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# Localization of the Forskolin Labeling Sites to Both Halves of P-Glycoprotein: Similarity of the Sites Labeled by Forskolin and Prazosin

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## SUMMARY

An iodinated derivative of forskolin, 6-*O*-[[2-[3-(4-azido-3-[$^{125}$I] iodophenyl)propionamido]ethyl]carbamyl]forskolin ([$^{125}$I]6-AIPP-Fsk), photolabels the multidrug efflux pump P-glycoprotein in membranes prepared from the multidrug-resistant cell lines KB-V1 and KB-C1. The labeling site for [$^{125}$I]6-AIPP-Fsk was localized by immunoprecipitation of tryptic fragments of P-glycoprotein labeled in KB-C1 membranes. A 6-kDa, photolabeled, tryptic fragment was immunoprecipitated by antiserum raised against residues 348–419 of P-glycoprotein, PEPG9, but not by antisera raised against flanking regions PEPG7 and PEPG11. A peptide that corresponds to residues 343–359 of P-glycoprotein inhibited immunoprecipitation of the 6-kDa fragment by antiserum against PEPG9 but had no effect on the immunoprecipitation of photolabeled fragments by antiserum against PEPG7. A second peptide, corresponding to residues 360–376, had no effect on the immunoprecipitation by antiserum against PEPG9. [$^{125}$I]6-AIPP-Fsk labels the carboxyl-terminal half of P-glycoprotein, because low molecular mass tryptic fragments were immunoprecipitated by three carboxyl-terminal antisera. Therefore, [$^{125}$I]6-AIPP-Fsk labels both halves of P-glycoprotein, and labeling in the amino-terminal half can be localized to residues 291–359, which span proposed transmembrane regions 5 and 6. KB-V1 membranes photolabeled with [$^{125}$I]6-AIPP-Fsk and [$^{125}$I]iodoarylazidoprazosin were digested with either *Staphlyococcus aureus* V8 protease or chymotrypsin and had similar digestion patterns, suggesting that the two drugs label the same sites on P-glycoprotein.

P-glycoprotein is a membrane-bound glycoprotein that acts as an energy-dependent efflux pump by transporting drugs out of cells (1). This protein is responsible for conferring MDR, a phenomenon in which tumor cells acquire resistance to a variety of structurally and functionally unrelated cytotoxic drugs (2–5). Most of the drugs that interact with P-glycoprotein are amphipathic with a planar aromatic ring structure, but the only feature found in all of the drugs is that they are hydrophobic (6). In addition to many anticancer drugs, such as doxorubicin, vinblastine, and etoposide, peptides are also substrates for the multidrug transporter (7). P-glycoprotein has a reported molecular mass of 130–180 kDa, and the predicted structure of P-glycoprotein, based on its amino acid sequence, consists of two similar domains, each containing six transmembrane regions and an ATP binding site (8–10).

There are a number of different photolabeled analogs of drugs that bind to P-glycoprotein, including vinblastine (10, 11), azidopine (12), verapamil (13, 14), prazosin (15, 16), colchicine (17), and daunomycin (18, 19). The ability of different drugs to compete with the binding of these photolabels to the P-glycoprotein is consistent with the ability of these drugs to be transported. Different photolabeled derivatives of drugs that bind to P-glycoprotein have been used to elucidate the locations of drug binding sites on the human and mouse P-glycoprotein. In these studies P-glycoprotein was photolabeled and digested either enzymatically or chemically into peptide fragments, and the radiolabeled peptide fragments of the protein were identified using antisera raised against specific regions of P-glycoprotein. These studies have been carried out with [$^{3}$H]azidopine (20–22) and [$^{125}$I]iodoarylazidoprazosin (15, 23) and demonstrate that drug binding sites are found on both halves of the protein. The binding of [$^{125}$I]iodoarylazidoprazosin to the carboxyl-terminal half of P-glycoprotein has been hypothesized to be in close proximity to transmembrane domains 11 and 12 (23) and has more recently been localized to a 4-kDa region within, or immediately carboxyl-terminal to, transmembrane domain 12 (24). The binding of [$^{3}$H]azidopine to the amino-terminal half of P-glycoprotein has been localized to a 25-kDa

**ABBREVIATIONS:** MDR, multidrug resistance; [$^{125}$I]6-AIPP-Fsk, 6-*O*-[[2-[3-(4-azido-3-[$^{125}$I]iodophenyl)propionamido]ethyl]carbamyl]forskolin; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

cyanogen bromide fragment including transmembrane regions 3–6 and the associated cytoplasmic regions (21).

Regions of the protein that affect drug binding have been identified. Studies of naturally occurring mouse P-glycoprotein mutants suggest that transmembrane domain 11 is a putative drug binding site. Serine to phenylalanine substitutions at amino acids 941 and 939 in mouse mdr1b and mdr2, respectively, had little effect on vinblastine resistance but strongly modulated resistance to colchicine and Adriamycin (25). Mdrla and mdrlb are also known as mdr3 and mdr1, respectively (4). Further studies suggest that these mutations result in reduced drug transport by decreasing the initial drug binding to the protein (26). Site-directed mutagenesis, changing prolines to alanines in transmembrane domains 4 and 11 in mouse NIH/3T3 cells, significantly reduced the ability of the protein to confer resistance to colchicine, Adriamycin, or actinomycin D but had no effect on vinblastine transport (27). These results suggest there are differences in the requirements for the binding of different substrates to P-glycoprotein.

Forskolin, a natural product diterpene, interacts with different membrane proteins including adenylyl cyclase (28), the glucose transporter (29, 30), the voltage-gated potassium channel (31), and ligand-gated ion channels (32, 33). The predicted structures of these proteins are similar to that of P-glycoprotein in that all of the proteins are composed of domains of six transmembrane regions. Wadler and Wiernik (34) first demonstrated that forskolin could affect drug transport in MDR cell lines. Forskolin and 1,9-dideoxyforskolin were shown to partially reverse Adriamycin resistance in a human MDR ovarian cancer cell line (SKVLB) but not in its parental cell line (35). An iodinated derivative of forskolin, [$^{125}$I]6-AIPP-Fsk, photolabeled P-glycoprotein in SKVLB membranes (35). Labeling of P-glycoprotein by [$^{125}$I]6-AIPP-Fsk was effectively competed with 1,9-dideoxyforskolin, verapamil, diltiazem, nifedipine, and vinblastine and was partially competed with forskolin, colchicine, and cytocholasin B. This work has now been extended to localize the labeling of forskolin on P-glycoprotein. Data are also presented that compare the labeling site of [$^{125}$I]6-AIPP-Fsk with that of [$^{125}$I]iodoarylazidoprazosin.

