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USE OF RECOMBINANT P-GLYCOPROTEIN FRAGMENTS TO PRODUCE ANTIBODIES TO THE MULTIDRUG TRANSPORTER

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Multidrug-resistance of human cancer cells may result from expression of a 170,000 dalton multidrug efflux pump called P-glycoprotein. To identify this multidrug transporter, and to study its structure and function, we have generated polyclonal rabbit antibodies against the amino-terminal and carboxy-terminal halves of the molecule using recombinant protein fragments produced in *Escherichia coli*. Two recombinant P-glycoprotein fragments, representing amino acids 140-228 and 919-1280, were overproduced in *Escherchia coli* by an inducible T7 expression system, gel-purified and injected into rabbits. Both antisera specifically immunoprecipitate ^{3}H-azidopine and ^{35}S-methionine labeled P-glycoprotein from multidrug-resistant cells and detect P-glycoprotein on Western blots with high sensitivity. Because these antisera were raised against epitopes in the amino-and carboxy-terminal halves of P-glycoprotein, they should be useful as research tools to define the function of these two halves of the molecule.

In cultured human cancer cells, one of the major mechanisms for resistance to natural product cytotoxic drugs is the expression of a 170,000 dalton plasma membrane-associated glycoprotein, called P-glycoprotein or multidrug transporter (reviewed in 1). This protein functions as an energy-dependent efflux pump for diverse natural product cytotoxic drugs such as vinblastine, vincristine, doxorubicin (Adriamycin), daunorubicin, actinomycin D, VP-16, VM-26, and taxol. P-glycoprotein consists of two homologous halves, each of which contains an ATP-binding site and six transmembrane regions (2,3). The multidrug transporter is found in normal tissues in transporting epithelia of the liver, kidney, colon and small intestine, in specialized capillary endothelial cells in brain and testis, and in the adrenal (4-6). According to a recent study of more than 400 human cancers, RNA for the multidrug transporter is expressed in many human tumors, especially adenocarcinomas derived from the epithelia enumerated above, and in many other cancers exposed to chemotherapy (7). These results suggest that expression of the multidrug transporter is also a major mechanism by which human cancers evade chemotherapy with natural product cytotoxic drugs.

To design strategies to circumvent this form of multidrug resistance, it is essential to be able to identify P-glycoprotein with high specificity, and to generate reagents which can be used to explore the structure and function of this intriguing transporter. This work describes the use of recombinant amino- and carboxy-terminal P-glycoprotein fragments to raise specific and sensitive polyclonal rabbit antisera as tools for immunoprecipitation or Western blot analyses.

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MATERIALS AND METHODS

Construction of *E. coli* Expression Vectors

Fragments of a cloned human *MDR*1 cDNA were introduced into plasmid pAR2106 and transformed into *E. coli* BL21 carrying the chromosomal lambda derivative DE3 which encodes T7 RNA polymerase controlled by the lac UV5 promoter. In this expression system, originally described by Studier (8,9), DNA sequences inserted in pAR2106 downstream from a T7 polymerase sensitive promoter are expressed at high levels after induction of the T7 RNA polymerase by IPTG. *MDR*1 cDNA fragments were obtained from the plasmid pMDR2000XS (10,11).

The fragment encoding amino acids 919-1280 from the eleventh transmembrane region to the carboxy terminus (2) was excised from pMDR2000XS using *Nde*I and *Bam*HI and inserted into pAR2106 cut with *Nde*1 and *Bam*H1 to produce expression vector pEXC30 (see Fig.1 bottom left). A 267 nucleotide *Pvu*II fragment encoding amino acids 140-228, which represents the second through the fourth transmembrane region including the first cytoplasmic and the second extracytoplasmic loop (2), was inserted into pAR2106 after this vector was cut with *Bam*H1 and blunt-ended with T4 DNA polymerase to produce the expression vector pEXP185 (see Fig. 1, bottom right). pEXP185 translation starts at an ATG codon derived from T7 protein 10, and therefore produces a fusion protein containing 13 amino acids from T7 protein 10 at its N-terminus. pEXC30 translation initiates at an internal ATG in the *MDR*1 cDNA (amino acid 919).

