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Expression of the Human Multidrug Transporter in Insect Cells by a Recombinant Baculovirus

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ABSTRACT: The plasma membrane associated human multidrug resistance (*MDR*1) gene product, known as the 170-kDa P-glycoprotein or the multidrug transporter, acts as an ATP-dependent efflux pump for various cytotoxic agents. We expressed recombinant human multidrug transporter in a baculovirus expression system to obtain large quantities and further investigate its structure and mechanism of action. *MDR*1 cDNA was inserted into the genome of the *Autographa californica* nuclear polyhedrosis virus under the control of the polyhedrin promoter. *Spodoptera frugiperda* insect cells synthesized high levels of recombinant multidrug transporter 2–3 days after infection. The transporter was localized by immunocytochemical methods on the external surface of the plasma membranes, in the Golgi apparatus, and within the nuclear envelope. The human multidrug transporter expressed in insect cells is not susceptible to endoglycosidase F treatment and has a lower apparent molecular weight of 140 000, corresponding to the nonglycosylated precursor of its authentic counterpart expressed in multidrug-resistant cells. Labeling experiments showed that the recombinant multidrug transporter is phosphorylated and can be photoaffinity labeled by [^{3}H]-azidopine, presumably at the same two sites as the native protein. Various drugs and reversing agents (e.g., daunomycin > verapamil > vinblastine ≈ vincristine) compete with the [^{3}H]azidopine binding reaction when added in excess, indicating that the recombinant human multidrug transporter expressed in insect cells is functionally similar to its authentic counterpart.

The development of simultaneous resistance to multiple drugs represents a major obstacle to successful cancer chemotherapy and is often associated with enhanced expression of the human multidrug resistance (*MDR*1) gene [reviewed in Gottesman and Pastan (1988)]. The *MDR*1 gene encodes a 170-kDa protein, termed P-glycoprotein or the multidrug transporter, which is found abundantly on the external surface of multidrug-resistant cells (Willingham et al., 1987). The transfer of a cloned *mdr* cDNA into drug-sensitive cells is sufficient to confer the complete multidrug resistance phenotype (Gros et al., 1986a; Guild et al., 1988; Pastan et al., 1988; Ueda et al., 1987). On the basis of the *MDR*1 cDNA sequence, a structural model has been proposed which predicts a 1280 amino acid polypeptide chain containing 12 transmembrane regions, 2 nucleotide binding sites, and 3 potential N-linked glycosylation sites (Chen et al., 1986; Gros et al., 1986b).

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Recently, biochemical analysis indicated that the human multidrug transporter is synthesized as a 140-kDa precursor which is slowly converted to its mature form by N-linked glycosylation (Richert et al., 1988). P-Glycoprotein was also found to be posttranslationally modified by phosphorylation (Garman et al., 1983; Richert et al., 1988; Hamada et al., 1987).

Both the cellular location and the structural model of the multidrug transporter are consistent with its suggested function as an ATP-driven efflux pump to decrease drug accumulation in multidrug-resistant cells. Various photoaffinity labeling reagents such as analogues of vinblastine and verapamil, as well as [^{3}H]azidopine, have been used in binding and competition assays to demonstrate that the multidrug transporter interacts directly with drug substrates and agents which inhibit its pump function (Akiyama et al., 1988; Chen et al., 1986; Cornwell et al., 1987a; Nogae et al., 1989; Safa, 1988; Safa et al., 1987). Labeling experiments involving 8-azido-[^{32}P]-ATP have shown evidence for the presence of ATP binding sites (Cornwell et al., 1987b). Membrane vesicles prepared from multidrug-resistant cells, but not from drug-sensitive cells, have been reported to bind [^{3}H]vinblastine (Cornwell et al., 1986a) and transport it in an ATP-dependent manner (Horio et al., 1988). The transport process is competitively inhibited by various drugs, including agents which reverse drug resistance in vitro (Horio et al., 1988). Recently, P-glycoprotein has been purified by affinity chromatography and found to exhibit ATPase activity (Hamada & Tsuruo, 1988a,b; Riordan & Ling, 1979). Reconstitution of drug transport with a purified active multidrug transporter, which would be essential to prove its suggested function and would allow its mechanism of action to be studied, has not yet been accomplished.

Both reconstitution experiments and further protein structure studies require large amounts of isolated multidrug transporter. Thus, we attempted to overproduce the *MDR*1 gene product in the helper-independent baculovirus expression system (Smith et al., 1983a,b). Abundant expression of a great variety of biologically active recombinant proteins has been achieved by using this approach [reviewed in Luckow and Summers (1988)]. Since baculovirus-infected *Spodoptera frugiperda* (Sf9) cells are able to perform many of the higher eukaryotic posttranslational modifications (including glycosylation and phosphorylation), we expected that they would produce a recombinant multidrug transporter which was antigenically and functionally similar to its authentic eukaryotic counterpart. Here we report that upon infection of insect cells with a recombinant *Autographa californica* nuclear polyhedrosis virus, carrying the human *MDR*1 gene under the control of the strong polyhedrin promoter, high-level expression of a nonglycosylated 140-kDa multidrug transporter was achieved. The recombinant protein is recognized specifically by various antibodies raised against different regions of the human multidrug transporter. The membrane-associated 140-kDa protein is also able to bind [^{3}H]azidopine, and various drugs compete for this reaction. Although viral infection kills the host cells, making it impossible to detect the ability of the recombinant transporter to confer drug resistance, these biochemical data suggest that the recombinant protein should be functional. Thus, this baculovirus expression system can be used for large-scale production of human multidrug transpoter for further biochemical and mechanistic studies, and to serve as an antigen for antibody production.

