Pgp recognizes and translocates such a broad spectrum of chemical compounds is presently unclear. Changes in single amino acid residues have been reported throughout the linear sequence of the Pgp molecule that resulted in alteration of the substrate specificity and drug-resistance profile (10–15). It is yet to be determined whether these residues directly take part in substrate recognition by forming contact sites for the drug molecules, or their change indirectly affects substrate interaction through a change in the overall structure of the protein.

To map the major areas of contact for the substrates within Pgp, photoaffinity analogs of various drugs have been used (16–19). Independent studies with Pgp involving different drug analogs have identified two major regions of photoaffinity labeling, one within each half of the protein (20–22). In the linear sequence of Pgp they correspond to areas in and around the putative TM5 and 6 in the N-terminal half, and TM11 and 12 in the C-terminal half (22, 23). Site-directed mutagenesis and domain-swapping experiments have also emphasized the role of these two regions in determining substrate specificity of Pgp (ref. 24; C. A. Hrycyna, P. Wu, U. A. Germann, I.P., and M.M.G., unpublished data). However, a major question that remains unanswered is whether the covalent binding regions identified in each half of Pgp are part of a single drug binding site or whether they represent two independent drug-interaction domains. Data regarding the number of drug-interaction sites within Pgp and their spatial arrangement within the drug-translocating pathway are essential to understand the mechanism of action of Pgp.

In this study using a photoaffinity substrate analog, [125I]iodoarylazidoprazosin ([125I]IAAP), and a known modulator of Pgp function, cis(Z)-flupeptixol, we provide direct evidence in favor of two nonidentical drug-interaction sites within Pgp and propose a model for the role of these two sites in drug translocation.

MATERIALS AND METHODS

Chemicals. cis(Z)-Flupentixol was bought from Research Biochemicals (Natick, MA). Vinblastine and prazosin were supplied by Sigma. Cyclosporin A was purchased from Calbiochem. Bodipy FL-prazosin and [125I]IAAP (2,200 Ci/mmol; 1 Ci = 37 GBq) were bought from Molecular Probes and DuPont/NEI, respectively. Pgp-specific mAb C219 (25) was a gift from Centocor.

Cell Lines and Baculovirus Expression Systems. Parent mouse cell line NIH 3T3 and NIH 3T3 transfected with wild-type human MDR1 cDNA, NIH-MDR1 (26), were grown in monolayer in 10594
DMEM with 10% fetal bovine serum as described (27). The recombinant baculovirus carrying either the human MDR1 gene, with 6× His-tag at the C-terminal end, BV-MDR1(H6) (M.R., S.V.A., D. Chen, C. A. Hrycyna, S.D. M.M.G., and I.P., unpublished data), or the MDR2 gene, BV-MDR2 (provided by U. A. Germann, Vortex Pharmaceuticals, Cambridge, MA) was used to infect High Five insect cells grown in serum free Ex-cell 400 medium as described (28). Cells were grown at 27°C in monolayer to confluency, infected with the recombinant baculovirus with multiplicity of infection of 10, and harvested 3 days postinfection.

Preparation of Crude Membranes from Insect Cells. Infected cells were scraped off and washed two times in PBS containing 1% aprotinin and incubated on ice for 45 min in lysis buffer (50 mM Tris-HCl, pH 7.5/50 mM mannitol/2 mM EGTA/1 mM DTT/1 mM 4- (aminoethyl) benzenesulfonfyl fluoride (AEBSF)/1% aprotinin). Cells were disrupted by repeated strokes of a Dounce homogenizer. Following lysis, undisrupted cells and nuclei were removed by centrifugation at 100,000 × g for 12 min. The supernatant was diluted with resuspension buffer containing 50 mM Tris (pH 7.5), 300 mM mannitol, 1 mM EGTA, 1 mM DTT, 1 mM AEBSF, and 1% aprotinin and centrifuged at 100,000 × g at room temperature for 16 h and then diluted to 15 ml with aprotinin and centrifuged at 100,000 × g.