CΔ23 was made by first nibbling the *MDR*1 cDNA from the 3' end by Bal 31 exonuclease followed by ligation of an universal termination linker (GCTTAATTAATTAAGC), Pharmacia LKB Biotechnology, Inc.) The translation termination resulted in the loss of 23 amino acids with replacement of four residues (RLIF)(25).

Synthesis of Recombinant P-Glycoprotein Fragments and Preparation of Membrane Fractions

Plasmids pEXP185 and pEXC30 grown in ampicillin-selected *E. coli* DH5 after transformation by standard techniques, were prepared by the alkaline lysis method (12). Strain

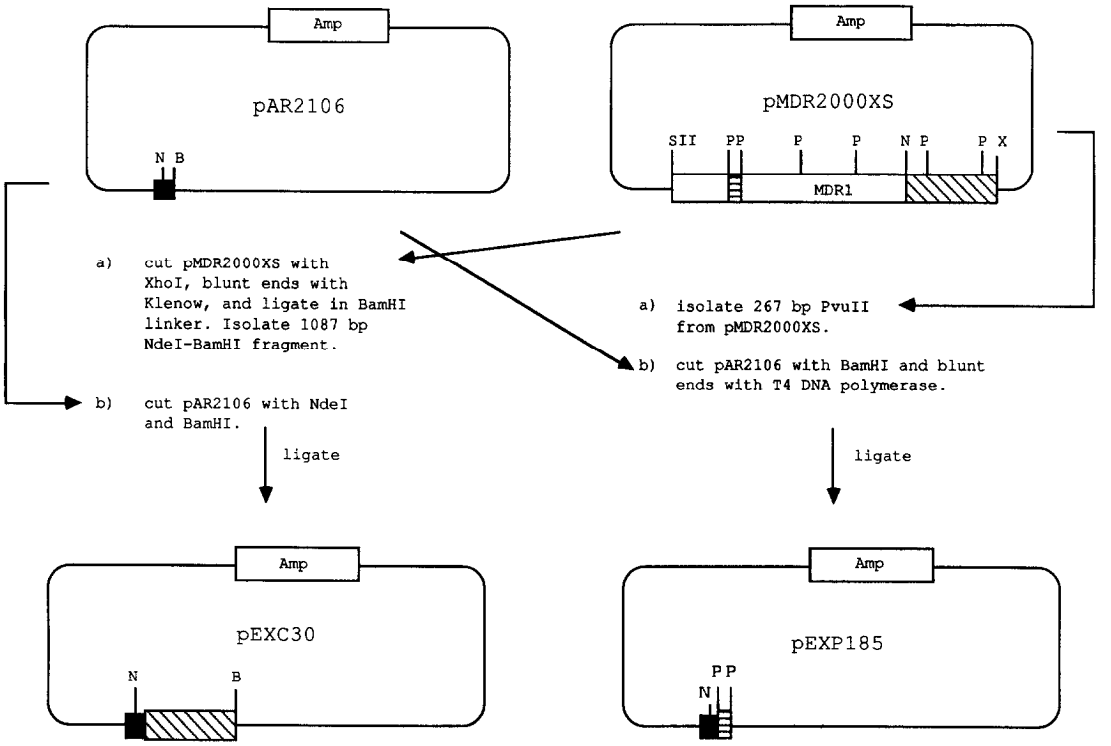

Figure 1: Schematic diagram showing the construction of the P-glycoprotein expression vectors used in this work. N, *Nde*I; B, *Bam*HI; SII, *Sac*II; P, *Pvu*II; X, *Xho*I. The filled in box represents the T7 expression and cloning sequences described in (9).