Experimental Procedures

Materials. The transfer vector pAC373, wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV), and the host cell line *Spodoptera frugiperda* (Sf9) were kindly provided by Dr. P. Howley (NCI, NIH, Bethesda, MD). Insect culture media were from Gibco. Enzymes and oligodeoxynucleotides used for recombinant DNA techniques were from BRL, Pharmacia LKB Biotechnology Inc., and New England Biolabs. C219 (Kartner et al., 1985) (Centocor, Malvern, PA) is a monoclonal mouse anti-hamster multidrug transporter antibody, and MRK16 (Hamada & Tsuruo, 1986) (a gift from Dr. T. Tsuruo, Japanese Foundation of Cancer Research, Tokyo, Japan) is a mouse anti-human multidrug transporter monoclonal antibody. 4007 is a polyclonal rabbit antiserum raised against a carboxy-terminal fragment of the human multidrug transporter (Tanaka et al., 1990). Rhodamine-labeled affinity-purified goat anti-mouse IgG and affinity-purified mouse anti-ferritin were from Jackson ImmunoResearch (Avondale, PA). Protein A–Sepharose was from Pharmacia LKB Biotechnology. Translabel (a mixture of about 70% [^{35}S]methionine and about 15% [^{35}S]cysteine, specific activity about 1100 Ci/mmol) was purchased from ICN Biomedical Inc. (Irvine, CA). Carrier-free [^{32}P]orthophosphate and [^{3}H]azidopine (specific activity 40 Ci/mmol) were from Amersham. Endoglycosidase F was from Boehringer Mannheim. BCA protein assay reagents were from Pierce. The VECTASTAIN Western blot developing kit was from Vector Labs (Burlingame, CA). Ferritin, vinblastine, daunomycin, vincristine, verapamil, quinidine, nifedipine, diltiazem, and actinomycin D were from Sigma.

Construction of Combinant Transfer Vector pAC-MDR1/A. Standard recombinant DNA techniques were used (Maniatis et al., 1982). Basically, a 4-kb human *MDR*1 cDNA fragment, containing the full-length coding region flanked by a 10 bp untranslated region at the 5′ end and a 110 bp untranslated region at the 3′ end, was inserted into the AcNPV-derived transfer vector pAC373 downstream from the polyhedrin promoter (Smith et al., 1985; Summers & Smith, 1987). To this end we introduced a *Bam*HI site into the single *Xho*I site at the 3′ end of the *MDR*1 cDNA in plasmid pMDR 2000XS with the poly(A) addition site deleted (Pastan et al., 1988). An approximately 4-kb *Bst*UI–*Bam*HI fragment, carrying the human *MDR*1 cDNA, was then isolated and ligated with phosphorylated *Bam*HI linkers and plasmid pUC18 (Yamisch-Perron et al., 1985) that had been double-digested using *Bam*HI and *Hin*dII. Finally, *MDR*1 cDNA was isolated as a 4-kb *Bam*HI fragment and inserted into the dephosphorylated, single *Bam*HI site of vector pAC373 to produce pAC-MDR1/A (Figure 1).

Production of Recombinant Baculovirus BV-MDR1. To generate recombinant baculovirus carrying the human *MDR*1 gene, the permissive host cell line Sf9 was cotransfected with pAC-MDR1/A (2 μg) and wild-type AcNPV viral DNA (1 μg) by the calcium phosphate coprecipitation method as described (Summers & Smith, 1987). Six days later, virus was harvested from the culture medium. Recombinant viruses (BV-MDR1) were identified by plaque assays (Summers & Smith, 1987) and hybridization to a ^{32}P-labeled *MDR*1-specific probe (Germann et al., 1989). Five rounds of plaque purification by hybridization screening were performed to eliminate wild-type AcNPV.

Viral Infections and Metabolic Labeling of Cells. Sf9 cells were seeded and infected with wild-type AcNPV or recombinant BV-MDR1 by using the media and protocols described by Summers and Smith (1987). Virus was usually added to a multiplicity of infection of ≥4 PFU per cell.

Metabolic labeling of Sf9 cells, unless otherwise noted, was carried out 45 h postinfection. Cells (initially 2.5 × 10^6 seeded

in a 60-mm tissue culture dish) were labeled for 3 h with 250 μCi/mL Translabel in Grace's insect culture medium supplemented with 5% (v/v) fetal bovine serum. Alternatively, cells were labeled for 1.5 h with 200 μCi/mL [^{32}P]orthophosphate in the same culture medium.

Immunofluorescence. All procedures were performed at room temperature. Infected Sf9 cells were harvested 2.5 days postinfection, washed 3 times with Dulbecco's phosphate-buffered saline (DPBS), and seeded in DPBS on poly(L-lysine)-coated 35-mm plastic culture dishes. Thirty minutes later, the plates were carefully rinsed 5 times with DPBS containing 2 mg/mL bovine serum albumin (DPBS/BSA). Then the cells were incubated for 15 min with the monoclonal antibody MRK16 (10 μg/mL in DPBS/BSA). After five washes with DPBS and one wash with DPBS/BSA, affinity-purified goat anti-mouse IgG conjugated to rhodamine (50 μg/mL in DPBS/BSA) was added and incubated for 15 min. Finally, the cells were washed again 5 times with DPBS, fixed with 3.7% (v/v) formaldehyde in DPBS, mounted in buffered glycerol under a 1 coverslip, viewed, and photographed as described (Willingham et al., 1987).

Electron Microscopic Immunocytochemistry. Immunocytochemical detection of multidrug transporter was performed as previously reported (Pastan et al., 1988; Willingham, 1980; Willingham et al., 1987) by incubation of formaldehyde-fixed Sf9 cells sequentially with the monoclonal mouse antibody MRK16, goat anti-mouse IgG, affinity-purified mouse anti-ferritin, and horse spleen ferritin in the cell-permeabilizing buffer system NGG-sap-PBS composed of 4 mg/mL normal goat globulin, 0.1% (w/v) saponin, 1 mM EGTA, and phosphate-buffered saline (without Ca^{2+} and Mg^{2+}). Ferritin bridge labeling of random samples was quantitated as previously described (Pastan et al., 1988).

Immunoprecipitations. Metabolically labeled or [^{3}H]azidopine-labeled cells were washed 3 times with Dulbecco's phosphate-buffered saline (without Ca^{2+} and Mg^{2+}) and lysed in a plastic minitube on ice in 0.5 mL of BMR [20 mM MOPS, pH 7.0, 150 mM NaCl, 1 mM EDTA, 1% (w/v) deoxycholate, 1% (v/v) Triton-X-100, and 0.1% (w/v) SDS]. After centrifugation in an Eppendorf centrifuge at 4 °C for 10 min at 15000*g*, 150-μL aliquots of the extracts were immunoprecipitated as previously described (Germann et al., 1989) by adding 250 μL of RIPA [20 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% (v/v) Triton X-100, 1% (w/v) deoxycholate, 0.1% (w/v) SDS, and 1% (w/v) aprotinin] and incubating at 4 °C for 14–16 h with 4 μL of polyclonal rabbit anti-P-glycoprotein antiserum 4007. Immunocomplexes were precipitated for 2 h at room temperature with 40 μL of protein A–Sepharose [20% (w/v) in PBS]. After washes in RIPA and RIPAK (RIPA containing 2.5 M KCl), samples were eluted with Laemmli sample buffer at 37 °C for 15 min and analyzed by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) and subsequent fluorography (Bonner & Laskey, 1974).

