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FUNCTIONALLY ACTIVE HOMODIMER OF P-GLYCOPROTEIN IN MULTIDRUG-RESISTANT TUMOR CELLS*

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Abstract: P-glycoprotein plays a key role in multidrug resistance of tumor cells. In order to elucidate the possible quarternary structure/function relationship of P-glycoprotein, we treated multidrug-resistant human leukemia K562/ADM cells with the crosslinking reagent, disuccinimidyl suberate. In addition to 180K P-glycoprotein, a 340K protein was immunoprecipitated with an anti-P-glycoprotein monoclonal antibody, MRK-16. The 340K protein is most probably a dimeric P-glycoprotein, since only the 180K P-glycoprotein was immunoprecipitated with MRK-16 when K562/ADM cells were treated with the cleavable crosslinking reagent, dithiobis(succinimidylpropionate), and analysed under reduced conditions. The dimeric P-glycoprotein was photolabeled with [^{3}H]azidopine like the 180K monomeric P-glycoprotein and the photolabeling was inhibited by excess amount of vincristine and verapamil. The dimeric P-glycoprotein could be a functionally active form of the protein involved in the transport of antitumor agents.

A mechanism of multidrug resistance is attributed to the reduced accumulation of antitumor agents in resistant cells as compared with their drug sensitive cells (1-5). A membrane glycoprotein termed P-glycoprotein is overexpressed in multidrug-resistant cells (5-8). P-glycoprotein can specifically bind antitumor agents (9, 10), Ca^{2+} channel

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Abbreviations used are : DSS, disuccinimidyl suberate; PBS, phosphate buffered saline; ADM, adriamycin; VCR, vincristine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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blockers (11-13) and progesterone (14). The purified P-glycoprotein has an ATPase activity (15, 16), and plasma membrane vesicles from multidrug-resistant cells containing P-glycoprotein accumulate antitumor agents in a ATP-dependent manner (17, 18). These results indicate that the P-glycoprotein is the transporter of various antitumor agents in the plasma membrane of multidrug-resistant cells.

The amino acid sequence and the secondary structure of P-glycoprotein has been determined from the cloned human mdr1 gene (19). P-glycoprotein is a tandemly duplicated molecule, with each half containing six hydrophobic transmembrane helices and a large hydrophilic domain with a consensus sequence for ATP-binding (19). On the other hand, the tertiary and quarternary structure of P-glycoprotein is not understood. Studies utilizing radiation inactivation analysis has suggested a dimerization of P-glycoprotein in the membrane (20). However, there has been no direct evidence for the dimeric form of P-glycoprotein, and as a result, no functional analysis have been initiated.

Chemical crosslinking is a good method to determine the subunit structure of protein complexes. The high affinity interleukin-2 receptor complex (21-23) and the dimeric structure of mutated transferrin receptor (24) were clarified by crosslinking methods. In order to study the possible quarternary structure of P-glycoprotein, we analysed the chemical crosslinked products of P-glycoprotein. We report here that a homodimer of P-glycoprotein is present in multidrug-resistant cells and that this P-glycoprotein dimer is functionally active.

Materials and Methods

Chemicals: Disuccinimidyl suberate (DSS) was obtained from Pierce Chemicals, Rockford. [^{3}H]Azidopine (1.85 TBg/mmol) and Amplify was from Amersham Japan, Tokyo. Acrylamide/Bis (29:1) was from Bio-Rad Laboratories, Richmond. Cross-Linked Phosphorylase b SDS Molecular Weight Marker was from Sigma Chemicals, St. Louis. 2D-Silver Stain II "DAIICHI" was from Daiichi Pure Chemicals, Tokyo. 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) was from Dojindo Laboratories, Kumamoto.