BL21/DE3 was transformed with 50 ng of purified plasmid immediately before each preparation of recombinant P-glycoprotein fragment, a single transformed ampicillin-resistant colony was picked and 5 ml of an inoculum was grown overnight in L broth containing 50 μg/ml ampicillin. For preparation of membrane fractions, 2 ml of the overnight inoculum were added to 200 ml of L broth + ampicillin. At an O.D. 600 of 0.3, IPTG was added to a final concentration of 0.4 mM, and the bacteria were incubated at 37° C for 90 min to allow for expression of the P-glycoprotein fragments. The induced bacteria were harvested by centrifugation at 2000 xg for 10 min and washed with a solution containing 20% sucrose, 5 mM Tris, pH 7.5, 1 mM EDTA before suspension in TE solution (10 mM Tris, pH 7.6, 1 mM EDTA). The suspended bacteria were sonicated at 100 watts for 30 secs three times with a Tekmar sonic disruptor sonicator. The insoluble membrane-containing fraction was collected by centrifugation at 36,000 xg for 2 hr.

Preparation of Rabbit Anti-P-Glycoprotein Antibodies

Membrane fractions of *E. coli* carrying pEXP185 and pEXC30 were fractionated on 12.5% or 10% SDS polyacrylamide gels (13), respectively. Bands corresponding to the predicted molecular weights of the recombinant P-glycoprotein fragments (14 kDa or 40 kDa) were detected by Commassie Blue staining and were not seen in either control transformations with the pAR2106 vector alone, or in uninduced preparations. Gel slices containing 85 μg of P-glycoprotein peptides, as estimated from Coomassie blue staining of parallel controls, were lyophilized, pulverized with a mortar and pestle, and resuspended in phosphate buffered saline. These preparations were emulsified in complete Freund's adjuvant and each was injected intradermally in multiple sites in two New Zealand rabbits. An additional 4 injections of antigen in incomplete Freund's adjuvant was used per rabbit, given every 2-3 weeks. Rabbit 4077 had the highest titer against the amino-terminal fragment and rabbit 4007 had the highest titer against the carboxy-terminal fragment.

^{3}H-Azidopine and ^{35}S-Methionine Labeling and Immunoprecipitation

Multidrug resistant KB-C1 cells were harvested, washed and resuspended in PBS at 1 x 10^{6} cells per 100 μl with 1 μCi ^{3}H-azidopine (40 Ci/mmol, Amersham) and irradiated as previously described (14). P-glycoprotein was labeled with ^{35}S-methionine and immunoprecipitated from multidrug resistant NIH 3T3 cells transfected with the pHaMDR1 retroviral vector (10) as previously described (15).

Western Blot Analysis

Crude membrane fractions were prepared from analyzed cells by differential centrifugation as previously described (16). Proteins were separated on an SDS-7% polyacrylamide gel (13) and electrophoretically transferred to nitrocellulose membranes (0.2 μm, Schleicher and Schuell, Inc., Keene, NH) (17). The nitrocellulose was blocked for 1 hr in (10% (w/v) Carnation™ in PBS). Reaction with primary antiserum (diluted 1:500 in blocking solution) was done overnight at 4°C. After three washes with PBST (PBS supplemented with 0.1% (v/v) Tween-20) and one wash with PBS, the blots were incubated with biotinylated goat-anti rabbit IgG (Vector Laboratories, Burlingame, CA) (4 μg/ml in blocking solution) for 1 h at room temperature. Then the nitrocellulose was washed again three times with PBST, once with PBS, and finally stained using the Vectastain™ ABC kit, and the substrate diaminobenzidine tetrahydrochloride (Sigma) according to the protocol of the manufacturer (Vector Laboratories, Burlingame, CA).