Immunoblot Analysis. Proteins separated on an SDS–polyacrylamide gel were electrophoretically transferred to nitrocellulose membranes in Towbin buffer (Towbin et al., 1979) [25 mM Tris-HCl, pH 8.3, 192 mM glycine, and 20% (v/v) methanol] for 2 h at constant voltage (100 V) and 4 °C. After transfer, the nitrocellulose membranes were treated for 1 h with blocking solution [10% (w/v) Carnation nonfat dry milk in phosphate-buffered saline (PBS)] and subsequently incubated with primary antibody [monoclonal C219 (2 μg/mL) or polyclonal 4007 antiserum, diluted 1:500] in blocking solution at 4 °C overnight. After three washes in PBS containing 0.1% (v/v) Tween-20 (PBST) and one wash in PBS, the blots were reacted with secondary antibody (biotinylated goat anti-mouse IgG or goat anti-rabbit IgG, 4 μg/mL in blocking solution) for 1 h at room temperature. Finally, immunoblots were washed again 3 times with PBST, once with PBS, and developed with the VECTASTAIN ABC kit (involving immunoperoxidase staining using an avidin/horseradish peroxidase H complex and the oxidizable substrate diaminobenzidine tetrahydrochloride) according to the protocol given by the manufacturer (Vector Labs).

Endoglycosidase F Reactions. Immunoprecipitated multidrug transporter was eluted from protein A–Sepharose with 400 μL of elution buffer [0.1 M Tris-HCl, pH 8.0, 2% (w/v) SDS, and 5% (v/v) β-mercaptoethanol] for 30 min at room temperature. Acetone precipitation was performed by adding 0.1 volume 50% (w/v) sucrose and 1 volume of prechilled acetone and incubating overnight at −20 °C. Vacuum-dried, acetone-precipitated pellets were eluted with 5 μL of ELU-A [100 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1% (v/v) β-mercaptoethanol, and 2% (w/v) SDS] and diluted with 95 μL of 100 mM Tris-HCl, pH 7.5, and 0.75% (v/v) Triton-X-100. After addition of 2 μL of 100 mM *o*-phenanthroline (dissolved in methanol), the mixture was incubated for 4 h at 37 °C with 0.05 unit of endoglycosidase F. The reaction was stopped by acetone precipitation as described above.

Cellular Fractionation and [^{3}H]Azidopine Labeling. Cells were harvested in phosphate-buffered saline (PBS), washed twice with PBS, resuspended in prechilled homogenizing buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, and 1 mM $MgCl_2$), and swollen on ice for 10 min. They were disrupted by 20 strokes in a tight-fitting Dounce homogenizer. Cell debris, as well as nuclei, were removed by centrifugation at 400*g* for 10 min at 4 °C. The low-spin supernatant was fractionated by subsequent centrifugation at 30000*g* for 30 min at 4 °C. The high-spin pellet was resuspended in homogenizing buffer and considered as the crude membrane fraction, the supernatant as the cytosolic fraction.

Protein concentrations in the cellular fractions were determined by the bicinchoninic acid (BCA) assay according to the protocol of the manufacturer (Pierce). Cell fractions containing 25 μg of protein were incubated in 50 μL of homogenizing buffer with 50 μM [^{3}H]azidopine for 1 h in the dark at room temperature and then photoactivated on ice for 20 min as described (Germann et al., 1989). Competition assays were performed by the addition of 1 μL (a 200-fold or a 400-fold molar excess) of the corresponding drug stock solution.

Results

Production of a Recombinant Baculovirus Containing a Human MDR1 cDNA. To introduce the human *MDR*1 gene into the genome of wild-type AcNPV, the recombinant transfer vector pAC-MDR1/A (Figure 1) was constructed as outlined under Experimental Procedures. Plasmid pAC-MDR1/A contains an *MDR*1 cDNA, including the full-length coding region, which is flanked by 10 nucleotides of the 5′ untranslated leader and 100 nucleotides of the 3′ untranslated region, under the control of the strong polyhedrin promoter. Plasmid pAC-MDR1/A and wild-type AcNPV DNA were used in cotransfection of the permissive host cell line Sf9 (Summers & Smith, 1987). Homologous recombination between the AcNPV sequences present in the transfer vector and wild-type AcNPV DNA gave rise to recombinant BV-MDR1 baculoviruses. These were identified by plaque assays and hybridization screening (Summers & Smith, 1987) using a human *MDR*1-specific DNA probe. The initial screening showed that approximately 1 out of 10^6 total viruses produced was a re-

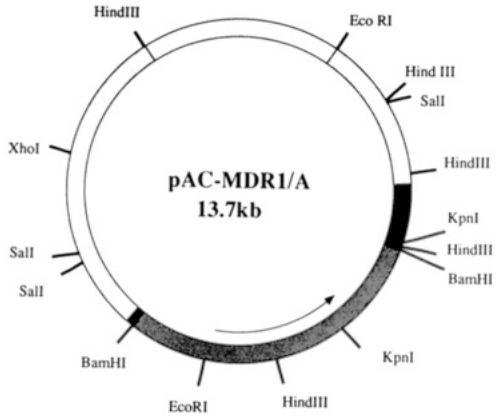

FIGURE 1: Restriction map of baculovirus expression vector pAC-MDR1/A. The cross-hatched region represents human *MDR*1 cDNA, the black regions represent AcNPV polyhedrin promoter and structural gene, the dotted regions represent flanking AcNPV genomic DNA sequences, and the white region represents pUC8 plasmid DNA. The direction of transcription of the *MDR*1 cDNA is indicated by the arrow.