## Experimental Procedures

**Materials.** [$^{125}$I]6-AIPP-Fsk was prepared as described previously (36). [$^{125}$I]Iodoarylazidoprazosin (specific activity, 2200 Ci/mmol) was obtained from DuPont-New England Nuclear. *N*-Tosyl-L-phenylalanine chloromethyl ketone-treated trypsin, *Staphylococcus aureus* V8 protease, 1,9-dideoxyforskolin, and PMSF were obtained from Sigma. Aprotinin and soybean trypsin inhibitor were obtained from Boehringer Mannheim. Protein A-Sepharose was obtained from Pierce. $^{14}$C-labeled high and low molecular weight standards were obtained from GIBCO-BRL.

**Cell lines.** KB-C1 and KB-V1 cells, which overexpress P-glycoprotein, were derived from a parent cell line, KB-3-1, and have been described previously (37, 38). The KB-C1 cells overexpress a form of the protein with a valine in place of glycine at position 185. KB-C1 and KB-V1 cells were selected with colchicine and vinblastine, respectively, and are maintained with 1 μg/ml drug.

**Antisera.** Six antisera were prepared using fusion proteins between *Pseudomonas exotoxin* and specific regions of the P-glycoprotein, i.e., PEPG7, PEPG8, PEPG9, PEPG10, and PEPG11 (39). The P-glycoprotein amino acid residues from which these fusion proteins were derived are 231–296, 874–939, 348–419, 991–1062, and 436–529, respectively. The 4007 antiserum was raised against amino acid residues 919–1280 of P-glycoprotein (40).

**Peptides.** Two peptides corresponding to different regions of the fusion protein for PEPG9 were synthesized. Peptide 1 and peptide 2 correspond to amino acids 343–359 (FSVGQASPSIEAFANAR) and 360–376 (GAAYEIFKIIDNKPSID), respectively. The sequence of a control peptide, peptide 3, is CHARQVDVKLRLD. Peptide 1 and peptide 3 were synthesized using *N*-*t*-butoxycarbonyl chemistry and peptide 2 was synthesized using 9-fluorenylmethoxycarbonyl chemistry, with an Applied Biosystems 430A peptide synthesizer. Peptides were purified using reverse phase high performance liquid chromatography and their purity was confirmed by amino acid analysis and mass spectrometry.

**Membrane preparations.** KB-C1 and KB-V1 cells grown in several T-150 flasks to produce $10^7$ to $10^8$ cells were harvested with trypsin and then rinsed with growth medium. The cells were centrifuged at 1000 rpm and washed three times with PBS. The cells were rinsed at $10^7$ cells/ml in ice-cold hypotonic lysis buffer (10 mM Tris·HCl, pH 8.0, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM PMSF, 1 μg/ml aprotinin) and then resuspended in the same buffer for 20 min before homogenization (20 strokes) in a Dounce homogenizer. The extract was transferred to an ice-cold centrifuge tube and centrifuged for 10 min at 2000 rpm in a Sorvall SS34 rotor. The supernatant was centrifuged for 30 min at 16,000 rpm. The supernatant was discarded and the membrane pellet was resuspended to $10^8$ cell equivalents/ml in ice-cold PBS. The protein concentration was 2–5 mg/ml.

**Photolabeling of membranes with [$^{125}$I]6-AIPP-Fsk and subsequent digestion with trypsin.** Membrane samples (45–55 μg) in 100 μl of PBS were incubated on ice in the dark with $2 \times 10^6$ cpm of [$^{125}$I]6-AIPP-Fsk and were photolabeled in a UV Stratagene Stratalinker as described previously (35). One modification to the protocol was that the photolabeling was not quenched with β-mercaptoethanol, to prevent interference of the β-mercaptoethanol with the immunoprecipitation experiments. Membranes were centrifuged and the pellets were resuspended by vortex-mixing in 18 μl of 50 mM Tris, pH 8.5. Trypsin (2 μl of a 0.5–2 mg/ml solution dissolved in 1 mM HCl; final concentration of trypsin, 0.05–200 μg/ml) was added to each membrane sample and samples were incubated at 37° for 2.5 hr. The samples were prepared for either gel electrophoresis or immunoprecipitation.

**Electrophoresis.** For electrophoretic separation of the radiolabeled protein fragments, an equal volume of 2× NOVEX sample buffer (3.0 mM Tris·HCl, pH 8.45, 24% glycerol, 8% SDS, 4% β-mercaptoethanol) was added to each sample, and the samples were heated for 10 min at 37°. The samples were applied to a 10% NOVEX tricine gel and run at a constant 95 V. The gels were stained with Coomassie blue according to the NOVEX protocol and dried, and the radiolabeled proteins were detected by autoradiography using Kodak XAR-5 film. The $^{14}$C-labeled molecular mass standards were myosin (200 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18.4 kDa), lysozyme (14.3 kDa), and bovine trypsin inhibitor (6.2 kDa).

**Immunoprecipitation.** An immunoprecipitation protocol described previously was followed, with some modifications (41). Briefly, the trypsin-digested membrane samples were diluted with 20 μl of 2% SDS, 2 mg/ml bovine serum albumin, 50 mM Tris, pH 8.5, and then diluted with 5 volumes of 1.25% Triton X-100, 190 mM NaCl, 50 mM Tris, pH 7.4, with the inhibitors PMSF, aprotinin, and soybean trypsin inhibitor. The samples were incubated overnight with 5 μl of antiserum and then for 2 hr with 65 μl of a 50% solution of Pierce Protein A-Sepharose that had been resuspended previously in PBS. The samples were washed and the bound protein was eluted with 35 μl of 2× NOVEX sample buffer, with 15 min of rapid shaking. The samples were microfuged for 1 min and the supernatants containing the radiolabeled protein fragments were removed, heated for 10 min at 37°, and applied to 10% NOVEX tricine gels as described above.

**Competition experiments with 1,9-dideoxyforskolin.** KB-C1 membranes were incubated in the presence and absence of 100 μM 1,9-dideoxyforskolin for 30 min on ice. The samples were photolabeled with [$^{125}$I]6-AIPP-Fsk and digested with 0, 1, 5, or 10 μg/ml trypsin as

described above. The samples were then immunoprecipitated with antiserum against PEPG9 and were resolved on gels after the procedure described above for immunoprecipitation.

**Competition experiments with peptides.** KB-C1 membranes (50 μg) were photolabeled and digested with 10 μg/ml trypsin as described above. Immunoprecipitation buffers (10 μl) of peptide (0–10 μg/ml) dissolved in 0.5% acetic acid and 5 μl of antisera were added to trypsin-digested samples. The samples were then treated as described above for immunoprecipitation.

