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Dimerization of the P-glycoprotein in membranes

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Plasma membranes from a CHO cell line, CH^RC5, which exhibits multidrug resistance was studied using radiation inactivation analysis. The P-glycoprotein content of the membrane was determined by Western blots. Irradiation resulted in the loss of P-glycoprotein. The dependence of this loss on radiation dose corresponded to a target size of 250 kDa which is the molecular mass of a dimer of the P-glycoprotein. This is strong evidence to indicate that the P-glycoprotein self associates in the membrane.

Introduction

Treatment of cancer patients with cytotoxic agents often results in the development of tumor cells which are multidrug resistant. This phenomenon is believed to be a major factor limiting the efficacy of cancer chemotherapy [1,2]. A feature of many drug resistant tumor cells is the presence of an overexpressed membrane protein, the P-glycoprotein [3,4]. This protein is believed to have ATPase activity [5] and membrane vesicles enriched in this protein can transport drugs in an ATP-dependent manner [6]. Cells have been made multidrug resistant through infection with a retrovirus carrying the cDNA for the P-glycoprotein gene, MDR1 [7] or by transfection with the MDR genes [8]. The P-glycoprotein has been identified by photoaffinity labelling as the protein that binds to vinblastine [9,10], a drug believed to be transported by this protein. Photoaffinity labelling has also been used to demonstrate binding to the P-glycoprotein of verapamil [11] and of azidopine [12,13], drugs which reverse the multidrug resistance phenotype. In the present work, we measure the radiation inactivation target size of the P-glycoprotein immunoreactivity. Target size analysis has been successfully used for studying the structural basis of specific functions within complex biological structures including membranes [14,15].

Experimental Procedures

Materials

Tissue cultures products were from Gibco (Grand Island, NY). All other chemicals were purchased from Sigma.

Methods

Cell cultures

The adenosine-thymidine-glycine-requiring auxotroph Chinese hamster ovary (CHO) cells (AB_1) and a colchicine-resistant line derived from these cells (CH^RC5) were cultured in α-Minimal Essential Medium (with ribonucleosides and deoxyribonucleosides) supplemented with 7% fetal calf serum plus antibiotics (streptomycin and penicillin). These cell lines, obtained from Dr. Ling of the Ontario Cancer Institute, Toronto, were grown as monolayer cultures at 37°C, in a humidified atmosphere of 5% CO_2/95% air.

Membrane isolation

Cells were washed with phosphate-buffered saline (PBS) and suspended in hypotonic lysis buffer (10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol). The cell suspension was then incubated for 20 min in an ice bath. The cells were disrupted using a tightly fitting Dounce homogenizer and the nuclei were removed by centrifugation at ($300 \times g$) for 10 min. Then, the following differential centrifugation steps were

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applied: mitochondrial spin, (4000 × *g*) for 10 min and microsomal spin, (35 000 × *g*) for 30 min. The microsomal pellet was resuspended in PBS and layered onto a 16%/31%/45% discontinous sucrose gradient in 10 mM Tris-HCl (pH 7.4) and centrifuged for 15 h at (100 000 × *g*) in an SW 25.2 rotor. Vesicles at the two interfaces were collected, diluted in 10 mM Tris-HCl (pH 7.4)/0.25 M sucrose (buffer A) and centrifuged for 60 min at (100 000 × *g*). The vesicle pellet was then resuspended in buffer A at a concentration of 0.7–1.5 mg of protein/ml and stored in liquid nitrogen prior to use. Relative to the homogenate, the activity of 5′-nucleotidase in the plasma membrane vesicle fraction ('16/31' interface) was enriched 14-fold and in the rough microsome fraction ('31/45' band) it was enriched 2-fold. Both the plasma membrane and rough microsome fractions gave identical results in radiation inactivation studies. Plasma membrane fractions were used for the results reported below.

Irradiation procedure

The samples for irradiation were spread as a thin film in aluminum trays and frozen at −70°C overnight. Frozen aliquots of membrane preparations were irradiated with a 1.5 MeV fast electron beam produced by a Van de Graaff accelerator (High Voltage Engineering Corp., Model GS) at −45°C. The control samples were run concurrently under the same conditions but without irradiation. The irradiated membrane preparations were either used immediately or stored at −70°C for up to 4 days.