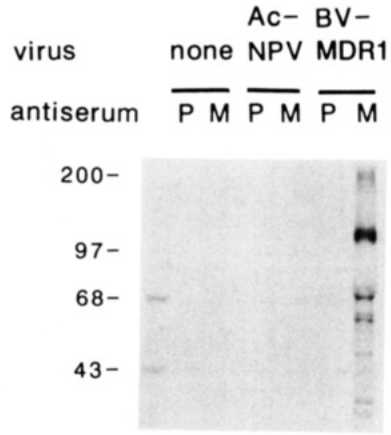

FIGURE 2: Immunoprecipitations of [^{35}S]methionine-labeled Sf9 cell extracts. AcNPV, BV-MDR1, or noninfected cells were labeled for 3 h at 45-h postinfection, total extracts were prepared, immunoprecipitated with either preimmune serum (P) or multidrug transporter-specific antiserum 4007 (M), and analyzed by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970), followed by fluorography (Bonner & Laskey, 1974). Sizes of molecular weight markers are given $\times 10^{-3}$.

combinant one. Five rounds of hybridization screening were performed before the recombinant BV-MDR1 virus was considered plaque-purified. Finally, selected recombinant plaques were also identified visually by their occlusion-negative phenotype due to the absence of polyhedrin synthesis (Summers & Smith, 1987).

Synthesis of Recombinant Multidrug Transporter in BV-MDR1-Infected Sf9 Cells. Antisera known to react with different epitopes of the human multidrug transporter were used to identify the recombinant human *MDR*1 gene product expressed in BV-MDR1-infected insect cells. In a first experiment, BV-MDR1-infected Sf9 cells, as well as noninfected or wild-type AcNPV-infected control cells, were metabolically labeled with [^{35}S]methionine, and total lysates were immunoprecipitated with the polyclonal rabbit antiserum 4007. Immune complexes were precipitated by protein A–Sepharose and analyzed by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) and subsequent fluorography (Bonner & Laskey, 1974). As shown in Figure 2, a prominent protein of approximately 140 kDa was immunoprecpitated by 4007 antiserum from BV-MDR1-infected Sf9 cells. This protein was not detected in noninfected or wild-type AcNPV-infected Sf9 cells. The human *MDR*1 gene product, therefore, is expressed in the baculovirus expression system. The recombinant human multidrug transporter synthesized in insect cells, however, has a lower apparent molecular weight (140000) than its authentic counterpart produced in multidrug-resistant human cells (170000) (Gottesman & Pastan, 1988).

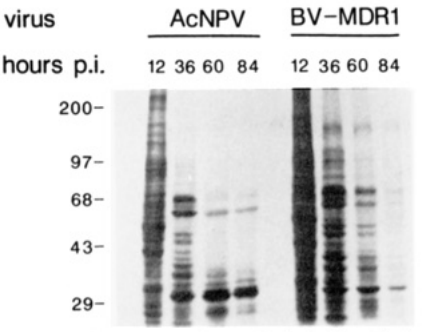

FIGURE 3: Analysis of total protein synthesis in baculovirus-infected Sf9 cells. Host cells were labeled with [^{35}S]methionine for 3 h at different times after infection with either AcNPV or BV-MDR1. Total cell extracts from an equal number of cells were resolved by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) and analyzed by fluorography (Bonner & Laskey, 1974). On the left, sizes of standards are indicated in kilodaltons.

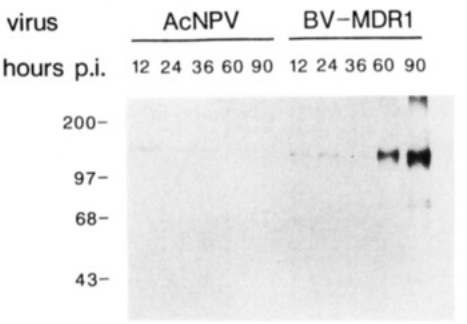

FIGURE 4: Recombinant multidrug transporter production in baculovirus-infected Sf9 cells. Total cell extracts were prepared from AcNPV- or BV-MDR1-infected Sf9 cells at different times after infection, resolved by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970), electrophoretically transferred to nitrocellulose (Towbin et al., 1979), and immunostained using anti-multidrug transporter-specific antiserum 4007. On the left, the sizes of molecular weight standards are indicated $\times 10^{-3}$.

The time course of multidrug transporter synthesis in BV-MDR1-infected Sf9 cells was investigated. BV-MDR1-infected Sf9 cells or AcNPV-infected control cells were metabolically labeled with [^{35}S]methionine for 3 h at different times after infection. Total cell lysates were size-fractionated on an SDS–polyacrylamide gel (Laemmli, 1970). As shown in Figure 3, no 140-kDa protein was produced in AcNPV-infected Sf9 cells. In BV-MDR1-infected cells, however, synthesis of the recombinant multidrug transporter started within 36-h postinfection and continued through the time period examined. By 60–90 h after infection, this protein was one of the major labeled species. At this time, however, the overall protein synthesis rate, as measured by [^{35}S]methionine uptake, was markedly decreased due to the lytic cycle of viral infection. Thus, labeling was most efficient between 36- and 60-h postinfection.

To monitor the accumulation of multidrug transporter produced in BV-MDR1-infected Sf9 cells, total lysates from 2.5×10^5 cells were analyzed at different times after infection by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) followed by Western blotting and immunostaining using the monoclonal anti-multidrug transporter antibody C219 (Kartner et al., 1985). As presented in Figure 4, increasing quantities of the 140-kDa recombinant multidrug transporter