Trypsin Digestion of Pgp Containing Membranes. The photoaffinity compound [125I]IAAP, a derivative of the α1-adrenoreceptor antagonist prazosin. Prazosin stimulates ATP hydrolysis in isolated membranes from High Five insect cells infected with a recombinant baculovirus, BV-MDR1(H6) (Fig. I4), with an apparent Km of 20 µM. The ability of Pgp to transport prazosin was also studied by fluorescence-activated cell sorter analysis in drug-resistant NIH-MDR1 cells using a Bodipy FL derivative of the drug (Fig. I5). When incubated with 0.5 µM Bodipy FL-prazosin, NIH-MDR1 cells showed reduced intracellular accumulation compared with that observed in drug-sensitive NIH 3T3 cells. Reduced accumulation was completely reversed in the presence of 10 µM cyclosporin A, indicating Pgp-mediated extrusion of Bodipy FL-prazosin from NIH-MDR1 cells.

The photoaffinity analog of prazosin [125I]IAAP has been shown to bind Pgp with high affinity in both intact cells and in membrane preparations, which is inhibited by the agents that interact with Pgp, including prazosin (19, 21). After photoaffinity labeling of crude membranes was carried out according to Bruggemann et al. (20), with slight modifications. An aliquot of membranes (10 µg protein) was incubated for 10 min with indicated concentrations of [125I]IAAP at room temperature (± 1 mCi/ml) containing 1% aprotinin. Cells were centrifuged at 500 × g for 5 min, resuspended in 300 µl PBS containing 0.1% BSA, and analyzed with a FACSort flow cytometer equipped with CELL QUEST software (Becton Dickinson).

Measurement of ATPase Activity. Pgp-associated ATP hydrolysis was measured by determining the release of inorganic phosphate from MgATP in the presence and absence of 0.3 mM sodium orthovanadate using a colorimetric assay as described by Sarkadi et al. (8), with minor modifications.

Photoaffinity Labeling with [125I]IAAP. Photoaffinity labeling of crude membranes was carried out according to Bruggemann et al. (20), with slight modifications. An aliquot of membranes (10 µg protein) was incubated for 10 min with indicated concentrations of [125I]IAAP at room temperature (± 1 mCi/ml) containing 1% aprotinin. Cells were centrifuged at 500 × g for 5 min, resuspended in 300 µl PBS containing 0.1% BSA, and analyzed with a FACSort flow cytometer equipped with CELL QUEST software (Becton Dickinson).

RESULTS
Prazosin and Its Analogs Are Substrates of Pgp. To investigate the number of drug-interaction sites on Pgp, we used the photoaffinity compound [125I]IAAP, a derivative of the α1-adrenoreceptor antagonist prazosin. Prazosin stimulates ATP hydrolysis in isolated membranes from High Five insect cells infected with a recombinant baculovirus, BV-MDR1(H6) (Fig. I4), with an apparent Km of 20 µM. The ability of Pgp to transport prazosin was also studied by fluorescence-activated cell sorter analysis in drug-resistant NIH-MDR1 cells using a Bodipy FL derivative of the drug (Fig. I5). When incubated with 0.5 µM Bodipy FL-prazosin, NIH-MDR1 cells showed reduced intracellular accumulation compared with that observed in drug-sensitive NIH 3T3 cells. Reduced accumulation was completely reversed in the presence of 10 µM cyclosporin A, indicating Pgp-mediated extrusion of Bodipy FL-prazosin from NIH-MDR1 cells.

The photoaffinity analog of prazosin [125I]IAAP has been shown to bind Pgp with high affinity in both intact cells and in membrane preparations, which is inhibited by the agents that interact with Pgp, including prazosin (19, 21). After photoaffinity labeling of crude membranes was carried out according to Bruggemann et al. (20), with slight modifications. An aliquot of membranes (10 µg protein) was incubated for 10 min with indicated concentrations of [125I]IAAP at room temperature (± 1 mCi/ml) containing 1% aprotinin. Cells were centrifuged at 500 × g for 5 min, resuspended in 300 µl PBS containing 0.1% BSA, and analyzed with a FACSort flow cytometer equipped with CELL QUEST software (Becton Dickinson).

Quantitation of Radioactivity in Protein Bands. Gels were fixed with a solution containing 50% methanol and 10% acetic acid for 15 min, treated with Novex gel drying solution for 10 min, and air dried between Dryease mini-cellophane for 16–18 h. Dried gels were exposed to Kodak Biomax-MR X-ray films at −80°C. The radioactive bands were cut out from the gel and soaked in 1 ml of tissue solubilizer (Solvable) from Packard at room temperature for 16 h and then diluted to 15 ml with Biosafe II scintillation fluid. Radioactivity associated with each band was determined in a scintillation counter.