Chemical crosslinking: 5 x 10^8 cells of an Adriamycin-resistant variant of human leukemia (K562/ADM) were treated with 1 mM DSS in 10 ml of phosphate buffered saline (PBS) for 30 min at 25°C. The cells were pelletted and solubilized with 2.5 ml of 10 mM Tris-HCl, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.02 mM phenylmethylsulfonyl fluoride and 1 % CHAPS. To the supernatant of the cell lysate, 20 μg of MRK-16 was added and incubated for 1 hr at 4°C with constant mixing. Then 100 μl of Protein A-sepharose CL-4B (20 % by volume in PBS) was added and further incubated for 2 hr at 4°C with constant mixing. The precipitates were washed ten times with PBS containing 1 % CHAPS, and the final precipitates were boiled for 5 min

in 30 µl of SDS-urea sample buffer. The electrophoresis of the samples in 3 % polyacrylamide cylindrical gels were carried out according to the manufacturers specifications (Sigma Chemicals). After electrophoresis the gels were stained with 2D-Silver Stain "DAIICHI" according to instructions with some modifications. For photoaffinity labeling experiments, K562/ADM cell membranes (200 µg) were photolabeled with 10 pmol of [^{3}H]azidopine in 25 µl of reaction mixture as described previously (25). The labeled membranes were treated with 1 mM DSS for 30 min at 25°C, and the electrophoresis was carried out as above or in 3.5 % polyacrylamide slab gels. Since the low concentration of acrylamide in phosphate buffer did not polymerize around the teeth of the teflon well former, Tris buffer was used in the preparation of slab gels, which might result in a change of P-glycoprotein mobility as described below. After the electrophoresis, the gels were fixed in 25 % isopropyl alcohol and 10 % acetic acid, treated with the fluorographic reagent Amplify for 30 min, dried and then exposed to X-ray film for three weeks at -70 °C as described previously (25).

Results and Discussions

Fig. 1 documents the immunoprecipitation of P-glycoprotein from K562/ADM cells with the monoclonal antibody, MRK-16. As reported previously (15) and shown in control lanes (a and b) the MRK-16 precipitated the 180K P-glycoprotein. A faint band of approximately 130K was also detected. This 130K protein is probably a protease-sensitive, degradation product of intact P-glycoprotein (26), since MRK-16 recognizes the large fragment (about 130K) of P-glycoprotein (unpublished

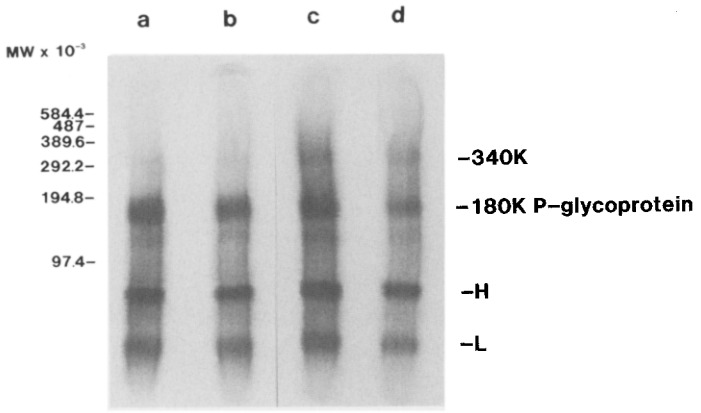

Fig. 1. Immunoprecipitation of dimeric P-glycoprotein. K562/ADM cells were treated with 1 mM DSS (gels c and d) or not (gels a and b), solubilized in 1 % CHAPS and immunoprecipitated with MRK-16. The proteins were separated by SDS-PAGE in 3 % polyacrylamide cylindrical gels and silver stained. Proteins from 10^8 cells (a and c) or 5 x 10^7 cells (b and d) were applied. H and L represent the heavy chain and the light chain of MRK-16, respectively.

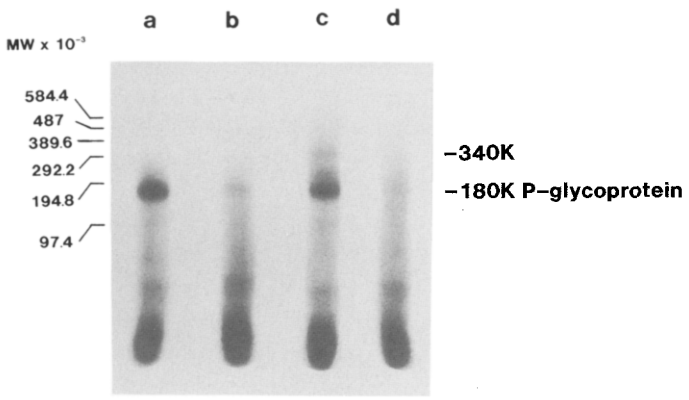

Fig. 2. Photoaffinity labeling with [^{3}H]azidopine and crosslinking with DSS of K562/ADM cell membranes.