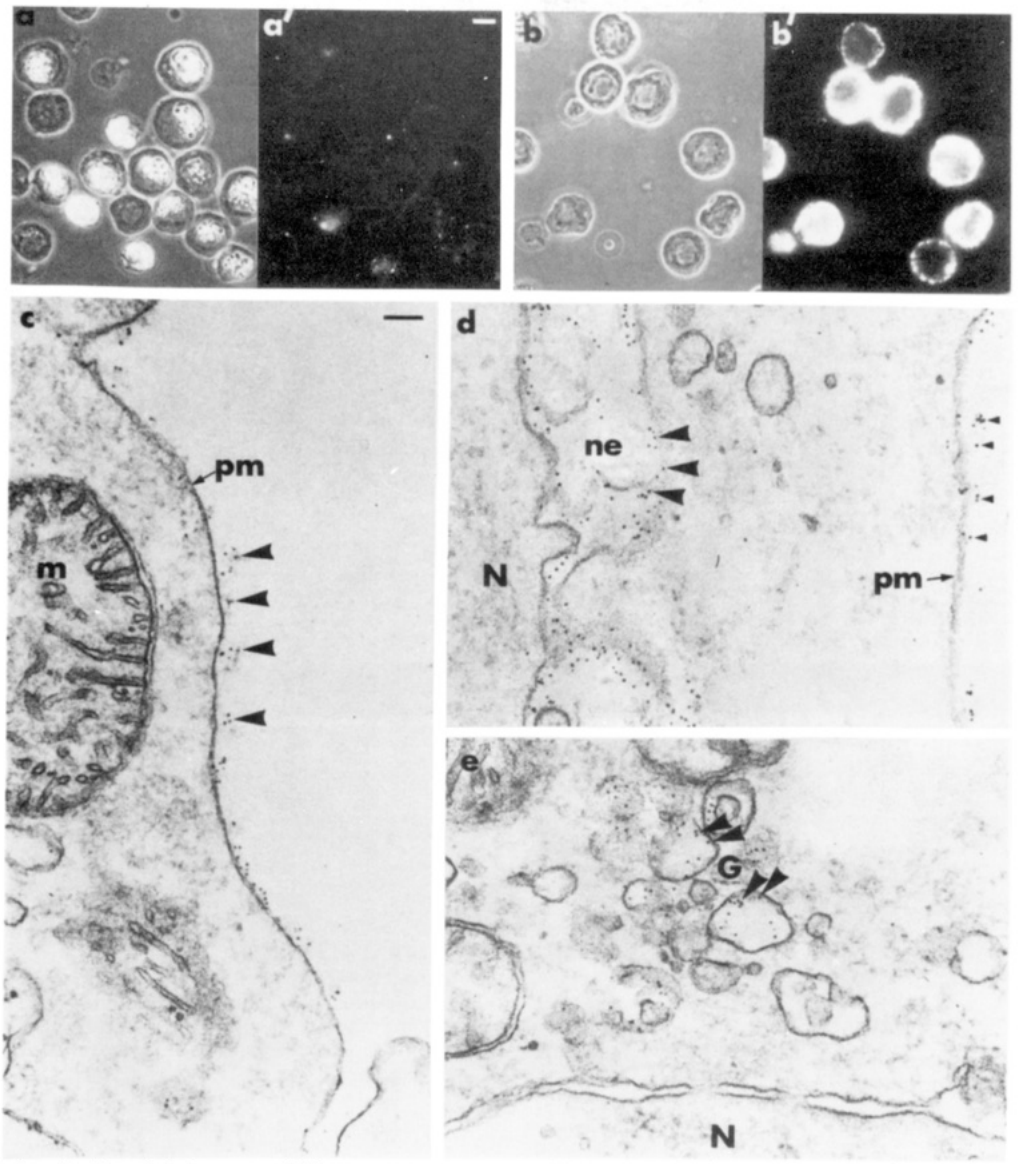

FIGURE 5: Cellular localization of the human multidrug transporter expressed in baculovirus-infected Sf9 cells. A human P-glycoprotein-specific monoclonal antibody (MRK16; Hamada & Tsuruo, 1986) was used to examine the extracellular surface of AcNPV- (a′) or BV-MDR1-infected (b′) Sf9 cells for the presence of recombinant multidrug transporter by immunofluorescence (Willingham et al., 1987). Panels a and b show the corresponding phase-contrast image of the same fields shown in a′ and b′. Immunohistochemistry [using monoclonal antibody MRK16 (Hamada & Tsuruo, 1986) and ferritin bridge labeling (Pastan et al., 1988; Willingham, 1980; Willingham et al., 1987)] was used to determine the intracellular localization of the recombinant protein. Major sites of localization (arrowheads) were found on the extracellular site of the plasma membranes (pm), in the nuclear envelope (ne), and in the Golgi apparatus (G). No protein was detected in other organelles such as the nucleus (N) or the mitochondria (m).

could be detected immunologically during the lytic cycle of infection, indicating that the protein was stable. Cells harvested 4-days postinfection contained the most recombinant multidrug transporter and were, therefore, considered to be the most suitable for protein isolation and characterization.

Localization of the Recombinant Human Multidrug Transporter Produced in BV-MDR1-Infected Insect Cells. The human multidrug transporter found in high levels in human multidrug-resistant cells is a transmembrane protein present in large amounts in the plasma membrane and in small amounts in Golgi stack membranes (Willingham et al., 1987). The mouse monoclonal antibody MRK16 (Hamada & Tsuruo, 1986) recognizes a human-specific epitope of the multidrug transporter on the external surface of the plasma membrane and the lumenal face of the Golgi stacks (Willingham et al., 1987). To examine whether the recombinant multidrug transporter synthesized by insect cells is also expressed on the cell surface, immunofluorescence studies were performed. Viable cells infected with either the wild-type AcNPV or the BV-MDR1 were harvested 60 h after infection and stained with the monoclonal antibody MRK-16 (Hamada & Tsuruo, 1986) and affinity-purified goat anti-mouse IgG conjugated to rhodamine as described under Experimental Procedures. As shown in Figure 5b′, a bright cell surface fluorescence was detected only in BV-MDR1-infected cells. No cell surface labeling could be observed in wild-type AcNPV-infected cells (Figure 5a′), which expressed large amounts of polyhedrin, as obvious from the viral occlusions visible in Figure 5a. Similarly, a negative control antibody gave no fluorescence staining of BV-MDR1-infected cells (data not shown).