**Photolabeling of KB-V1 membranes with [$^{125}$I]6-AIPP-Fsk and [$^{125}$I]iodoarylazidoprazosin.** KB-V1 membranes were prepared by differential centrifugation and sucrose gradient separation according to previously published methods (41). The membranes (100 μg) were incubated with $10^6$ cpm of [$^{125}$I]iodoarylazidoprazosin or $10^5$ cpm of [$^{125}$I]6-AIPP-Fsk for 1 hr at room temperature in the dark, as described previously for [$^{125}$I]iodoarylazidoprazosin (15). The samples were irradiated with UV light for 5 min at 4°. The proteins were solubilized in Laemmli buffer (42) and resolved on a 10% Laemmli gel in a Hoeffer gel apparatus run at 20-mA constant current. The gels were dried and exposed to Kodak XAR-5 film with an intensifier screen for 1 hr.

**Digestion of photolabeled KB-V1 membranes with either *S. aureus* V-8 protease or chymotrypsin.** The membranes were photolabeled with [$^{125}$I]6-AIPP-Fsk and [$^{125}$I]iodoarylazidoprazosin as described above and the proteins were resolved on 10% Laemmli gels. The gels were immediately dried, with no fixation, and were exposed to X-ray film. The radioactive fragments were identified on the gel and cut from the gel, and the fragments were sliced into about eight pieces. The sliced fragments were coelectrophoresed with 0, 0.02, 0.2, 2, or 20 μg of *S. aureus* V8 protease or 0, 0.2, or 2 μg of chymotrypsin on gels prepared according to the method of Cleveland *et al.* (43). The resultant digests were detected by autoradiography of the Cleveland gels.

## Results

**Labeling of P-glycoprotein in KB-C1 and KB-V1 membranes.** KB-C1 and KB-V1 are MDR cell lines that overexpress P-glycoprotein and are derived from the KB-3–1 drug-sensitive KB (HeLa) human carcinoma cell line. KB-C1 and KB-V1 cell lines were selected with colchicine and vinblastine, respectively, and are maintained with 1 μg/ml drug (38). KB-C1 cells have a single point mutation in the amino acid sequence of the P-glycoprotein, with a valine in place of a glycine at amino acid residue 185 (44).

KB-C1 and KB-V1 membranes were digested with 0–200 μg/ml trypsin to determine the overall pattern of digestion. The membranes were photolabeled with [$^{125}$I]6-AIPP-Fsk, digested with trypsin, and separated on tricine gels as described in Experimental Procedures. Photolabeling of P-glycoprotein in membranes that were not treated with trypsin is represented in Fig. 1, *lanes 0*. The major protein photolabeled in these lanes was a 170-kDa protein, similarly to photolabeling of the P-glycoprotein in SKVLB membranes (35). Forskolin also competed for labeling of the 170-kDa protein in both KB-C1 and KB-V1 membrane preparations (data not shown). The digestion patterns of radiolabeled fragments from the two cell lines were very similar and were reproducible, with the major fragments being initially generated at 1 μg/ml trypsin (Fig. 1). Neither digestion went to completion; however, the major fragment generated in both was a 6-kDa fragment.

**Immunoprecipitation of photolabeled tryptic fragments by amino-terminal P-glycoprotein antisera.** Membranes from KB-C1 cells were photolabeled with [$^{125}$I]6-AIPP-Fsk and digested with trypsin, and the photolabeled P-glycoprotein fragments were immunoprecipitated with different P-glycoprotein antisera to either the amino-terminal or carboxyl-terminal half of the protein. The locations of the peptide regions of P-glycoprotein used to prepare antisera are shown in Fig. 2. Two amino-terminal antisera, those against PEPG7 and PEPG9, immunoprecipitated similar photolabeled fragments (Fig. 3). Two fragments that were immunoprecipitated, of 97 and 70 kDa, were similar to the major amino-terminal digestion fragments previously reported in mouse *mdr1b*-expressing cells (23) and KB-C1 cells (20), respectively, using different amino-terminal antisera. There was some immunoprecipitation of a 55-kDa fragment at all concentrations of trypsin. However, there were no major fragments detected between 43 and 18 kDa with either antiserum. A major fragment of about 14 kDa and two minor fragments that migrated between the 14.3-kDa and 18.4-kDa markers, produced by trypsin concentrations of >1 μg/ml, were immunoprecipitated by both antisera (Fig. 3). The major difference between anti-PEPG9 and -PEPG7 antisera was the ability of the anti-PEPG9 antiserum to immunoprecipitate a 6-kDa fragment (Fig. 3). The gels in Fig. 3 were overexposed to show that antiserum against PEPG7 did not immunoprecipitate even small amounts of a 6-kDa fragment. The 6-kDa fragment is the same size as the major photolabeled fragment produced in the tryptic digest of the KB-C1 and KB-V1 membranes in Fig. 1. Preimmune serum for PEPG9 did not immunoprecipitate any small tryptic fragments after digestion of KB-C1 membranes with 1, 5, or 10 μg/ml trypsin (data not shown).

A similar immunoprecipitation experiment was carried out with antiserum against PEPG11, which contains part of the first ATP binding region of the P-glycoprotein and is adjacent to PEPG9. Antiserum against PEPG11 immunoprecipitated 97-, 70-, and 55-kDa fragments at all concentrations of the trypsin digestion (Fig. 4). Minor fragments were detected between the 43- and 29-kDa markers. However, there was no evidence of a 6-kDa fragment even when the gel was overexposed.

A drug competition experiment was performed to demonstrate that the 6-kDa fragment immunoprecipitated by antiserum against PEPG9 was a proteolytic fragment of P-glycoprotein labeled specifically by [$^{125}$I]6-AIPP-Fsk. KB-C1 membranes were pretreated with no drug or with 100 μM 1,9-dideoxyforskolin before incubation with [$^{125}$I]6-AIPP-Fsk. The membranes were then digested with 0, 1, 5, or 10 μg/ml trypsin and the samples were immunoprecipitated with antiserum against PEPG9. 1,9-Dideoxyforskolin competed for the labeling of intact P-glycoprotein and most of the 6-kDa fragment (Fig. 5). In a similar drug competition experiment, vinblastine (55 μM) also inhibited labeling of P-glycoprotein and the 6-kDa fragment (data not shown).