Trypsin Digestion of Pgp Containing Membranes. To generate the N- and C-terminal halves of Pgp, membranes were incubated at 37°C with 0.75–1.5× trypsin (by weight) for 15 min. Reactions were stopped by addition of 5-fold excess of soybean trypsin inhibitor followed by SDS/PAGE sample buffer. Samples were subjected to separation by SDS/PAGE using 8% gel, and the photolabeled fragments were detected by autoradiography.

SDS/PAGE and Immunoblot Analysis. Electrophoresis and immunoblot analysis were performed as described (30).

Fig. 1. Interaction of prazosin and its analogs with Pgp. (A) Stimulation of Pgp-mediated ATP hydrolysis by prazosin. The vandate-sensitive Pgp-ATPase activity in the presence of indicated concentrations of prazosin in High Five insect membranes was assayed as described. (B) Pgp-mediated extrusion of Bodipy FL-prazosin from NIH-MDR1 cells. Accumulation of Bodipy FL-prazosin was measured in drug-sensitive NIH 3T3 and drug-resistant NIH-MDR1 cells. After incubating in glucose containing DMEM with 0.5 µM of Bodipy FL-prazosin in the presence or absence of cyclosporin A, cells were pelleted and analyzed for intracellular accumulation (fluorescence intensity) of the prazosin derivative by fluorescence-activated cell sorter. NIH 3T3 and NIH-MDR1 cells in the absence (.......) of the prazosin derivative by fluorescence-activated cell sorter.

Autoradiogram of 3 mCi of cyclosporin A. (C) Photoaffinity labeling of Pgp in insect cell membranes by [125I]IAAP. Membranes isolated from High Five insect cells, infected with recombinant virus BV-MDR1(H6) or BV-MDR2 were photoaffinity labeled with 2 mCi of [125I]IAAP in presence and absence of 25 µM cis(Z)-flupentixol as described. (Left) Autoradiogram of 3 µg of membrane protein/lane separated in an 8% gel by SDS/PAGE. (Right) Immunoblot analysis of the same samples using Pgp-specific mAb C219.
immunoblot). Immunoprecipitation with polyclonal antibody was confirmed by immunoblot analysis using mAb C219 (Fig. 1). Unlike most reversal agents, Flupentixol, an antipsychotic drug, has been shown to be a potent agent for reversing Pgp-mediated drug resistance (32). Flupentixol-labeled membranes were incubated with 7.5 nM [125I]IAAP in intact cells (33), in plasma membrane preparations (Fig. 1C, autoradiogram), and in reconstituted proteoliposomes (unpublished data). Prior studies using photoaffinity analogs of different drug molecules demonstrated the presence of two major photolabeled regions, one on each half of the protein (20–22). These two areas included TMs 5 and 6 in the N-terminal half and TMs 11 and 12 in the C-terminal half of Pgp (22, 23).

To determine the effect of cis(Z)-flupentixol on [125I]IAAP labeling of each of these two halves, we have taken advantage of a highly susceptible trypsin cleavage site in the linker region between the two halves of Pgp (20, 34). Incubation of insect cell membranes with trypsin, in a protein to trypsin ratio of 1:0.75–1.5 (wt/wt), allowed separation of the two halves by SDS-PAGE. In an 8% gel, the N- and C-terminal halves of the protein were detected as 70- and 60-kDa bands, respectively (Fig. 2). Identity of the two halves was determined by immunoblot analysis using polyclonal antibodies PEPG 13 and 4007, specific for the N- and C-terminal halves of Pgp, respectively. The arrow with long tail, the arrowhead with short tail, and the arrowhead show the position of Pgp, N-half, and C-half, respectively.

cis(Z)-Flupentixol Preferentially Stimulates [125I]IAAP Labeling to the Site on C-Terminal Half of Pgp. cis(Z)-Flupentixol, an antipsychotic drug, has been shown to be a potent agent for reversing Pgp-mediated drug resistance (32).

Unlike most reversal agents, cis(Z)-flupentixol enhanced photoaffinity labeling of Pgp with [125I]IAAP in intact cells (33), in plasma membrane preparations (Fig. 1C, autoradiogram), and in reconstituted proteoliposomes (unpublished data). Prior studies using photoaffinity analogs of different drug molecules demonstrated the presence of two major photolabeled regions, one on each half of the protein (20–22). These two areas included TMs 5 and 6 in the N-terminal half and TMs 11 and 12 in the C-terminal half of Pgp (22, 23).