Electron microscopy immunocytochemistry including ferritin bridge labeling (Willingham, 1980; Willingham et al., 1987) was used to determine more precisely the location of the recombinant multidrug transporter in BV-MDR1-infected cells. Quantitation of the immunocytochemical localization of the human multidrug transporter in plasma membranes of Sf9

Table I: Quantitation of Immunocytochemical Localization of the Human Multidrug Transporter in Plasma Membranes of Sf9 Insect Cells Infected with Wild-Type AcNPV or BV-MDR1

virus	antibody	total area examined (mm^2)	no. of ferritin cores counted	cores per mm^2 (±SD)
AcNPV	control	1.72	5	3 (±3)
AcNPV	MRK16	1.57	7	4 (±2)
BV-MDR1	control	2.33	22	9 (±6)
BV-MDR1	MRK16	2.39	1723	715 (±57)

cells infected with wild-type AcNPV or recombinant BV-MDR1 is summarized in Table I. Clearly, high levels of the recombinant human *MDR*1 gene product are expressed only in BV-MDR1-infected cells. Figure 5c–e demonstrates the presence of large amounts of ferritin-labeled recombinant multidrug transporter (arrowheads) on the external surface of the plasma membranes (pm), in the Golgi apparatus (G), and within the nuclear envelope (ne) of BV-MDR1-infected Sf9 cells.

Recombinant Human Multidrug Transporter Produced in the Baculovirus Expression System Is Phosphorylated, but Not Glycosylated. Labeling experiments and glycosidase digestions have shown that the human multidrug transporter present in human multidrug-resistant KB-V1 cells is N-glycosylated (Richert et al., 1988). Endoglycosidase F treatments convert the glycosylated 170-kDa protein into a lower molecular weight form that comigrates with the 140-kDa nonglycosylated precursor on an SDS–polyacrylamide gel. As shown in Figure 6A, the recombinant multidrug transporter produced in insect cells comigrates with the endoglycosidase F treated form of the authentic human counterpart. Furthermore, endoglycosidase F treatment does not affect its apparent molecular weight. It is, therefore, suggested that the recombinant *MDR*1 gene product synthesized by insect cells is not glycosylated.

The native human multidrug transporter produced by multidrug-resistant human cells has been reported to be phosphorylated (Richert et al., 1988). Metabolic labeling of BV-MDR1-infected Sf9 cells in the presence of [^{32}P]orthophosphate showed that the recombinant multidrug transporter was also phosphorylated (Figure 6B).

The Multidrug Transporter Expressed in Insect Cells Is Functionally Similar to Its Authentic Human Counterpart. [^{3}H]Azidopine photoaffinity labeling (Safa et al., 1987) was used to compare the functional similarity of the recombinant human multidrug transporter expressed in insect cells with its authentic counterpart present in multidrug-resistant human KB-V1 cells (Akiyama et al., 1985). Cell extracts were fractionated by differential centrifugation, and a low-speed pellet (containing cell debris and nuclear fractions), a high-speed pellet (enriched in crude membranes), and a high-speed supernatant (cytosolic fraction) were labeled by [^{3}H]azidopine. As shown in Figure 7C, both the 170-kDa multidrug transporter in the pellet fractions of KB-V1 cells and the 140-kDa multidrug transporter in the pellet fractions of Sf9 cells were labeled by this reagent. The native protein was more efficiently labeled than the recombinant one, since the signal obtained from the 170-kDa band was more intense than the one from the 140-kDa band, although somewhat less multidrug transporter was detected by immunoblotting (Figure 7B). Various substrates of the multidrug transporter added in excess (daunomycin > verapamil > vinblastine ≈ vincristine) were found to inhibit the [^{3}H]azidopine labeling of the recombinant protein produced in insect cells as summarized in Table II. Some compounds, however, showed a slightly different relative

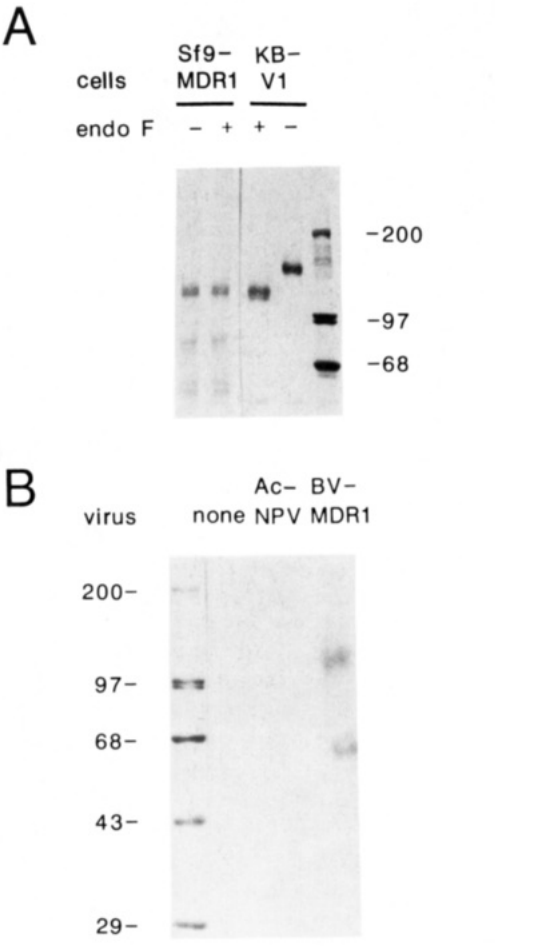

FIGURE 6: Posttranslational modifications of recombinant multidrug transporter. (A) Endoglycosidase F treatment of multidrug transporter produced in BV-MDR1-infected Sf9 cells and multidrug-resistant KB-V1 cells. BV-MDR1-infected Sf9 cells were metabolically labeled with [^{35}S]methionine for 3 h at 45-h postinfection, and total extracts were prepared and treated with endoglycosidase F for 4 h. Viable KB-V1 cells were labeled with [^{3}H]azidopine. Total cell extracts were immunoprecipitated with multidrug transporter-specific antiserum 4007 and analyzed by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970), and fluorography (Bonner & Laskey, 1974). Sizes of standards are indicated in kilodaltons. (B) Phosphorylation of the recombinant multidrug transporter expressed in BV-MDR1-infected Sf9 cells. BV-MDR1-infected, wild-type AcNPV-infected, and noninfected Sf9 cells were labeled with [^{32}P]orthophosphate for 1.5 h at 45-h postinfection. Total cell extracts were prepared and immunoprecipitated using multidrug transporter-specific antiserum 4007. Immunoprecipitates were analyzed by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970), followed by fluorography (Bonner & Laskey, 1974). Sizes of standards are given in kilodaltons.