Peptide competition experiments were performed to localize the 6-kDa photolabeled fragment on P-glycoprotein. A 7.2-kDa fragment is predicted to be produced by complete tryptic digestion of P-glycoprotein. This fragment would be generated by digestion at $Lys^{291}$ and $Arg^{359}$ (Fig. 6). A 17-amino acid peptide, peptide 1, that corresponds to amino acid residues 343–359 of P-glycoprotein was synthesized. Peptide 1 corresponds to the carboxyl-terminal 17 amino acids of the 7.2-kDa fragment, which overlaps the amino-terminal 12 amino acids of PEPG9, residues 348–359 (see Fig. 6). KB-C1 membranes were photolabeled and digested with 10 μg/ml trypsin. Photolabeled tryptic fragments were immunoprecipitated with antisera against PEPG7 and PEPG9 in the presence of increasing concentra-

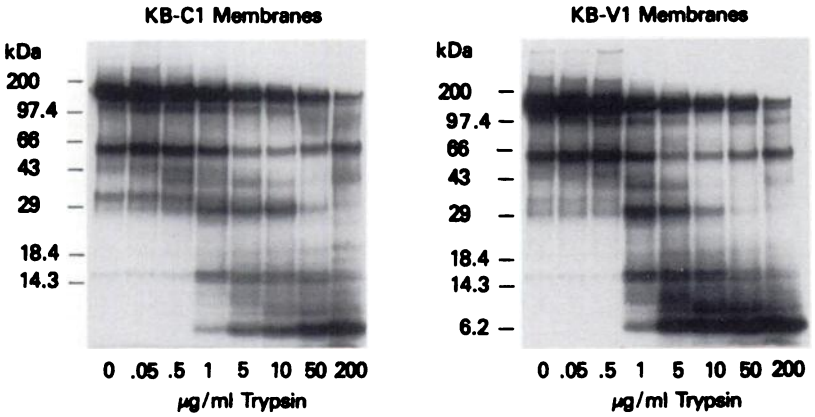


**Fig. 1.** Trypsin digestion of photolabeled KB-C1 and KB-V1 membranes. Membranes (55 μg) were incubated with [$^{125}$I]6-AIPP-Fsk, photolabeled, and digested with 0–200 μg/ml trypsin as described in Experimental Procedures. The radiolabeled protein fragments were solubilized in NOVEX sample buffer and the fragments were separated on 10% NOVEX tricine gels. The gels were stained, dried, and exposed to Kodak XAR-5 film.

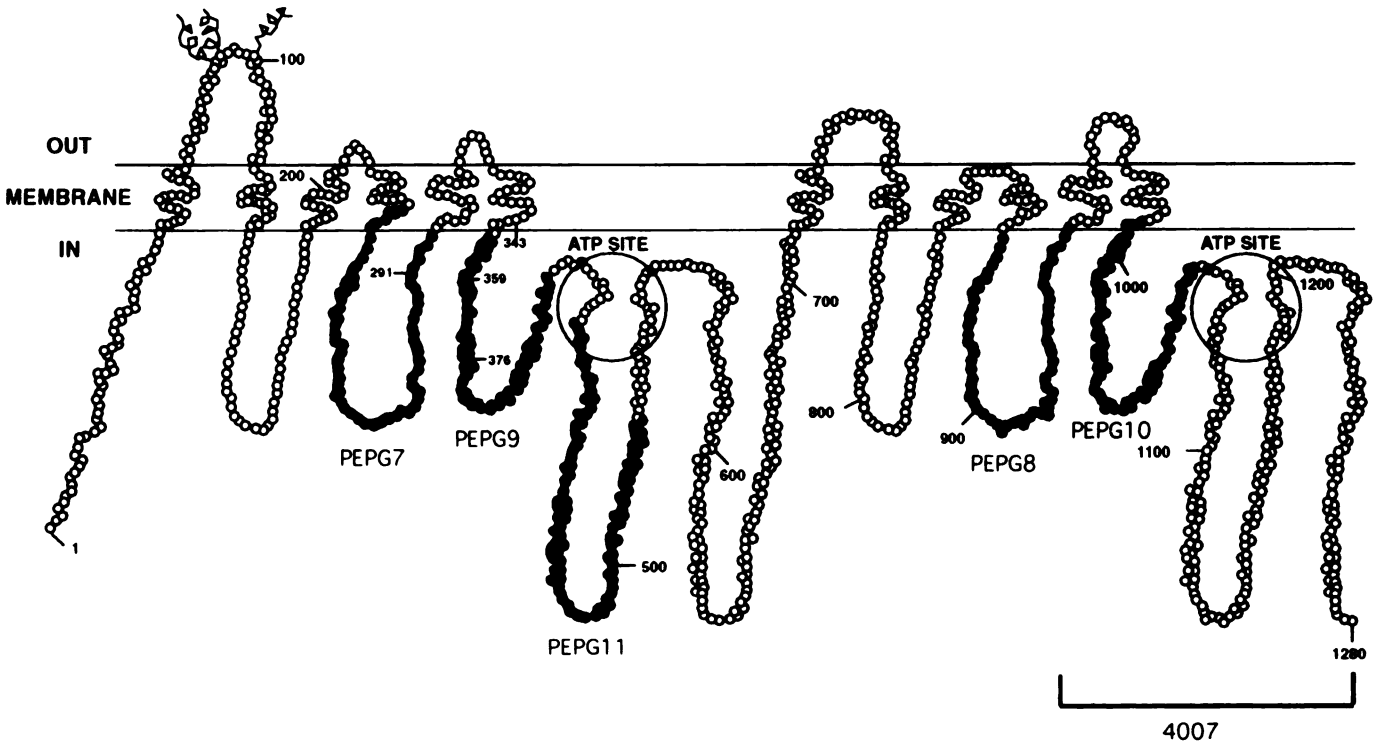


**Fig. 2.** Structure of P-glycoprotein. A predicted structure of P-glycoprotein is shown, and the regions of the protein from which the fusion proteins for the antisera were produced are *highlighted*.

tions of peptide 1 (Fig. 7). Peptide 1 did not inhibit the immunoprecipitation of intact P-glycoprotein or any photolabeled tryptic fragments by antiserum against PEPG7. In contrast, peptide 1 inhibited the immunoprecipitation of the 6-kDa fragment, fragments between the 14- and 18-kDa markers, and intact P-glycoprotein at concentrations of peptide 1 of >0.016 μg/ml. Peptide 2, generated against amino acid residues 360–376 of P-glycoprotein (see Fig. 6), did not compete with the immunoprecipitation of the 6-kDa or 14–18-kDa fragments by antiserum against PEPG9 at the same concentrations of peptide tested for peptide 1 (data not shown). In addition, a control, unrelated, 13-amino acid peptide, peptide 3, also failed to compete with the immunoprecipitation of P-glycoprotein or any photolabeled fragments by antiserum against PEPG9 (data not shown).