Immunoblotting

After irradiation, membrane samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the polypeptides were transferred to nitrocellulose paper at 140 mA for 14 h [16]. The nitrocellulose blot was incubated for several hours at 4°C in PBS containing 5% bovine serum albumin, followed by incubation for 3 h with anti-P-glycoprotein monoclonal antibody C219 (1:1000 dilution). The C219 is a monoclonal antibody which is specific for an epitope in the C-terminal region of the P-glycoprotein [17]. After three washes with PBS containing 0.5% Tween 20 (pH 8.2), the blot was incubated 2 h at room temperature with ^{125}I-labeled *S. aureus* protein A, diluted in PBS ($1 \cdot 10^6$ cpm/ml, prepared by the Iodogen method). The blot was washed to remove unbound ^{125}I-protein A with PBS containing 0.5% Tween 20 (pH 8.2) wrapped in Saran Wrap and exposed at −70°C to Kodak XAR film utilizing an intensifying screen. The amount of ^{125}I-protein A bound to the nitrocellulose blot was quantified by scanning the autoradiogram in a Hoefer GS-300 scanning densitometer. The data was analyzed using the GS-350 data system (IBM version). Reduced α_2-macroglobulin (170 kDa) and phosphorylase *b* (97.4 kDa) were used as molecular mass markers. It has recently been emphasized that computer-aided quantitation of autoradiograms may not give peak areas which are proportional to radioactivity [18]. We have confirmed the linear dependence of the peak areas of our western blots with the amount of unirradiated membrane subjected to the above procedure (Fig. 1).

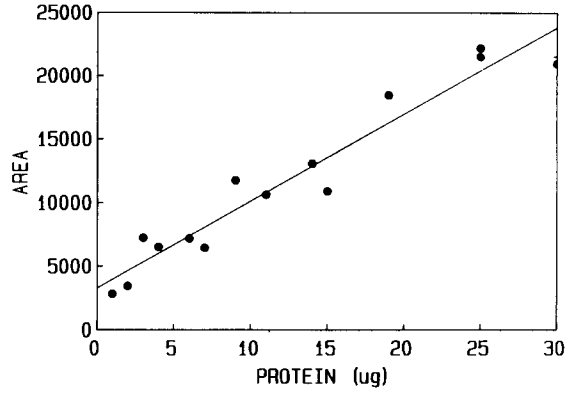

Fig. 1. Relationship of peak area (arbitrary units) measured by computer-aided densitometry with amount of membrane protein originally applied to SDS gels.

Data analysis

The logarithm of the fraction of 170 kDa band intensity was plotted as a function of radiation dose. All data were fitted to a straight line by a least square analysis. The dose, D_{37}, at which the fractional inactivation equals $1-(1/e)$, was determined from this plot and related to the target mass (m) by the empirical equation $m = 6.4 \times 10^5/D_{37}$ (Mrad), where Mrad is the radiation dose in megarads. Radiation doses were measured by blue cellophane dosimetry as detailed elsewhere [14]. Since irradiation of samples and blue cellophane were carried out at −45°C, no correction for temperature effects was made in this calculation.

Results

Membranes were irradiated and the proteins separated by SDS-PAGE. Proteins with molecular masses greater than 60 kDa are progressively destroyed by the radiation, whereas low molecular mass proteins were less affected (not shown). The proteins were blotted on nitrocellulose paper and incubated first with the monoclonal antibody C219 against P-glycoprotein and then with ^{125}I-protein A. Only SDS-PAGE gels from unirradiated or irradiated CH^RC5 membranes, but not AB_1 membranes, showed immunoreactive bands whose decay could be analyzed. The degree of radioactivity associated with the higher molecular mass bands decreased as the the radiation dose increased (Fig. 2). The immunoreactive band exhibits heterogeneity even in the