To determine the effect of cis(Z)-flupentixol on [125I]IAAP labeling of each of these two halves, we have taken advantage of a highly susceptible trypsin cleavage site in the linker region between the two halves of Pgp (20, 34). Incubation of insect cell membranes with trypsin, in a protein to trypsin ratio of 1:0.75–1.5 (wt/wt), allowed separation of the two halves by SDS-PAGE. In an 8% gel, the N- and C-terminal halves of the protein were detected as 70- and 60-kDa bands, respectively (Fig. 2). Identity of the two halves was determined by immunoblot analysis using polyclonal antibodies PEPG 13 and 4007, specific for the N- and C-terminal halves of Pgp, respectively. The arrow with long tail, the arrowhead with short tail, and the arrowhead show the position of Pgp, N-half, and C-half, respectively (Fig. 2). Identification of the two halves was determined by immunoblot analysis using polyclonal antibodies PEPG 13 and 4007 [specific for the N- and C-terminal half, respectively (31, 35); Fig. 2B]. An additional band of 65 kDa was also photoaffinity labeled but was not detected by antibodies against Pgp. Labeling of this endogenous insect cell membrane protein was not inhibited by vinblastine or cyclosporin A (see Fig. 4).

A total of 5 nM [125I]IAAP labeled both N- and C-terminal halves. Radioactivity associated with the C-terminal half was about 1.5- to 2-fold higher than that of the N-terminal half. However, incubation of the membranes with cis(Z)-flupentixol for 3 min at 21–23°C before addition of 5 nM [125I]IAAP dramatically enhanced labeling of the C-terminal half of the protein without any significant effect on the N-terminal half (Fig. 2A). The total radioactivity associated with the two major bands and undigested Pgp equals the amount associated with the intact Pgp molecule before digestion (data not shown). For convenience, we named the [125I]IAAP binding site that photoaffinity labeled the N-terminal half, the N-site, and the one that photoaffinity labeled the C-terminal half, the C-site. However, this does not imply that the sites are exclusively formed by the respective halves.

cis(Z)-Flupentixol-Mediated Stimulation Was Due to Increased Affinity of the C-Site for [125I]IAAP. To determine the affinity of the two sites for [125I]IAAP, photoaffinity labeling was carried out in the presence of varying concentrations of [125I]IAAP ranging from 0 to 40 nM in the presence and absence of 10 μM cis(Z)-flupentixol. In the absence of cis(Z)-flupentixol, the apparent KD for binding of [125I]IAAP to the N- and C-sites were 42 and 20 nM, respectively (Fig. 3). In the presence of cis(Z)-flupentixol, the apparent KD of binding to the C-site was preferentially reduced to 6 nM without significant change in the Kd for the N-site (46 nM; Fig. 3). The change in KD also indicated that cis(Z)-flupentixol-mediated enhancement of [125I]IAAP labeling to the C-site was not due to an increase in the efficiency of the photoactivation step, but that it represented a change in the binding affinity for the drug analog.

Vinblastine and Cyclosporin A Inhibit [125I]IAAP Labeling of Both the Sites in Presence and Absence of cis(Z)-Flupentixol. To confirm that the binding of [125I]IAAP to the N- and C-sites represented functionally specific interaction with the drug binding sites, insect cell membranes were pre-incubated either with varying concentrations of vinblastine, a Pgp substrate, or with cyclosporin A, an inhibitor of Pgp function, before addition of 5 nM [125I]IAAP. Vinblastine and cyclosporin A effectively inhibited [125I]IAAP labeling to both sites (Fig. 4A and B Upper). The apparent IC50 (concentration required for 50% inhibition) values for inhibition of binding to the N-site were 0.5 μM for vinblastine and 0.05 μM for cyclosporin A. IC50 values for binding to the C-site were 2-fold less for both vinblastine (0.25 μM) and cyclosporin A (0.025 μM) than for the N-site.