**Immunoprecipitation of photolabeled tryptic fragments by carboxyl-terminal P-glycoprotein antisera.** Antiserum 4007 was prepared against a very large carboxyl-terminal peptide that extends from amino acid 919 (just before transmembrane domain 11) to amino acid 1280 of the carboxyl terminus of P-glycoprotein (see Fig. 2). KB-C1 membranes were photolabeled and digested with 0–200 μg/ml trypsin (Fig. 8). At all concentrations of trypsin a 55-kDa fragment was the major fragment immunoprecipitated. Minor radiolabeled fragments that migrated between the 14- and 18-kDa markers were immunoprecipitated in addition to fragments of 40 and 30 kDa. Even at a very high concentration of trypsin (200 μg/ml), a 6-kDa photolabeled fragment was not detected. Similar results were observed with antisera generated against peptides from the carboxyl-terminal half of P-glycoprotein, PEPG8 and PEPG10, that correspond to the amino-terminal peptides PEPG7 and PEPG9, respectively (see Fig. 2). Digestions with these two carboxyl-terminal antisera were carried out with 0–10 μg/ml trypsin (data not shown).

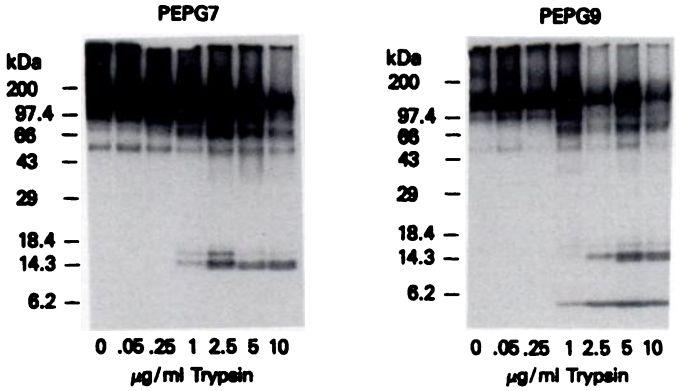


Fig. 3. Immunoprecipitation of photolabeled, trypsin-digested KB-C1 membranes by antisera against PEPG7 and PEPG9. KB-C1 membranes (50 μg) were incubated with 2 × $10^6$ cpm of [$^{125}$I]6-AIPP-Fsk, photolabeled, and digested with 0–10 μg/ml trypsin. The samples were incubated with antisera and then Protein A-Sepharose and were washed as described in Experimental Procedures. The proteins were eluted from the Sepharose with NOVEX sample buffer and applied to 10% NOVEX tricine gels. The gels were stained, dried, and exposed to Kodak XAR-5 film.

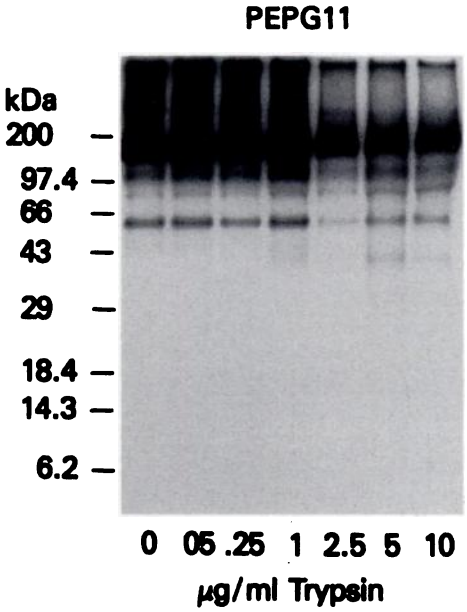


Fig. 4. Immunoprecipitation of photolabeled, trypsin-digested KB-C1 membranes by antiserum against PEPG11. The KB-C1 membranes (50 μg) were photolabeled and digested with 0–10 μg/ml trypsin, and the radiolabeled fragments from P-glycoprotein were immunoprecipitated as described for Fig. 3.

**Comparison of the ability of [$^{125}$I]6-AIPP-Fsk and [$^{125}$I]iodoarylazidoprazosin to photolabel the P-glycoprotein in KB-V1 membranes.** [$^{125}$I]Iodoarylazidoprazosin is a photoactive drug that has been shown to photolabel both halves of P-glycoprotein in mouse and human cell lines (15, 23). The abilities of [$^{125}$I]iodoarylazidoprazosin and [$^{125}$I]6-AIPP-Fsk to label the P-glycoprotein in KB-V1 membranes were compared. Membranes were photolabeled with $10^5$ cpm of [$^{125}$I]6-AIPP-Fsk or $10^6$ cpm of [$^{125}$I]iodoarylazidoprazosin, following the protocol for photolabeling of P-glycoprotein with [$^{125}$I]iodoarylazidoprazosin described previously (15). The intensity of the labeling of P-glycoprotein by [$^{125}$I]6-AIPP-Fsk ($10^5$ cpm) was similar to that produced by a 10-fold higher

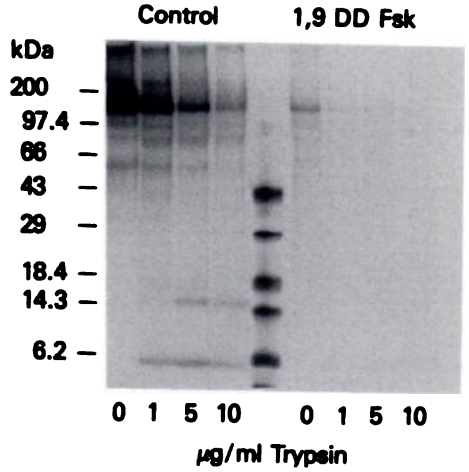


Fig. 5. Immunoprecipitation of photolabeled, trypsin-digested KB-C1 membranes by antiserum against PEPG9 after competition with 1,9-dideoxyforskolin. KB-C1 membranes (50 μg) were incubated with either PBS or 100 μM 1,9-dideoxyforskolin (*1,9 DD Fsk*) for 30 min before incubation with [$^{125}$I]6-AIPP-Fsk. The samples were photolabeled and digested with 0–10 μg/ml trypsin. The digested fragments were incubated with antiserum and immunoprecipitated as described for Fig. 3.

concentration of [$^{125}$I]iodoarylazidoprazosin ($10^6$ cpm) (Fig. 9). This suggests that [$^{125}$I]6-AIPP-Fsk was about 10 times more efficient at photolabeling P-glycoprotein than was [$^{125}$I]iodoarylazidoprazosin and that [$^{125}$I]6-AIPP-Fsk labeled fewer nonspecific proteins (Fig. 9).

KB-V1 membranes were photolabeled with both photolabels and then digested with either *S. aureus* V8 protease or chymotrypsin. Fragments of 45 and 35 kDa were generated for both photolabels with 0.02 μg of *S. aureus* V8 protease and were further digested to a 6-kDa fragment with higher concentrations of protease (Fig. 10). Similar results were observed for both photolabels using chymotrypsin. At low concentrations of chymotrypsin (0.2 μg), there were several digested fragments similar to those generated from the *S. aureus* V8 protease digestion, with major fragments being detected at 35 and 6 kDa. There was no further digestion of these fragments with 2 μg of chymotrypsin. These results suggest that forskolin and prazosin label the same site.

