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Specific drug binding by purified lipid-reconstituted P-glycoprotein: dependence on the lipid composition

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We fused P-glycoprotein with β-galactosidase at the C-terminus aiming to study the mechanism of drug binding of P-glycoprotein in reconstitution experiments. Expression of the fusion protein in NIH 3T3 cells conferred a multidrug-resistant phenotype, suggesting that β-galactosidase fusion at the C-terminus does not affect the functions of P-glycoprotein. The fusion protein was partially purified by simple immunoprecipitation with anti-β-galactosidase polyclonal antibody, and its [^{3}H]azidopine binding property was investigated in the presence of various compositions of liposomes. The purified P-glycoprotein, after reconstitution into liposomes, was capable of binding [^{3}H]azidopine. When the cholesterol content of liposomes was increased to a weight ratio of 20%, the specific binding activity of the partially purified fusion protein was stimulated, and when the cholesterol content was increased higher, the binding activity decreased. The binding was specifically decreased by competition with vinblastine. Stigmasterol was less effective, and ergosterol was the least effective in stimulating the specific binding.

Introduction

P-glycoprotein, which is involved in the typical multidrug resistance of cancer cells, binds structurally and functionally varying anticancer drugs [1–3], and pumps them out of the cells [4–6]. A photoaffinity dihidropyridine analog azidopine specifically binds to P-glycoprotein and competes with anticancer drugs in binding to P-glycoprotein [7], suggesting that azidopine and anticancer drugs share a common binding site in P-glycoprotein. [^{3}H]Azidopine photoaffinity labels two different regions of P-glycoprotein corresponding to transmembrane domains 1–6 and 7–12 [8,9], suggesting that P-glycoprotein contains either two binding sites for azidopine or a single site formed by the two homologous halves of the protein. It is also reported that one amino acid substitution at position 185 (which is between transmembrane domains 2 and 3) affects the drug-binding site and alters the relative resistance to drugs, especially to colchicine [10,11]. The drug-binding site affected by the amino acid substitution at position 185 is proposed not to be the initial binding site but another site, associated with release of bound drug to the outside of the cell. These results, however, still do not describe how P-glycoprotein recognizes many different molecules that share no obvious structural or functional similarity.

We previously reported that human P-glycoprotein expressed in yeast cells did not function as a drug transporter, although some of the molecules seemed to be inserted correctly in the yeast plasma membrane [12]. We studied the effects of membrane components of yeast cells on the activity of P-glycoprotein, and found that ergosterol, a major sterol component of yeast plasma membrane, competed with azidopine in binding to P-glycoprotein in membrane vesicles of multidrug resistant cells, while cholesterol, a main sterol component in the animal plasma membrane, had little effect.

To understand the mechanism of drug binding, reconstitution experiments with purified P-glycoprotein are necessary. Here we report azidopine binding of partially purified P-glycoprotein fused with β-galactosidase. Specific binding was dependent on the cholesterol content of the liposomes in which the fusion protein was expected to be reconstituted.

Experimental procedures

Materials. Anti-human P-glycoprotein monoclonal antibody MRK16 was kindly provided by Dr. Takashi Tsuruo. Anti-β-galactosidase polyclonal antibody was from Advance and protein A-Sepharose CL-4B was from Pharmacia. Anti-P-glycoprotein monoclonal anti-