When similar experiments were carried out in the presence of 10 μM cis(Z)-flupentixol, the IC50 values for inhibition by both vinblastine (0.75 μM) and cyclosporin A (0.05 μM) for the N-site were not significantly altered. However, a clear 5- to 6-fold increase in the concentration required for half maximal inhibition of the [125I]IAAP binding to the C-site was observed for both vinblastine (1.25 μM) and cyclosporin A (0.15 μM; Fig. 4A and B Lower). This preferential increase in the IC50 for inhibition of [125I]IAAP binding to the C-site, induced by interaction with cis(Z)-flupentixol, argues in favor of two discrete [125I]IAAP binding sites per Pgp molecule.

Differential Accessibility of the N- and C-Sites to [125I]IAAP After Vanadate Trapping. Inhibition of Chinese hamster Pgp ATPase activity by sodium orthovanadate resulted from trapping of a MgADP-Vi complex at the MgADP-P; binding site (36, 37). Urbatsch and Senior (38) have shown a 2-fold reduction in the [1H]azidopine labeling of Pgp in the presence of MgATP and vanadate. Recent studies, using purified and reconstituted Pgp, have shown that for vanadate-induced inhibition of drug binding,
ATP hydrolysis is essential (M.R., S.V.A., D. Chen, C. Hrycyna, S.D., M.M.G., and I.P., unpublished data). We therefore investigated the effect of vanadate on cis(Z)-flupentixol-mediated stimulation of $^{[125]}$IAAP labeling of the C-site. When Pgp containing membranes were incubated at 37°C for 10 min with 5 nM $^{[125]}$IAAP, 10 μM cis(Z)-flupentixol, 5 mM MgATP, and varying concentrations of vanadate, a dramatic decrease in the level of $^{[125]}$IAAP bound to both halves was observed (Fig. 5A). The inhibition was concentration-dependent, with a significant drop in labeling observed at 50 μM vanadate, the lowest concentration used in the experiment. When radioactivity associated with N- and C-sites was estimated, a clear difference in the extent of inhibition was observed between them. At 400 μM vanadate, the C-site retained only 10% of the $^{[125]}$IAAP that was bound in the absence of vanadate, whereas the N-site retained 85–95% of the binding. At this concentration vanadate completely inhibited Pgp-mediated ATP hydrolysis (data not shown). This result suggests a differential susceptibility of the two sites to MgADP-Vi induced conformational change in Pgp. No inhibition was evident when ATP was excluded from the reaction (Fig. 5A), indicating that vanadate by itself had no direct effect on $^{[125]}$IAAP labeling or on cis(Z)-flupentixol-mediated stimulation.

We further assessed the relative accessibility of the N- and C-sites to $^{[125]}$IAAP under conditions where MgADP-Vi has already been trapped within Pgp. Membranes were incubated with or without 400 μM vanadate in the presence of 5 mM MgATP and 10 μM cis(Z)-flupentixol for 10 min at 37°C. Following incubation, 2, 10, and 20 nM of $^{[125]}$IAAP were added separately to three different reactions and incubated at 37°C for 10 additional minutes, followed by UV irradiation for 5 min and analysis by SDS/PAGE. $^{[125]}$IAAP binding to the N-site was 30–45% of binding in the absence of vanadate, whereas binding to the C-site never exceeded 10–15% of the control (Fig. 5B). Therefore, the effect of vanadate-induced inhibition on the C-site was far more profound than on the N-site. These data also indicate that the N- and C-sites represented different $^{[125]}$IAAP interaction sites.

DISCUSSION

The unusually broad range of substrate specificity of human Pgp has stimulated interest in elucidating its underlying mechanism of drug recognition. A major step toward achieving this goal will be determining the number of substrate-interacting sites within the protein molecule. Over the years photoaffinity labeling has emerged as a powerful technique to study substrate and ligand binding properties of transporters and receptor proteins. Independent studies, using different photoaffinity analog of Pgp substrates, successfully detected two proteolytic fragments, one