## Discussion

A photoactive derivative of forskolin, [$^{125}$I]6-AIPP-Fsk, was previously shown to photolabel P-glycoprotein in a human carcinoma cell line that overexpresses P-glycoprotein. Several drugs known to bind to P-glycoprotein inhibited the ability of forskolin to label P-glycoprotein (35). Forskolin is a natural product diterpene that is hydrophobic, a common characteristic of all drugs that interact with P-glycoprotein; however, it has no charge at neutral pH and does not have any aromatic rings, making it relatively unique among P-glycoprotein-interacting agents. We employed this photolabel to localize the drug binding site(s) of forskolin on P-glycoprotein and to determine whether the drug binding site was similar to that identified with other labels. Our results detect labeling sites for a forskolin photaffinity label in both halves of P-glycoprotein, localize an amino-terminal labeling site to a 6-kDa fragment between amino acid residues 291 and 359, and demonstrate equivalent labeling sites for forskolin and prazosin photoaffinity labels.

[$^{125}$I]6-AIPP-Fsk photolabels P-glycoprotein in both KB-V1

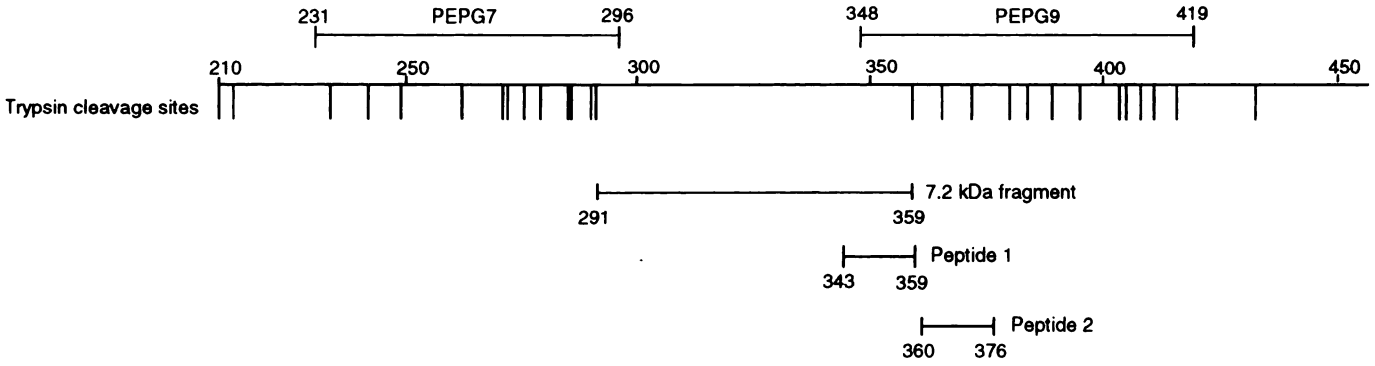


**Fig. 6.** Portion of P-glycoprotein from amino acid 210 to amino acid 450; *vertical lines*, potential trypsin cleavage sites. Locations of peptide 1 and peptide 2 in relation to this sequence of P-glycoprotein and the overlap of PEPG7 and PEPG9 peptides are shown. A 7.2-kDa fragment of P-glycoprotein is predicted from a trypsin limited digest at amino acids 291 and 359. Peptide 1 overlaps the carboxyl-terminal 17 amino acids of this 7.2-kDa fragment and the amino-terminal 12 amino acids of PEPG9. Peptide 2 starts immediately carboxyl-terminal to the 7.2-kDa fragment and overlaps 17 amino acids of PEPG9.

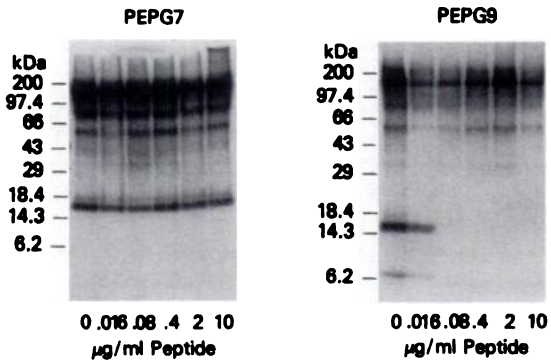


**Fig. 7.** Peptide competition with the immunoprecipitation of photolabeled, trypsin-digested KB-C1 membranes by antisera against PEPG7 and PEPG9. KB-C1 membranes (50 μg) were photolabeled with [$^{125}$I]6-AIPP-Fsk and then digested with 10 μg/ml trypsin. The digested fragments were incubated with 0–10 μg/ml peptide 1 and 5 μl of antiserum and then immunoprecipitated as described for Fig. 3.

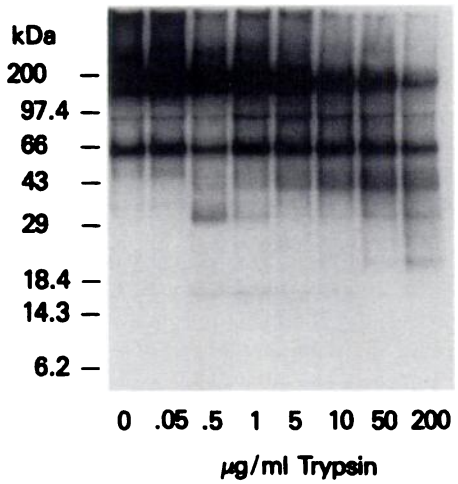


**Fig. 8.** Immunoprecipitation of photolabeled, trypsin-digested KB-C1 membranes by antiserum 4007. The KB-C1 membranes (50 μg) were photolabeled and digested with 0–200 μg/ml trypsin, and the radiolabeled fragments from P-glycoprotein were immunoprecipitated as described for Fig. 3.