Table II: Inhibition of [^{3}H]Azidopine Photoaffinity Labeling of Human Multidrug Transporter in Plasma Membranes of Multidrug-Resistant Human KB-V1 Cells and BV-MDR1-Infected Sf9 Insect Cells

drug	KB-V1	Sf9-MDR1
vinblastine	+++[a]	+[b]
daunomycin	+++	+++
colchicine	–[c]	–
vincristine	++[d]	+
verapamil	++	++
quinidine	+	++
nifedipine	++	++
diltiazem	++	++
actinomycin D	+++	+++

[a] Means more than 80% inhibition. [b] Means 30–50% inhibition. [c] Refers to no detectable inhibition. [d] Means 50–80% inhibition.

inhibition of the [^{3}H]azidopine labeling of the recombinant multidrug transporter, when compared to the labeling of P-

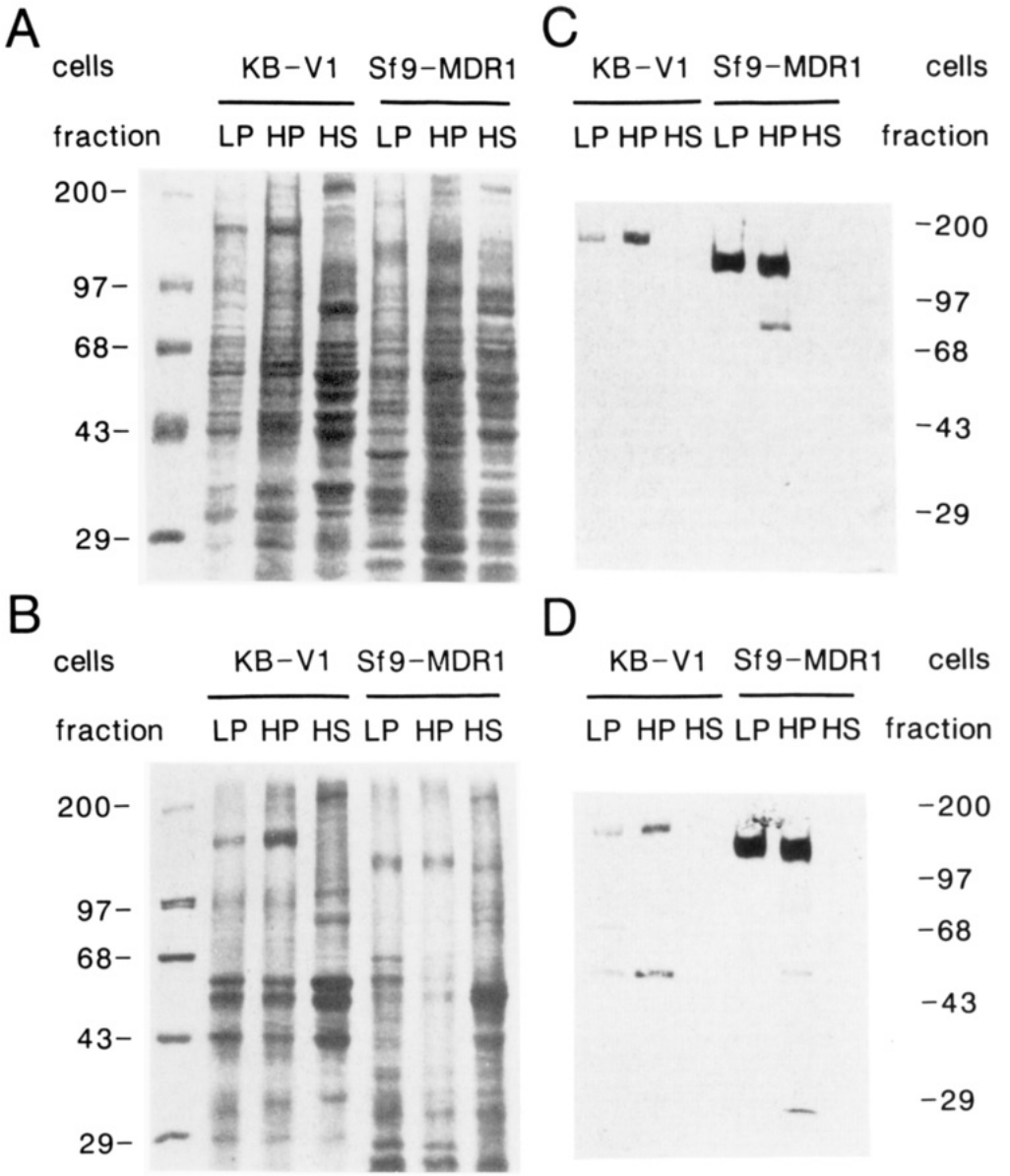

FIGURE 7: Comparison of multidrug transporter synthesis in BV-MDR1-infected Sf9 and multidrug-resistant human KB-V1 cells. KB-V1 cells and BV-MDR1-infected Sf9 cells were fractionated by differential centrifugation. Low-speed pellets (LP) containing nuclei and cell debris, high-speed pellets (HP) enriched in crude membranes, and high-speed supernatants (HS) representing the cytosolic fractions were resolved by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970). Panel A shows total proteins stained by Coomassie Blue. Panel B represents a fluorogram of [^{3}H]azidopine-labeled proteins. Panels C and D show immunoblots developed with the monoclonal multidrug transporter-specific antibody C219 (Kartner et al., 1985) (C) or the polyclonal antiserum 4007 (Tanaka et al., 1990) (D), respectively. Sizes of standards are given in kilodaltons.

glycoprotein in multidrug-resistant KB-V1 cells.

Discussion

To establish a system useful for the large-scale production of normal and mutant multidrug transporter for projected structural and biochemical studies, a human *MDR*1 cDNA was expressed in insect cells using the baculovirus expression system. Here, we report the initial characterization of the synthesized recombinant protein.

Upon infection with the recombinant baculovirus BV-MDR1, Sf9 host cells produce high levels of recombinant multidrug transporter which accumulates for 4–5 days until the cells lyse. The kinetics of multidrug transporter synthesis in BV-MDR1-infected cells, therefore, resemble polyhedrin production in wild-type AcNPV-infected insect cells (Summers & Smith, 1987) and since the protein accumulates, it must be very stable. This finding is consistent with the previously reported long half-life (48–72 h) of the authentic multidrug transporter expressed in multidrug-resistant human cells (Richert et al., 1988).