**FIG. 3.** Concentration-dependent binding of $^{[125]}$IAAP to the N- and the C-terminal halves of Pgp in the presence and in absence of cis(Z)-flupentixol. (A) Pgp-containing insect cell membranes (10 μg of membrane protein) were photoaffinity labeled with concentrations of $^{[125]}$IAAP ranging from 1 to 50 nM with (Right) or without (Left) pre-incubation with 50 μM of cis(Z)-flupentixol. Photoaffinity labeled membranes were then treated with trypsin as described in the legend to Fig. 2, and samples were analyzed in an 8% gel by SDS/PAGE. (B) Photoaffinity labeled membranes were incubated with varying concentrations (0–10 μM) of vinblastine in the absence (Lower) or absence (Upper) of 10 μM cis(Z)-flupentixol. N- and C-terminal halves of Pgp were separated in an 8% gel by SDS/PAGE. $^{[125]}$IAAP binding to the N- and C-terminal halves of Pgp was quantified by measuring radioactivity associated with each fragment, as described. (B) Membranes were photoaffinity labeled as described above except with varying concentrations (0–10 μM) of cyclosporin A in the presence (Lower) and absence (Upper) of cis(Z)-flupentixol. Concentration of vinblastine and cyclosporin A required for half maximal inhibition (IC$_{50}$) was calculated from their respective inhibition curves.

**FIG. 4.** Effect of vinblastine and cyclosporin A on $^{[125]}$IAAP labeling of the N- and C-sites of Pgp in the absence and presence of cis(Z)-flupentixol. (A) Photoaffinity labeling of Pgp containing crude insect cell membranes were carried out with 5 nM of $^{[125]}$IAAP and with varying concentrations (0–10 μM) of vinblastine in the presence (Lower) and absence (Upper) of 10 μM cis(Z)-flupentixol. N- and C-terminal halves of Pgp were separated in an 8% gel by SDS/PAGE, and $^{[125]}$IAAP labeling was quantified by measuring radioactivity associated with each fragment, as described. (B) Membranes were photoaffinity labeled as described above except with varying concentrations (0–10 μM) of cyclosporin A in the presence (Lower) and absence (Upper) of cis(Z)-flupentixol. Concentration of vinblastine and cyclosporin A required for half maximal inhibition (IC$_{50}$) was calculated from their respective inhibition curves.

**FIG. 5.** Effect of vinblastine and cyclosporin A on $^{[125]}$IAAP labeling of the N- and the C-terminal halves of Pgp in the presence and in absence of cis(Z)-flupentixol. (A) Pgp-containing insect cell membranes (10 μg of membrane protein) were photoaffinity labeled with concentrations of $^{[125]}$IAAP ranging from 1 to 50 nM with (Right) or without (Left) pre-incubation with 50 μM of cis(Z)-flupentixol. Photoaffinity labeled membranes were then treated with trypsin as described in the legend to Fig. 2, and samples were analyzed in an 8% gel by SDS/PAGE. (B) Photoaffinity labeled membranes were incubated with varying concentrations (0–10 μM) of vinblastine in the presence of 50 μM vanadate, the lowest concentration used in the experiment. When radioactivity associated with N- and C-sites was estimated, a clear difference in the extent of inhibition was observed between them. At 400 μM vanadate, the C-site retained only 10% of the $^{[125]}$IAAP that was bound in the absence of vanadate, whereas the N-site retained 85–95% of the binding. At this concentration vanadate completely inhibited Pgp-mediated ATP hydrolysis (data not shown). This result suggests a differential susceptibility of the two sites to MgADP-Vi induced conformational change in Pgp. No inhibition was evident when ATP was excluded from the reaction (Fig. 5A), indicating that vanadate by itself had no direct effect on $^{[125]}$IAAP labeling or on cis(Z)-flupentixol-mediated stimulation.
from each half of Pgp as major photoaffinity labeled sites (20, 22, 23, 39). Competition studies also suggested the presence of more than one drug binding site (40). However, no study until now has conclusively answered the question whether the two photolabeled peptides are part of a common drug binding site or whether they represent two separate drug-interaction domains. In this work we show that there are at least two distinguishable drug-interaction sites of Pgp.

To study this question we made use of the photoaffinity compound, \([^{125}\text{I}]\)IAAP, which is a derivative of the \(\alpha_1\) adrenoreceptor antagonist prazosin. Both prazosin and its fluorescent analog, Bodipy FL-prazosin, are substrates of Pgp (Fig. 1A and B). The photoaffinity analog, \([^{125}\text{I}]\)IAAP, specifically labels Pgp but not the MDR2 gene product, which is closely related but functions as a phosphatidylcholine translocase (ref. 41; Fig. 1C). We photoaffinity labeled the intact Pgp molecule and then, taking advantage of a highly susceptible trypsin cleavage site in the linker region, separated the two halves by SDS/PAGE (Fig. 2).