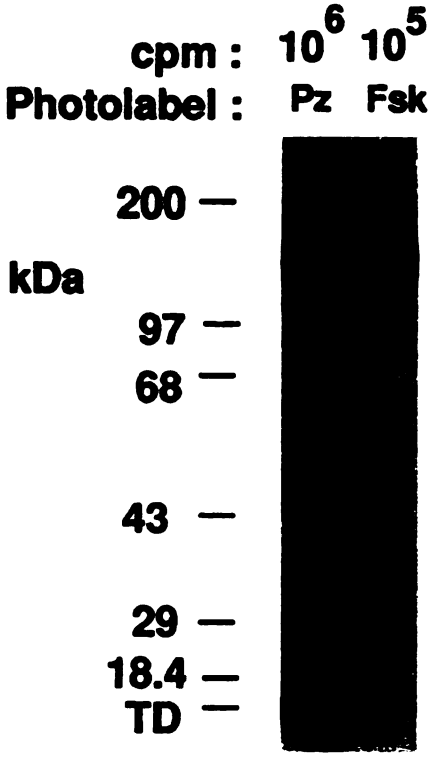


**Fig. 9.** Comparison of the ability of [$^{125}$I]6-AIPP-Fsk and [$^{125}$I]iodoarylazidoprazosin to photolabel P-glycoprotein in KB-V1 membranes. KB-V1 membranes (100 μg) prepared by differential centrifugation and sucrose gradient centrifugation were incubated with $1 \times 10^5$ cpm of [$^{125}$I]6-AIPP-Fsk (*Fsk*) or $1 \times 10^6$ cpm of [$^{125}$I]iodoarylazidoprazosin (*Pz*) for 1 hr and irradiated under a UV lamp as described in Experimental Procedures. The proteins were solubilized in Laemmli sample buffer and resolved on 10% Laemmli gels. The gels were dried and exposed to Kodak XAR-5 film. *TD*, tracking dye.

and KB-C1 membranes. The trypsin digestion patterns of these photolabeled membranes were very similar, suggesting that forskolin labels similar regions of the protein regardless of the point mutation of P-glycoprotein from KB-C1 membranes. In another study, similar results were observed with KB-V1 and KB-C1 cells that were first photolabeled with [$^3$H]azidopine and then chemically digested with CNBr (21). However, when vinblastine was present during the photolysis with [$^3$H]azidopine, the azidopine labeling of the P-glycoprotein was inhibited

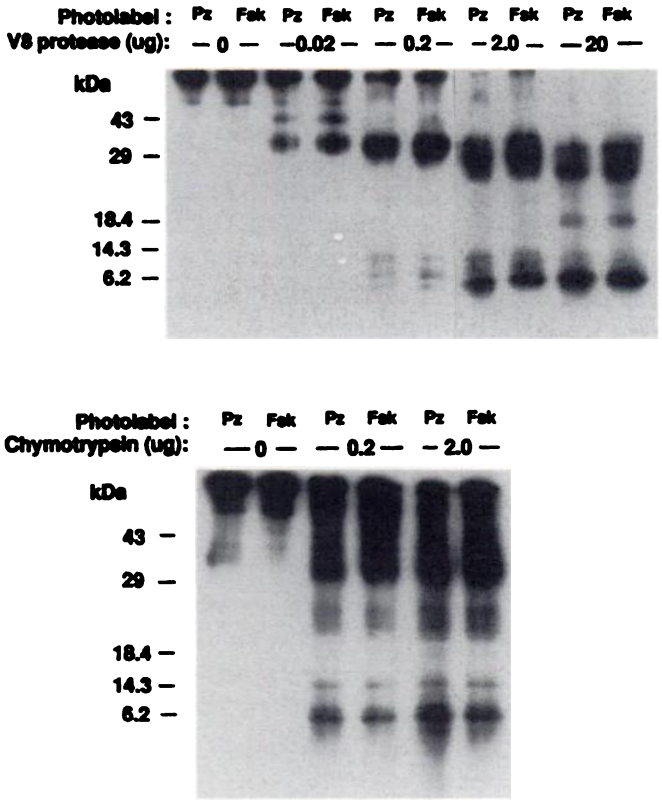


**Fig. 10.** Comparison of KB-V1 membranes photolabeled by [$^{125}$I]6-AIPP-Fsk (*Fsk*) or [$^{125}$I]iodoarylazidoprazosin (*Pz*) and then digested with either V-8 protease or chymotrypsin. KB-V1 membranes (100 $\mu$g) were incubated with the photolabels and resolved on gels as described for Fig. 9. After the gels were exposed to X-ray film the radioactive bands were identified, excised from the gels, and sliced. The samples were then coelectrophoresed with either V-8 protease or chymotrypsin on gels prepared according to the method of Cleveland *et al.* (43). The gels were dried and exposed to Kodak XAR-5 film.

in KB-C1 cells but not in KB-V1 cells, suggesting that the KB-C1 mutation at residue 185 can affect the interaction of P-glycoprotein with some drugs (21).

A putative labeling site for [$^{125}$I]6-AIPP-Fsk has been localized to amino acid residues 291–359 in the amino-terminal half of P-glycoprotein. This was determined by immunoprecipitation experiments with trypsin-digested fragments using several site-directed antibodies and peptide competition experiments. Antiserum against PEPG9 was raised against a fusion protein containing the peptide corresponding to amino acid residues 348–419 of the P-glycoprotein, a region immediately following transmembrane domain 6. This antiserum immunoprecipitated a 6-kDa radiolabeled tryptic fragment. This 6-kDa fragment was not immunoprecipitated by two other antisera against regions that closely border PEPG9, i.e., PEPG7 and PEPG11. Photolabeling of the 6-kDa tryptic fragment was substantially decreased when labeling was carried out in the presence of 1,9-dideoxyforskolin, consistent with this fragment being derived from forskolin labeling of P-glycoprotein.

A 7.2-kDa fragment, Lys$^{291}$ through Arg$^{359}$, is predicted to be generated in the amino-terminal half of P-glycoprotein by trypsin cleavage. This fragment spans transmembrane domains 5 and 6 and contains no other tryptic cleavage sites (see Figs. 2 and 6). This fragment potentially is the 6-kDa radiolabeled fragment that is specifically immunoprecipitated by antiserum against PEPG9, because the fusion protein used to generate antiserum against PEPG9 contains a peptide sequence that overlaps the 7.2-kDa fragment by 12 amino acids, from residue 348 to residue 359. Two peptides were synthesized to help determine the identity of this immunoprecipated fragment. The sequence of peptide 1 corresponds to the carboxyl-terminal 17 amino acids of the 7.2-kDa fragment, residues 343–359, and the amino-terminal 12 amino acids of the P-glycoprotein peptide sequence PEPG9, residues 348–359 (see Fig. 6). This peptide inhibited the ability of antiserum against PEPG9 to immunoprecipitate the 6-kDa photolabeled tryptic fragment and other photolabeled fragments migrating between the 14- and 18-kDa molecular mass markers. This inhibition was essentially complete, suggesting that the sequence of peptide 1 represents the only epitope of the 6-kDa fragment that overlaps PEPG9. The fragments migrating between the 14- and 18-kDa markers overlap a larger portion of the P-glycoprotein that is recognized by antisera against PEPG9 and PEPG7, because both antisera can immunoprecipitate these fragments. Peptide 1 had no effect on the ability of antiserum against PEPG7 to immunoprecipitate P-glycoprotein or any other radiolabeled fragments, including the fragments migrating between the 14- and 18-kDa markers. A second peptide corresponding to residues 360–376, carboxyl-terminal to peptide 1, did not inhibit the immunoprecipitation of the 6-kDa fragment by antiserum against PEPG9. Therefore, a fragment is photolabeled that migrates with a molecular mass of approximately 6 kDa and is immunoprecipitated by antiserum against PEPG9 but not by antisera against PEPG7 or PEPG11, and this immunoprecipitation is specifically inhibited by peptide 1 but not by peptide 2. These results suggest that this 6-kDa photolabeled fragment is the postulated 7.2-kDa fragment, Lys$^{291}$ through Arg$^{359}$. In support of this conclusion, a major 6-kDa fragment was produced when radiolabeled KB-C1 membranes were solubilized with SDS to unfold membrane proteins and were then digested with trypsin (data not shown). This fragment was immunoprecipitated by antiserum against PEPG9, similar to the immunoprecipitation of the 6-kDa radiolabeled fragment from membrane-digested P-glycoprotein presented in this paper.