K562/ADM cell membranes (200 μg) were photolabeled with [^{3}H]azidopine in the absence (gels a and c) or presence (gels b and d) of 100 μM vincristine, and then treated with 1 mM DSS (c and d) or not (a and b). The labeled membrane proteins were analysed by SDS-PAGE in 3 % polyacrylamide cylindrical gels and fluorography as described in Meterials and Methods. The 340K in the right indicate the 340K dimeric P-glycoprotein.

obvservation). When K562/ADM cells were treated with 1 mM DSS and immunoprecipitated with MRK-16 as above, a 340K band was observed in addition to the 180K and 130K bands (lanes c and d). The molecular mass of 340K is nearly two-fold that of the 180K P-glycoprotein. Moreover, when K562/ADM cells were crosslinked with the cleavable crosslinking reagent dithiobis(succinimidylpropionate), only the 180K of P-glycoprotein was precipitated with MRK-16 (27). These observations indicate that the 340K band is a homodimer of P-glycoprotein. Since the crosslinking of protein complexes is generally inefficient (21-24), the content of the dimeric P-glycoprotein in the cells is not clear. By radiation inactivation analysis, however, the target size of P-glycoprotein was reported to be 250K and is close to two times the molecular mass of the protein component of the P-glycoprotein (20). Therefore all the P-glycoprotein might exist in the dimeric state in the membrane of multidrug-resistant cells.

In order to examine whether the dimeric P-glycoprotein is functional, plasma membranes from K562/ADM cells were photolabeled with [^{3}H]azidopine and then crosslinked by DSS. The crosslinked membrane proteins were analysed by SDS-PAGE in 3 % polyacrylamide cylindrical gels and fluorography (Fig. 2). The photolabeled P-glycoprotein was observed as a 180K band (lane a), and after crosslinking with DSS a significant amount of a photolabeled 340K protein band was observed (lane c). The binding of [^{3}H]azidopine to 180K P-glycoprotein and 340K

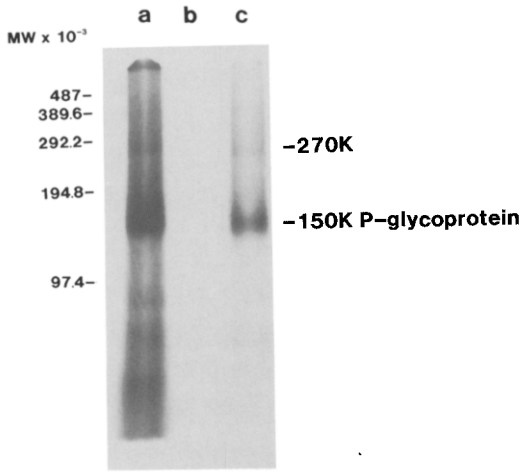

Fig. 3. Immunoprecipitation of [^{3}H]azidopine-labeled dimeric and monomeric P-glycoprotein with MRK-16.

K562/ADM cell membranes (400 μg) were photolabeled with 0.4 μM [^{3}H]azidopine, crosslinked with 1 mM DSS, solubilized in 1 % CHAPS buffer, and then immunoprecipitated with MRK-16 (lane c) or normal mouse serum (lane b). The photolabeled and crosslinked K562/ADM cell membrane without immunoprecipitation was loaded in lane a. The labeled proteins were analysed by SDS-PAGE in 3.5 % polyacrylamide slab gel and fluorography. The 270K in the right indicates the 270K dimeric P-glycoprotein.

protein was specific because 100 μM of vincristine added during the incubation abolished the both protein bands (Fig.2, lanes b and d).