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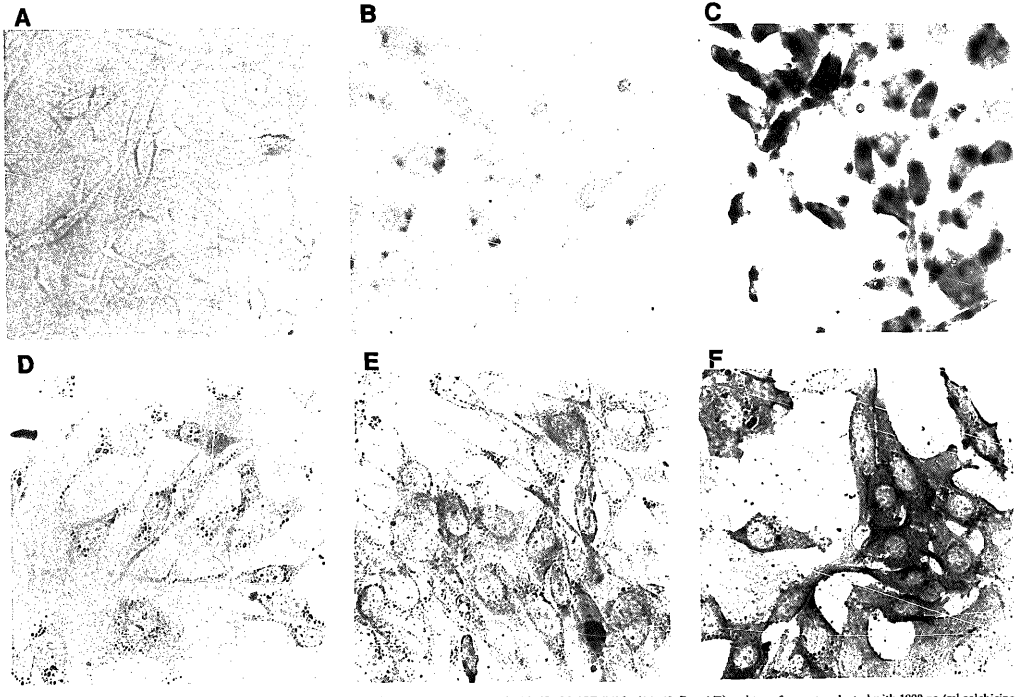

Fig. 1. X-gal staining and immunostaining. NIH 3T3 host cells (A and D), transformants selected with 40 nM dCF (NMgal14–40, B and E) and transformants selected with 1000 ng/ml colchicine after dCF selection, and maintained with 800 ng/ml colchicine (NMgal-C800, C and F) were stained with X-gal (A, B, and C) and reacted with MRK16 (D, E, and F).

body C219 was from Centocor. L-α-Phosphatidylcholine prepared from fresh turkey egg yolk (approx. 60%) was from Sigma. An immunostaining kit using alkaline phosphatase-labeled avidin biotin was from Dako. [^{3}H]Azidopine (specific activity 1 μCi/μl) was obtained from Amersham. 9-β-D-Xylofuranosyl adenine and 2′-deoxycoformycin were obtained from the National Cancer Institute, USA.

Plasmid and transfection. The plasmid pSKMgal, which is directed to express human P-glycoprotein fused with β-galactosidase at the C-terminus [13], was introduced into NIH 3T3 cells along with pSV2*neo* by the calcium phosphate coprecipitation method. Transfected cells were first selected in medium containing 0.8 mg/ml G418. G418-resistant cells were pooled and selected for the expression of the adenosine deaminase (*ADA*) gene which is on pSKMgal as another transcriptional unit in medium containing 9-β-D-xylofuranosyl adenine and 2′-deoxycoformycin (dCF).

X-gal staining. Cells were fixed with 2% formaldehyde and 0.2% glutaraldehyde in PBS at 4°C for 5 min, then washed three times with PBS. Fixed cells were reacted with 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM $MgCl_2$ in PBS at 37°C for more than 3 h, and washed with tap water.

Immunoprecipitation and reconstitution. Membrane vesicles prepared as reported previously [1] corresponding to 1 mg of protein were suspended in 1 ml of solubilizing buffer (50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 0.5% CHAPS, 2 mM DTT, 5% glycerol) for 1 h at 4°C with continual inversion, then insoluble materials were removed by centrifugation at 15000 × *g* for 10 min. Solubilized proteins were reacted with rabbit anti-β-gal polyclonal antibody on ice for 1 h, and then with 100–200 μl of protein A-Sepharose (50% v/v in solubilizing buffer) at 4°C for 1 h with continual inversion. Immune complex was precipitated by centrifugation and was washed four times with solubilizing buffer, and twice with solubilizing buffer containing various compositions of liposomes.

Photoaffinity labeling. Photoaffinity labeling was done as described previously [7] with minor modifica-

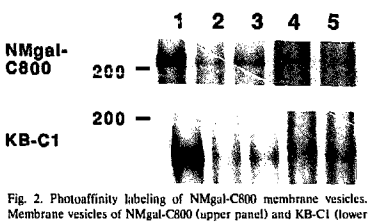

Fig. 2. Photoaffinity labeling of NMgal-C800 membrane vesicles. Membrane vesicles of NMgal-C800 (upper panel) and KB-C1 (lower panel) corresponding to 20 μg protein was photoaffinity labeled with [^{3}H]azidopine, and the effects of 1000-fold excess of various competitors were examined. Lane 1, no competitor; lane 2, vinblastine; lane 3, verapamil; lane 4, colchicine; lane 5, doxorubicin. Molecular size standards are indicated in kDa at the left.