The total \([^{125}\text{I}]\)IAAP bound to the intact Pgp molecule was distributed in a ratio of 2:3 between the N- and C-terminal halves, respectively, which was in close agreement with previous studies using \([^3\text{H}]\)azidopine (20). cis-(Z)-Flupentixol, a drug that reverses Pgp function (32), allosterically stimulated \([^{125}\text{I}]\)IAAP binding to Pgp (unpublished data). When the effect of cis(Z)-flupentixol on \([^{125}\text{I}]\)IAAP labeling of the two halves was studied, we found an 8- to 10-fold increase in photoaffinity labeling of the C-site without any significant change in the extent of labeling of the N-site (Fig. 2A). This preferential increase in binding was due to enhanced affinity of the C-site for \([^{125}\text{I}]\)IAAP as reflected by its reduced \(K_d\). On the other hand, the lack of effect of cis(Z)-flupentixol on \([^{125}\text{I}]\)IAAP binding to the N-site was evident by no noticeable change in the \(K_d\) of that site (Fig. 3). This result clearly suggests that photoaffinity labeling of the two halves represents two separate events of drug-interaction rather than two covalent attachment sites for the photoactive derivative within a single site.

It is worth pointing out that the terms N-site and C-site do not imply that the drug binding regions are exclusively formed by the N- and C-terminal halves, respectively. Two homologous halves of the human Pgp have been expressed separately in insect cells (42). Although both halves have low ATPase activity, neither of them exhibited drug-stimulated ATP hydrolysis, indicating a lack of proper interaction with drug substrates. When the half molecules were coexpressed, the drug-stimulated ATPase activity was recovered to a certain extent. Therefore, to achieve a functionally significant interaction with the substrate molecule, proper association between the two halves is necessary. We speculate that the photolabeled residues are located in two different halves of the protein with respect to its linear amino acid sequence, but that both halves come close together to form a drug translocating pathway within which N-site and C-site are spatially distinct substrate interaction pockets. This model is also supported by the evidence that mutations in the N-terminal TM 6 abrogate the ability of cis(Z)-flupentixol to stimulate labeling of the C-site (C. A. Hrycyna, S.D., S.V.A., I.P., and M.M.G., unpublished data).

Urbatsch et al. (37) proposed that inhibition of ATP hydrolysis by vanadate occurs through trapping of MgADP\(_\text{V}_6\), a stable analog of MgADP-P, in the catalytic site of Pgp. Based on the reduction in the level of \([^3\text{H}]\)azidopine photoaffinity labeling of Pgp in the presence of MgATP and vanadate, they suggested coupling between the catalytic site(s) and drug binding site(s) of Pgp (36, 38). Recently it has been observed that ATP hydrolysis, mediated by Pgp, is essential for vanadate-induced inhibition of drug binding (M.R., S.V.A., D. Chen, C. A. Hrycyna, S.D., M.M.G., and I.P., unpublished data). In addition, we have also shown that cis(Z)-flupentixol induced binding of \([^{125}\text{I}]\)IAAP can be similarly reversed by vanadate trapping (unpublished data). These studies suggest that a major conformational change is induced in the drug binding site of Pgp upon ATP hydrolysis. In investigating the effect of vanadate trapping on \([^{125}\text{I}]\)IAAP binding to the N- and C-sites (Fig. 5), we found that the C-site, to which binding was stimulated by cis(Z)-flupentixol, retained only 10–13% of its binding after vanadate treatment, whereas the N-site showed 40–45% residual binding. These data indicate a difference in coupling between the N and C drug-interaction sites and the catalytic domains, further emphasizing that these are distinct sites.

Based on these results and other studies, we propose a model on possible roles for the two nonidentical \([^{125}\text{I}]\)IAAP binding sites in the event of drug translocation (Fig. 6). This model is an extension of the catalytic scheme for ATP hydrolysis by Pgp originally proposed by Senior et al. (36). We suggest that the TM domains come close to each other and contribute to the formation of two spatially distinct substrate interaction sites, both of which are part of the drug-translocating pathway. The C-site, to which \([^{125}\text{I}]\)IAAP binding was stimulated by cis(Z)-flupentixol and which is more sensitive to vanadate-trapping, represents the initial drug-recognition site, or the ON-site (Fig. 6). This site is closer to the cytosolic phase of the membrane to recruit drug molecules (D) from the cytosol or from the inner leaflet of the lipid bilayer. The N-site, which is insensitive to cis(Z)-flupentixol and relatively less sensitive to vanadate trapping, functions as the OFF-site from which the drug molecule is finally released to the exterior. The ON- and OFF-sites are functionally equivalent to the inside-facing aspect and outside-facing aspect of