RESULTS

Using the Studier T7 expression system in *E. coli* it was possible to express milligram quantities of fragments of P-glycoprotein. As detailed in Materials and Methods, these fragments were purified on SDS polyacrylamide gels and served as potent antigens in rabbits. Figure 2 demonstrates that antisera 4007 and 4077 both specifically immunoprecipitate ^{3}H-azidopine-labeled P-glycoprotein from multidrug-resistant KB-C1 cells. Antiserum 4007 appears to immunoprecipitate a somewhat higher percentage of P-glycoprotein, and more readily detects higher molecular weight aggregates of this protein.

Figure 3 shows the specificity of these antibodies in immunoprecipitations using ^{35}S-methionine labeled cell extracts. Both antisera 4077 and 4007 (lanes 4, 5, 7 and 8) easily detect

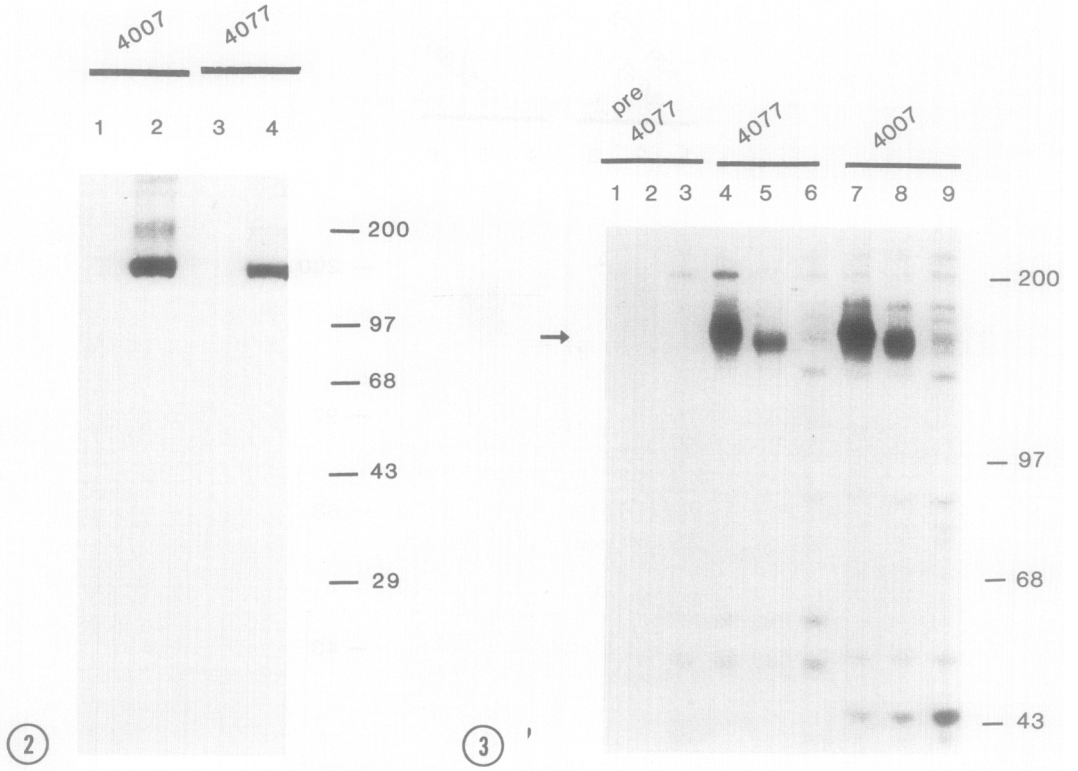

Figure 2: Immunoprecipitation of ^{3}H-azidopine-labeled P-glycoprotein from KB-C1 cells with antisera 4007 and 4077. Lanes 1 and 3 were immunoprecipitated from the drug-sensitive cell line KB-3-1, and lanes 2 and 4 were from the multidrug-resistant line KB-C1. M.W. standards in kDa are shown in the right margin.