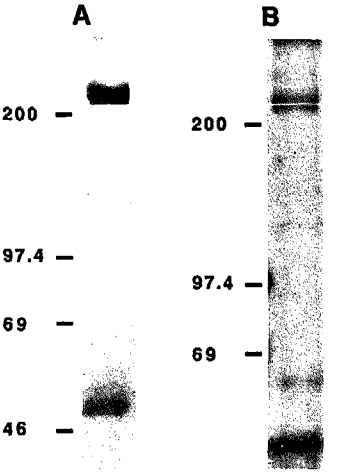

Fig. 3. Purification of the fusion protein. The fusion protein was purified by immunoprecipitation with anti-β-galactosidase polyclonal antibody from the membrane vesicles of NMgal-C800 cells. The immune complex was separated by SDS-PAGE and immunoblotted with anti-P-glycoprotein monoclonal antibody C219 (A) and Coomassie brilliant blue staining (B).

TABLE I

Relative resistance of the transformants expressing P-glycoprotein-β-galactosidase fusion protein

IC_{50} for NMgal14–40 and NMgal-C800 cells against colchicine (Col), vinblastine (Vbl), vincristine (Vcr), and doxorubicin (Dox) measured by MTT assay were compared with those for NIH 3T3 cells, and indicated by taking those for NIH 3T3 cells as 1.

	Relative resistance			
	Col	Vbl	Vcr	Dox
NIH 3T3	1	1	1	1
NMgal14–40	2.8	3.6	6.5	1.8
NMgal-C800	48.3	20.4	36.5	12.8

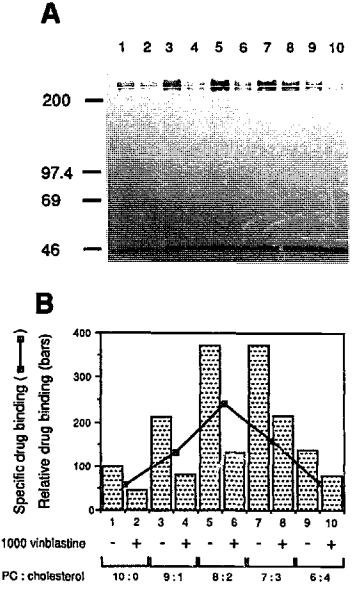

Fig. 4. Effects of cholesterol on azidopine binding of the partially purified fusion protein. (A) Photoaffinity labeling of the partially purified fusion protein reconstituted in liposomes containing no cholesterol (lanes 1 and 2), weight ratio of PC/cholesterol = 9:1 (lanes 3 and 4), PC/cholesterol = 8:2 (lanes 5 and 6), PC/cholesterol = 7:3 (lanes 7 and 8), and PC/cholesterol = 6:4 (lanes 9 and 10). Odd-numbered lanes, with no competitor; even-numbered lanes, in the presence of a 1000-fold excess of vinblastine. (B) The X-ray film was scanned by densitometer (Pharmacia, ULTROSCAN XL, LKB 2222-020) and intensities of the bands were calculated by taking lane 1 of each film as 100 (bars). Each bar is the average of duplicate measurements. Specific binding was calculated by subtracting drug binding in the presence of vinblastine from that in the absence of vinblastine and indicated as □——□. The numbers of each bar correspond to the lane numbers of A.

tions. Briefly, membrane vesicles corresponding to 20–40 μg of protein were reacted with 0.2 μM [^{3}H]azidopine in vesicle buffer (50 mM Tris-HCl (pH 7.5), 250 mM sucrose) at a final volume of 50 μl in the presence or absence of 1000-fold excess of competitors at room temparature for 20 min. The reaction mixture was then irradiated with a UV lamp (Ultra-Violet Products, Blak Ray Lamp Type XX-15L) for 30 min on ice. Labeled proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). When immunoprecipetated samples were used, 20 μl of the immune complex suspension (50% v/v) was reacted with [^{3}H]azidopine in vesicle buffer.