---

**Fig. 5.** Effect of vanadate trapping on \([^{125}\text{I}]\)IAAP photoaffinity labeling of the N- and C-terminal halves of Pgp. (A) Pgp-containing insect cell membranes were incubated at 37°C for 10 min with 4 nM \([^{125}\text{I}]\)IAAP, 10 \(\mu\text{M cis(Z)}\)-flupentixol, 5 mM MgCl\(_2\), and varying concentrations of vanadate in presence and absence of 2.5 mM ATP prior to UV exposure for 10 min. Photoaffinity labeled membranes were trypsinized to generate the N- and C-terminal fragments and analyzed by SDS/PAGE. (Lower) Radioactivity associated with the N- and C-halves in the presence and absence of ATP is shown. (B) Insect cell membranes were incubated at 37°C for 10 min with 10 \(\mu\text{M cis(Z)}\)-flupentixol, 2.5 mM ATP, and 5 mM MgCl\(_2\) in presence and absence of 400 \(\mu\text{M}\) of vanadate. After incubation, \([^{125}\text{I}]\)IAAP was added to final concentrations of 2, 10, and 20 nM, and Pgp was photoaffinity labeled the same way as above. (Lower) Recovery of radioactivity in both halves is shown. For other details, see legend to Fig. 2.
the drug binding site proposed by Senior et al. (36). Movement of the drug substrate from the ON-site to the OFF-site is unfavorable and rate-limiting for the drug to be translocated, but can be driven by the large free energy change that occurs during ATP hydrolysis (Fig. 6). This change involves generation of MgADP,zP, at the catalytic site and conversion of the ON-site to a low affinity site favoring movement of the drug molecule to the OFF-site. Once it is in the OFF-site, the drug molecule is released to the exterior. The release of drug from the OFF-site either precedes or takes place simultaneously with the release of Pz. This conformation of Pgp (PgpMgADP,zP) with low affinity for substrate can be stabilized by trapping MgADP, at the MgADP, site (Fig. 6, IV A). The relatively passive nature of the OFF-site (with lower affinity for substrate) is consistent with a single K, for the stimulation of ATP hydrolysis by substrates (9, 36).

We propose that cis(Z)-flupentixol interacts with an allosteric site of Pgp (unpublished data), which favors binding of the substrate to the ON-site by inhibiting translocation to the OFF-site. This allosteric site might overlap with either the ON or OFF-sites or be distinct. The identification of amino acid residue(s) cross-linked with [125I]IAAP and directed mutagenesis of these residues should help to elucidate the drug-interaction sites on Pgp.

We thank Drs. U. A. Germain for providing the BV-MDR2 construct, W. D. Stein for helpful discussions, and C. A. Hrycyna for the help with fluorescence-activated cell sorter analysis. We also thank members of the Multidrug Resistance group for comments on the manuscript.


FIG. 6. Schematic representation of the Pgp catalytic cycle and the possible functional role of the N- and the C-sites in drug translocation. In this model, the two TM domains and the ATP binding sites are represented by squares and circles, respectively. The model shows one cycle of ATP hydrolysis where the shaded circle represents the noncatalytic state. The two drug-interacting sites are along the drug-translocating pathway and are designated by two ellipses (shaded for ON-site and open for OFF-site). The cis(Z)-flupentixol (modulator) interaction site is depicted by the hexagon. The hatched ellipse indicates a conformational change in the drug-interaction site closer to the cytosolic phase of the lipid bilayer. The substrate molecule, cis(Z)-flupentixol and vanadate are shown as “D,” “F,” and “Vi,” respectively. The dark arrows represent favored reaction. Various states of Pgp during the catalytic/drug-translocation cycle are as follows: I, PgpMgATP; II, PgpMgATP-DON; IIA, PgpMgATP-DON-Pz; III, PgpMgADP-Pz; DOFF; IV, PgpMgADP-Pz; IVA, PgpMgADP-Vi.