Figure 3: Immunoprecipitation of P-glycoprotein from ^{35}S-methionine labeled extracts of multidrug-resistant cells. Lanes 1, 4 and 7 are from NIH 3T3 cells transfected with a human truncated *MDR*1 cDNA (CΔ23) (25); lanes 2, 5 and 8 are from transfections with the full length *MDR*1 cDNA; lanes 3, 6 and 9 are from the drug-sensitive parental cell line NIH 3T3. The arrow shows the position of P-glycoprotein. The CΔ23 molecule is 19 amino acids shorter than the full length *MDR*1 cDNA; its slower migration has been observed on multiple gels and remains unexplained.

P-glycoprotein in multidrug-resistant cell lines created by introducing the human *MDR*1 cDNA into mouse NIH 3T3 cells by transfection. With the long autoradiographic exposures used in this experiment, several other minor precipitated protein bands are seen. These bands are also seen in immunoprecipitates of the parental drug-sensitive cell line NIH 3T3 (lanes 6 and 9) or in the pre-immune reactions (lanes 1-3) and are therefore not specific to multidrug-resistant cells. P-glycoprotein was also detected by both antisera in KB-8-5 cells (18) which are only 3-6 fold multidrug-resistant and have low amounts of P-glycoprotein difficult to detect using other antisera (data not shown).

To test the species specificity of antiserum 4007 and to compare its efficiency to that of the anti-peptide antibody anti-P7 which we have previously described (19), immunoprecipitations of ^{35}S-methionine labeled cell proteins were done (Fig. 4). Antiserum 4007 precipitates P-glycoprotein of human (lane 2) and mouse origin (lane 5) with approximately equal efficiency,

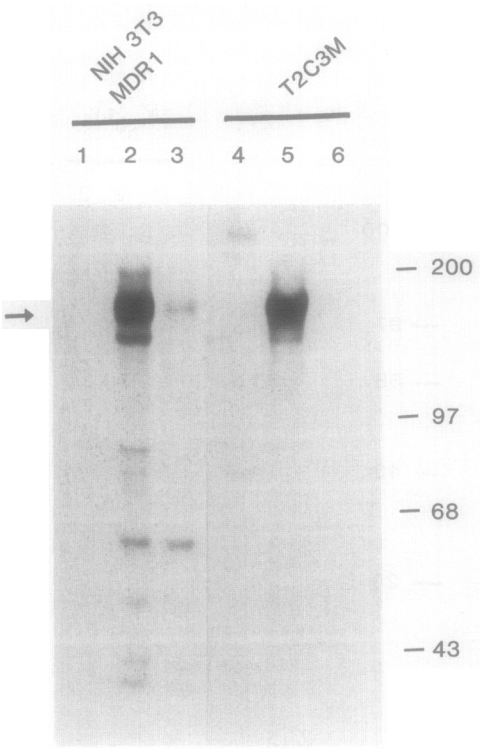

Figure 4: Immunoprecipitations of ^{35}S-methionine-labeled extracts from cells expressing human and mouse P-glycoprotein. NIH 3T3-*MDR*1 is the NIH 3T3 cell line transfected with a human *MDR*1 cDNA and T2C3M is an NIH 3T3 cell line selected for multidrug-resistance in 3 µg/ml colchicine which expresses the mouse P-glycoprotein. Preimmune reactions (lanes 1 and 4), and immunoprecipitations with 4007 (lanes 2 and 5) and anti-P7 (lanes 3 and 6) are shown. The arrow shows the position of P-glycoprotein.