Results

Expression of P-glycoprotein fused with β-galactosidase

NIH 3T3 cells were transfected with the expression vector that was directed to express P-glycoprotein fused with β-galactosidase, along with pSV2*neo* to select transfected cells initially with G418. Because the expression vector contains the *ADA* gene as another transcriptional unit, the G418-resistant cells were then selected with 10 nM deoxycoformycin (dCF). Expression of the fusion protein was monitored by staining the cells with X-gal, and one clone (NMgal14–40) expressing a large amount of the fusion protein was isolated. To amplify the expression of the fusion gene, NMgal14–40 cells were then cultured with increasing concentrations of dCF. After selection with 40 nM dCF, expression of the fusion gene was examined by staining the cells with X-gal (Fig. 1B), and by immunostaining with the monoclonal antibody MRK16 against human P-glycoprotein (Fig. 1E). Because MRK16 recognizes the extracellular region of P-glycoprotein, this result indicates the presence of the fusion protein in the plasma membrane of transformants. NIH 3T3 host cells were not stained by X-gal (Fig. 1A) nor did they react with MRK16 (Fig. 1D).

The fusion protein functioned as a multidrug transporter in NIH 3T3 cells

Transfected cells became 1.8–6.5-fold resistant to drugs after selection with 40 nM dCF (Table I). Because dCF does not relate to the MDR phenotype or induce endogenous mouse P-glycoprotein (Ueda, K., unpublished data), human P-glycoprotein fused with β-galactosidase is supposed to function as a multidrug transporter. But because selection with dCF gave a limited level of expression of the fusion protein, we proceeded to further selection with increasing concentrations of colchicine. NMgal-C800 cells that were selected with 1000 ng/ml colchicine and maintained with 800 ng/ml colchicine overexpressed the fusion protein (Figs. 1C and F), and became more resistant to drugs (Table I). Membrane vesicles of these transfectants were prepared and photoaffinity labeling with [^{3}H]azidopine was done. The fusion protein in membrane vesicles prepared from NMgal-C800 cells was labeled by [^{3}H]azidopine as efficiently as the authentic P-glycoprotein of KB-C1 (Fig. 2), and photoaffinity labeling competed with various substrates for P-glycoprotein in a similar pattern (Fig. 2). These data suggest that the fusion with β-galactosidase at the C-terminus does not affect the functions of P-glycoprotein.

Cholesterol-dependent specific azidopine-binding of partially purified fusion protein

The overexpressed fusion protein was partially purified by immunoprecipitation with anti-β-galactosidase polyclonal antibody (Figs. 3A and B), and azidopine-binding activity of the partially purified fusion protein was examined. The fusion protein was washed twice with solubilizing buffer containing liposomes of egg yolk phosphatidylcholine (PC) after immunoprecipitation. In this step, spontaneous reconstitution was expected. The partially purified fusion protein in PC liposomes was weakly photoaffinity labeled by [^{3}H]azidopine (Fig. 4A lane 1). But only 50% of azidopine binding was competed with excess vinblastine (Figs. 4A and B lane 2). Because our previous study suggested the involvement of membrane sterol in the function of P-glycoprotein [12], we examined the effects of cholesterol in azidopine binding of partially purified fusion protein. When the cholesterol content of liposome was increased to a PC/cholesterol weight ratio of 8:2, both of the total binding and the specific binding increased (Figs. 4A and B). When the cholesterol content was 7:3, the total binding was still high but the specific binding decreased. When the choles-