The localization of a forskolin labeling site in the amino-terminal half of P-glycoprotein is consistent with the labeling sites of other drugs in this same region. Antisera against PEPG9 immunoprecipitated a 5-kDa tryptic fragment from mouse mdr1b P-glycoprotein that was photolabeled with [$^{125}$I] iodoarylazidoprazosin (24). A 25-kDa CNBr fragment from KB-C1 cells was photolabeled by [$^{3}$H]azidopine and was immunoprecipitated by antisera against PEPG7 and PEPG9 (21), suggesting that the [$^{3}$H]azidopine labeling site was located between amino acid residues 198 and 440. [$^{3}$H]Azidopine binding to this 25-kDa fragment represented 60% of the total binding to P-glycoprotein, with the remaining [$^{3}$H]azidopine binding to the carboxyl-terminal half. It was also shown by functional analysis of chimeric genes that replacement of domains from the amino-terminal (transmembrane domains 5 and 6) or carboxyl-terminal (transmembrane domains 7 and 8) half of mdr1b by the homologous mdr2 regions destroyed the activity of mdr1b (45).

We were unable to localize the carboxyl-terminal labeling site of [$^{125}$I]6-AIPP-Fsk to a specific region, even though three different carboxyl-terminal antisera immunoprecipitated similar low molecular mass trypsin-digested fragments. Antisera

against PEPG8 and PEPG10 and antiserum 4007 immunoprecipitated radiolabeled fragments of 55 kDa, 40 kDa, and 30 kDa. Fragments with these molecular masses have previously been associated with a carboxyl-terminal drug labeling site (20). This indicates that part of a forskolin labeling site is associated with the carboxyl-terminal half of the molecule. A more specific proposal for the location of the forskolin labeling site can be obtained by comparison with data on the prazosin binding site on the mouse mdr1b form of P-glycoprotein. The carboxyl-terminal labeling site of [$^{125}$I]iodoarylazidoprazosin on the mouse mdr1b form of P-glycoprotein was localized to a 4-kDa fragment that is either within or immediately carboxyl-terminal to transmembrane domain 12 (24). The data in our paper demonstrate that [$^{125}$I]6-AIPP-Fsk and [$^{125}$I]iodoarylazidoprazosin label the same peptide fragments after proteolysis with V8 protease and chymotrypsin. This suggests that the forskolin labeling site may be localized near transmembrane domain 12, as described by Greenberger (24).

Both [$^{125}$I]6-AIPP-Fsk and [$^{125}$I]iodoarylazidoprazosin labeled P-glycoprotein in KB-V1 membranes; however, [$^{125}$I]6-AIPP-Fsk was about 10 times more efficient than [$^{125}$I]iodoarylazidoprazosin. Identical radioactive peptide profiles were produced by Cleveland mapping of photolabeled P-glycoprotein fragments from membranes made from J7.V1-1 mouse macrophage-like cells with [$^{3}$H]azidopine and [$^{125}$I]iodoarylazidoprazosin (15). Therefore, the three photolabels, i.e., [$^{125}$I]6-AIPP-Fsk, [$^{125}$I]iodoarylazidoprazosin, and [$^{3}$H]azidopine, probably label the same site or closely related sites on P-glycoprotein. These sites are closely associated with transmembrane domains 6 and 12. Drug binding to these regions represents either two distinct binding sites or a single drug binding site with interactions from both halves of the protein.

The results from inhibition studies of [$^{3}$H]azidopine labeling of P-glycoprotein by vinblastine suggest that P-glycoprotein has a single drug labeling site for azidopine and vinblastine that is composed of both halves of the protein (21). Studies using deletion and insertion mutations of P-glycoprotein have also concluded that both halves of the protein are important for drug binding and neither half can function alone (46). Our results with [$^{125}$I]-6-AIPP-Fsk are consistent with the conclusion that both halves are important. Although most substrates for P-glycoprotein appear to inhibit both photolabeling by a vinblastine analog, *N*-(*p*-azido-3-[$^{125}$I]-salicyl)-*N'*-$\beta$-aminoethylvindesine (47), and transport of vinblastine (48, 49), some kinetic studies suggest that azidopine and vinblastine might have distinct binding sites on P-glycoprotein (50). The present results and those of Greenberger *et al.* (23) suggest that azidopine, prazosin, and forskolin share the same binding site, but they do not address the binding site for vinblastine.

Labeling sites for other drugs on membrane proteins have been determined using protocols similar to those described in this paper. The binding site of another photoactive derivative of forskolin has been localized to transmembrane domain 10 of the glucose transporter (51). This protein contains two domains of six transmembrane regions and has two cytoplasmic loops, similarly to P-glycoprotein. The $\alpha$1 subunit of the L-type calcium channel consists of four domains. The covalent binding site of a drug, LU49888, has been localized to transmembrane region 6 of the fourth domain (52). Although the exact locations of these drug binding sites are slightly different, they all appear to be closely associated with the transmembrane segments, either within the membrane or carboxyl-terminal to the membrane.

Photolabeling experiments are difficult to interpret with respect to quantitation of the absolute levels of labeling at individual sites on a protein. This makes it difficult to determine unambiguously the number of interaction sites for drugs on a protein using only data from photoaffinity labeling experiments. However, it should be possible to identify the specific amino acid residues that are labeled by photoactive derivatives, thus providing better defined locations for drug interaction sites. The present study and others (24, 21) do demonstrate that at least three drugs, i.e., forskolin, prazosin, and azidopine, label similar peptides associated with the carboxyl- and amino-terminal halves of P-glycoprotein.

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