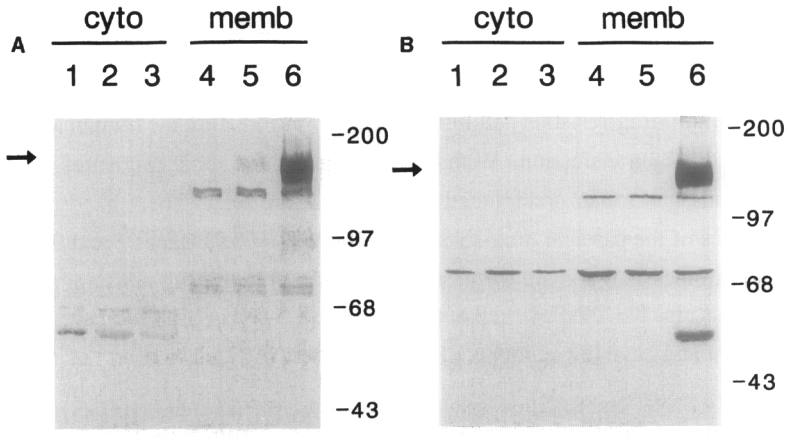

Figure 5: Western blot analysis of multidrug-resistant cells. Western blots were performed with antiserum 4077 (panel A) and 4007 (panel B) on cytoplasmic and membrane-containing fractions. Extracts were made from drug-sensitive KB-3-1 cells (lanes 1 and 4), low level multidrug-resistant KB-8-5 cells (lanes 2 and 5) and highly multidrug-resistant KB-C1 cells (lanes 3 and 6). The arrows show the position of P-glycoprotein.

indicating lack of species specificity of this antiserum. Similar results were obtained with antiserum 4077 and with both antisera using P-glycoprotein from hamster and dog cells (data not shown). In contrast, anti-P7 antiserum detects human P-glycoprotein with low efficiency (lane 3) and does not detect mouse P-glycoprotein at all (lane 6).

As shown in Fig. 5, both antisera 4077 and 4007 are very effective at detecting denatured P-glycoprotein in a Western blot. These antisera appear as efficient as monoclonal antibody C219 (20) (data not shown), since they detect P-glycoprotein in the low drug-resistant cell line KB-8-5 (lane 5 in A and B). The additional bands also seen in the cytoplasmic extracts (lanes 1-3) for each antiserum represent non-specific reaction with proteins also present in drug-sensitive cells (lanes 1 and 4). The additional band of molecular weight approximately 55 kDa seen in panel B, lane 6, is a proteolytic fragment of P-glycoprotein specifically detected by antiserum 4007 (21).

DISCUSSION

Antisera against specific regions of P-glycoprotein should prove to be very valuable reagents to identify and analyze the structure and function of P-glycoprotein. To date, several monoclonal and polyclonal antibodies have been prepared (19,20,22-24), the properties of which are summarized in Table I. The advantages of the antisera to recombinant P-glycoprotein peptides described in this report may be summarized as follows: (1) specificity for defined regions of P-glycoprotein (21); (2) high efficiency in immunoprecipitations; and (3) high efficiency on Western blots. In addition, the use of recombinant P-glycoprotein fragments means that there is essentially an unlimited supply of antigen for future immunizations.

The specificity of these antisera for amino- and carboxy-terminal fragments of P-glycoprotein should make it possible to use these antisera to identify specific domains of

Table I
Properties of Antibodies to P-Glycoprotein

Antibody	Source	Epitope	Uses	Reference
C219	mouse monoclonal	near ATP sites, intracellular peptide	immunohistochemistry; Western blots	(20)
MRK16	mouse monoclonal	extracellular	immunohistochemistry; species specific	(22)
JSB-1	mouse monoclonal	intracellular	immunohistochemistry	(23)
HYB 241	mouse monoclonal	extracellular	immunohistochemistry	(6)
HYB 612	mouse monoclonal	extracellular	immunohistochemistry	(6)
Anti-P-glycoprotein	rabbit polyclonal	multiple (P-glycoprotein antigen)	Western blots; immunoprecipitations	(24)
Anti-P7	rabbit polyclonal	peptide 28-38	immunoprecipitations	(19)
4077, 4007	rabbit polyclonal	peptides 140-228; 919-1280	immunoprecipitations; Western blots	This work

P-glycoprotein involved in drug and ATP-binding. The general structure of P-glycoprotein suggests that it is composed of two homologous halves (2,3). Using the antisera described in this report, we have recently found that each half of the molecule can be labeled with ^{3}H-azidopine (21).

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