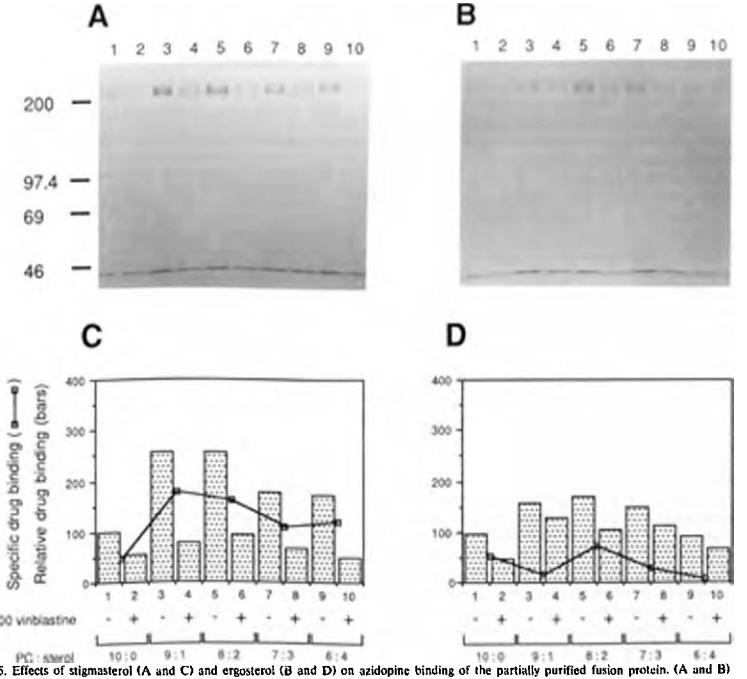

Fig. 5. Effects of stigmasterol (A and C) and ergosterol (B and D) on azidopine binding of the partially purified fusion protein. (A and B) Photoaffinity labeling of the partially purified fusion protein reconstituted in liposomes containing no sterol (lanes 1 and 2), weight ratio of PC/sterol = 9:1 (lanes 3 and 4), PC/sterol = 8:2 (lanes 5 and 6), PC/sterol = 7:3 (lanes 7 and 8), and PC/sterol = 6:4 (lanes 9 and 10). Odd-numbered lanes, with no competitor; even-numbered lanes, in the presence of a 1000-fold excess of vinblastine. (C and D) Relative drug binding (bars) and specific drug binding (□ ——— □) were calculated as described in the legends for Fig. 4. Each bar is the average of duplicate measurements.

terol content was 6:4, both the total binding and the specific binding decreased to the level without cholesterol.

Effects of stigmasterol and ergosterol on [^{3}H]azidopine binding

We examined if stigmasterol or ergosterol, which are the main sterols of plant and yeast plasma membrane, respectively, could substitute for cholesterol. Stigmasterol stimulated azidopine binding of the partially purified fusion protein, although less effectively than cholesterol. The highest total and specific bindings were observed when the stigmasterol content was a PC/stigmasterol weight ratio of 9:1 (Figs. 5A and C). Ergosterol was the least effective in stimulating the azidopine binding of the partially purified fusion protein (Figs. 5B and D).

Discussion

In this report, P-glycoprotein was expressed as a fusion protein with β-galactosidase at the C-terminus. After dCF selection, transformed cells showed a multidrug-resistant phenotype. Because dCF selection does not induce endogenous mouse P-glycoprotein, human P-glycoprotein fused with β-galactosidase is supposed to be functioning as a multidrug transporter. The fusion protein in membrane vesicles was efficiently labeled by [^{3}H]azidopine, and the pattern of competition by various drugs was similar to that of the authentic P-glycoprotein of multidrug-resistant KB-C1 cells. Moreover, the partially purified fusion protein has ATPase activity [13]. Thus, β-galactosidase fused at the C-terminus does not affect the functions of P-glycoprotein.

The fusion protein was partially purified by simple immunoprecipitation with anti-β-galactosidase polyclonal antibody. The photoaffinity labeling of the partially purified fusion protein reconstituted in liposomes by [^{3}H]azidopine indicates involvement of cholesterol in drug binding of P-glycoprotein. Cholesterol stimulated specific azidopine binding of the fusion protein when the PC/cholesterol weight ratio was 8:2. This ratio may be significant because cholesterol comprises 20–30% of the membrane lipids in the plasma membrane of human cells. Stigmasterol was less effective at stimulating the specific binding, and ergosterol scarcely stimulated the specific binding. These results are consistent with our previous report that P-glycoprotein expressed in yeast cells does not function as a multidrug transporter, probably due to the difference of the sterol component of the plasma membrane [12].

The mechanism of the stimulatory effect of cholesterol is not clear. One possibility is the influence of cholesterol on the motional order of liposomes. Another possibility is that cholesterol may interact directly with P-glycoprotein as an essential positive effector. It was recently reported that the membrane cholesterol content affects the activities of Na^+/K^+-ATPase [14] and UDP-glucuronosyltransferase [15]. In the former case ergosterol was the least effective at supporting the activity. These structural specificities for the sterol to be effective may suggest a direct interaction with the enzyme. Because P-glycoprotein directly interacts with progesterone at the drug binding site [16] and transports cortisol and aldosterone (Ueda, K., unpublished data), cholesterol may directly interact with the drug binding site of P-glycoprotein and assist the specific drug binding.

Acknowledgments

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