To confirm that the photolabeled 340K protein is the dimer of P-glycoprotein, the photolabeled and crosslinked membrane proteins were immunoprecipitated with MRK-16 and analysed by SDS-PAGE in 3.5 % polyacrylamide slab gel and fluorography. Of interest, in this 3.5 % polyacrylamide slab gel system, the previously described 180K and 340K proteins were identified as 150K and 270K, respectively (Fig. 3, lane a). The reason why the mobility of the proteins altered was not clear, but the change in buffer composition for the gels might be responsible. In addition to the 150K P-glycoprotein, the photolabeled 270K protein was precipitated with MRK-16 (Fig. 3, lane c). These results indicate that the photolabeled 270K protein in the 3.5 % polyacrylamide slab gel and the photolabeled 340K protein in the 3 % polyacrylamide cylindrical gel were the dimer of P-glycoprotein. This observation suggests that at least a significant amount of P-glycoprotein retains a dimeric state after [^{3}H]azidopine binding. Since the specific binding of [^{3}H]azidopine is a functional determinant of native P-glycoprotein (11) and that [^{3}H]azidopine-binding is closely correlated with the transport activity of antitumor agents by P-glycoprotein (28, 29), the dimeric P-glycoprotein could be functionally active in the transport of antitumor agents.

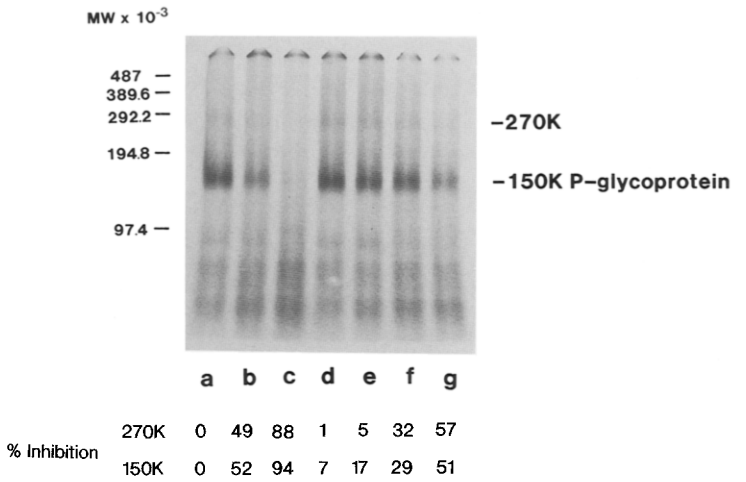

Fig. 4. Inhibitory effect of vincristine, ADM and verapamil on photolabeling of dimeric and monomeric P-glycoprotein by [^{3}H]azidopine.
K562/ADM cell membranes (200 μg) were photolabeled with 0.4 μM of [^{3}H]azidopine in the presence or absence of drugs and treated with 1 mM DSS. The labeled proteins were analysed by SDS-PAGE in 3.5 % polyacrylamide slab gel and fluorography. The extent to inhibition of drugs on the labeling of dimeric and monomeric P-glycoprotein was determined by scanning the film with Ultro Scan XL (Pharmacia). Lanes a: no drug, b: 10 μM vincristine, c: 100 μM vincristine, d: 10 μM ADM, e: 100 μM ADM f: 10 μM verapamil, and g: 100 μM verapamil. The 270K in the right indicates the 270K dimeric P-glycoprotein.

Fig. 4 shows the inhibition of [^{3}H]azidopine photolabeling of monomeric and dimeric P-glycoprotein by VCR, ADM and verapamil. VCR at 10 μM effectively reduced the photolabeling of both monomeric and dimeric P-glycoprotein by 52 and 49 %, respectively. VCR at 100 μM almost completely inhibited the photolabeling of both P-glycoproteins. The photolabeling of monomeric and dimeric P-glycoprotein was also reduced slightly by 100 μM of ADM and to a greater extent by 100 μM of verapamil. In all cases the extent of the reduction of the photolabeled band was similar in both the dimeric and monomeric P-glycoprotein, suggesting that functional differences between monomeric and dimeric P-glycoprotein may not exist.

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