Immunofluorescence analysis of intact BV-MDR1-infected Sf9 cells using a monoclonal mouse antibody recognizing an external domain of the human multidrug transporter (Willingham et al., 1987) revealed a bright surface fluorescence signal. It is, therefore, suggested that insect cells synthesize and process the multidrug transporter polypeptide in a manner similar to human cells and that the protein is inserted into the plasma membrane in the correct transmembrane orientation. This hypothesis was supported by a more precise localization involving electron microscopy immunocytochemistry. This analysis indicated the presence of the multidrug transporter in the Golgi apparatus and the plasma membranes of insect cells. Localization of the authentic counterpart in the same organelles in multidrug-resistant human cells has previously been described (Willingham et al., 1987). Surprisingly, in some insect cells, the recombinant protein was also found

within the nuclear envelope. We assume that this might result from overproduction of the multidrug transporter. In order to travel to the plasma membrane, this protein has to be translocated across the endoplasmic reticulum, which is continuous with the outer nuclear membrane. Due to overproduction, the translocation process might have become limiting, and some protein might have been inserted into the nuclear membranes. The fact that the recombinant multidrug transporter expressed in insect cells is not glycosylated could also account for this inefficiency of the translocation machinery.

The molecular size of the majority of the recombinant protein expressed in insect cells is 140 000 daltons and corresponds to the nonglycosylated form of the multidrug transporter produced in multidrug-resistant human cells (Richert et al., 1988). We were somewhat surprised to detect an absence of carbohydrates, since other recombinant human membrane proteins expressed in the baculovirus system [e.g., epidermal growth factor receptor (Greenfield et al., 1988), IL-2 receptor (Domingo & Trowbridge, 1988), and transferrin receptor (Domingo & Trowbridge, 1988)] have been reported to be partially glycosylated, lacking only complex-type oligosaccharides. According to recent studies investigating the pathway of protein glycosylation and secretion, Sf9 cells are not only able to perform N-glycosylation but also may even process N-linked oligosaccharides to a state of endoglycosidase H resistance (Jarvis & Summers, 1989). The sugar moiety of the authentic multidrug transporter expressed in multidrug-resistant cells has never been fully characterized, but a preliminary analysis has suggested that all oligosaccharides are N-linked and modified to an endoglycosidase H resistant form. Whereas in mammalian cells N-glycosylation usually occurs cotranslationally and is finished within approximately 15 min (Beguinot et al., 1985), the 140-kDa multidrug transporter precursor is only very slowly converted to the mature form (within 2–4 h) (Greenberger et al., 1987; Richert et al., 1988). Possible explanations were related to the extremely hydrophobic features of the polypeptide chain which seem to be important for the final tertiary structure, but might interfere with the rate of processing and intracellular transport (Richert et al., 1988). Similar reasons might account for the absence of carbohydrates in the recombinant protein produced in insect cells. Obviously, the presence of oligosaccharides is not required for the [^{3}H]azidopine and drug binding activity of the multidrug transporter. Similar conclusions were also reached when P-glycoprotein expressed in tunicamycin-treated cells (Beck & Cirtain, 1982) or in CHO glycosylation mutants (Ling et al., 1983) was found to confer drug resistance. Furthermore, similar data have been obtained from human *MDR*1 gene product expressed in *Saccharomyces cerevisiae*.[1] Yeast cells also synthesize a recombinant multidrug transporter which is not glycosylated, but inserted into the plasma membranes, and can be labeled with [^{3}H]azidopine, for which drugs added in excess compete (vinblastine > adriamycin > colchicine).

Both the multidrug transporter produced in insect cells and its authentic counterpart expressed in multidrug-resistant human cells (Richert et al., 1988) are phosphorylated. To date, neither the extent nor the site(s) of phosphorylation have been determined for any of the two proteins, and the role of this posttranslational modification is unknown.

Unfortunately, it was impossible to test whether BV-MDR1 infection conferred the multidrug-resistance phenotype to Sf9 cells, because they were committed to viral-induced lysis. Thus, [^{3}H]azidopine was used in binding and drug competition studies to test for the functional activity of the recombinant protein. [^{3}H]Azidopine is a photoreactive analogue of the dihydropyridine class of calcium channel blockers which has previously been described to label hamster, mouse, and human *mdr* gene products specifically (Germann et al., 1989; Safa et al., 1987; Yang et al., 1989). Recently, we have identified two sites in human multidrug transporter from colchicine-resistant KB-C1 cells, which are labeled by [^{3}H]azidopine: one is present in the amino-terminal half and the other in the carboxy-terminal half of the molecule (Bruggemann et al., 1989). [^{3}H]Azidopine also labels the recombinant multidrug transporter present in the plasma membranes from BV-MDR1-infected Sf9 cells. A comparison of tryptic digests of [^{3}H]azidopine-labeled proteins followed by immunoprecipitations with two different antisera, 4077 and 4007 (raised against epitopes in the amino- and carboxy-terminal half, respectively; Tanaka et al., 1990), revealed that the same two sites are labeled (data not shown) as in its authentic counterpart (Bruggemann et al., 1989).

The efficiency of the labeling reaction of the recombinant protein is markedly decreased when compared to the labeling of P-glycoprotein produced in multidrug-resistant human cells. Furthermore, although substrates for the multidrug transporter inhibit [^{3}H]azidopine photoaffinity labeling (Table II), their relative activity as inhibitors differs somewhat from that seen for P-glycoprotein expressed in human KB-V1 cells. These differences may be the result of an altered affinity for the corresponding substrate due to the large amounts of recombinant protein in the plasma membranes which could affect its tertiary structure. The absence of carbohydrates could also alter the tertiary structure. It is also possible that the recombinant multidrug transporter expressed in insect cells is less accessible for the photoaffinity labeling reagent due to virus-induced changes in the cell surface. Furthermore, the recombinant protein which is inserted in the nuclear envelope might not be accessible at all. Nevertheless, we could clearly demonstrate that the recombinant multidrug transporter retains its characteristic feature of interacting with structurally and functionally different drugs. It, therefore, seems that the baculovirus expression system will be useful for the large-scale production of recombinant multidrug transporter for structural and mechanistic studies, as well as for antibody production.

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Registry No. Daunomycin, 20830-81-3; verapamil, 52-53-9; vinblastine, 865-21-4; vincristine, 57-22-7.

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