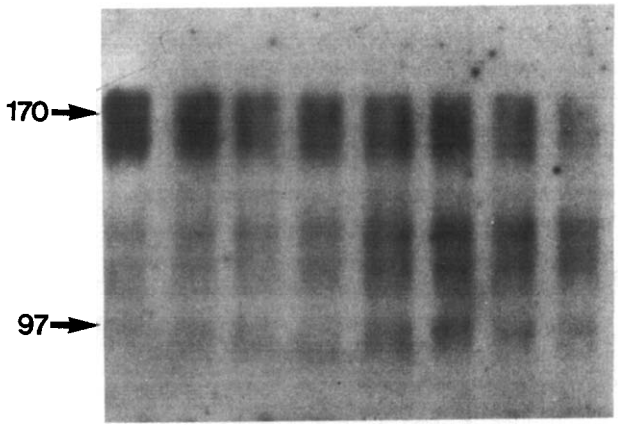

Fig. 2. Immunoblot of P-glycoprotein irradiated with doses up to 6 Mrad. Approximately 25 μg of membrane protein (plasma membrane fraction) was subjected to SDS-PAGE and immunoblotting both before and after irradiation as described in Experimental Procedures. The figure shows an autoradiograph exposed for 72 h to a Kodak XAR film. The irradiation doses, from left to right are: 0, 0.5, 1.0, 1.5, 2.0, 2.4, 2.8 and 3.3 Mrad. Arrows at left mark the position of the 170 and 97 kDa standards.

unirradiated sample. This behaviour has been previously observed using both this antibody and a different monoclonal antibody [19]. The double band has been ascribed to heterogeneity in N-linked carbohydrate [20] as well as to multiple forms of the P-glycoprotein [21]. The radiation sensitivity of the protein was visualized by densitometry and the logarithm of the intensity of the more intense higher molecular mass band of the doublet was plotted as a function of radiation dosage (Fig. 3). From this plot, combining data from four separate irradiation experiments, a $D_{37} = 2.57$ Mrad was calculated which corresponds to a molecular mass of 250 kDa. Each of the experiments analyzed individually fell into the molecular mass range 245–260 kDa. Analysis of the lower molecular mass band of the doublet also yielded a target size in this molecular mass

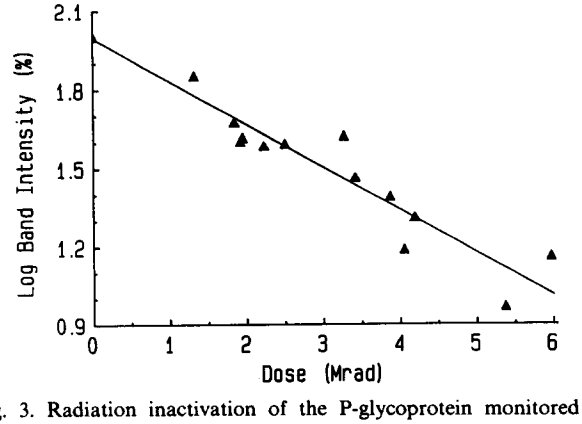

Fig. 3. Radiation inactivation of the P-glycoprotein monitored by measurement of the intensity of the band in the autoradiogram. The combined results from four separate irradiation experiments. The intensity of the highest molecular weight band decays in a monoexponential way as a function of the radiation dose.

range. The data on the decay of the higher molecular mass band is more reliable, since after irradiation the lower band may contain degradation products from the upper band.

Discussion

Target size analysis yielded a molecular mass of 250 kDa for the immunoreactive band in gel electrophoresis. This is close to two times the molecular mass of the protein component of the P-glycoprotein which has been estimated to be 120–125 kDa [21] and 140 kDa [22]. It has been shown that the molecular mass determined by radiation inactivation is dependent only on the protein portion and not on the covalently bound carbohydrate of a glycoprotein [23]. The results therefore suggest that the P-glycoprotein exists as a dimer in the membrane. However, the protein is detected as a monomer after dissociation in SDS and Western blot analysis. The immunoblots specifically detect the P-glycoprotein. The anti-P-glycoprotein monoclonal antibody we have used for the immunoblots is specific for an epitope on the carboxy-terminal portion of this protein [17]. The immunoreactive bands run close to a 170 kDa protein standard. The results of the Western blots for non-irradiated samples agree well with results previously reported for membranes containing the P-glycoprotein [19–21]. This is the case for gels run both with the purified plasma membrane vesicle fraction (the '16/31' interface) as well as those run with the less pure '31/45' interface. Thus, the presence of other membrane fractions does not increase the number of immunoreactive bands. Finally, the membrane fractions from the AB_1 cells did not show any immunoreactive bands, either before or after irradiation, demonstrating that these bands are associated with multidrug resistant cells which overexpress the P-glycoprotein. It is therefore highly probable that the immunoblots specifically detect the P-glycoprotein. The fact that the intensity of the initial P-glycoprotein band decays with a rate corresponding to a target size of 250 kDa is evidence that this protein is dimeric in the membrane.

This work presents strong evidence that the P-glycoprotein has a quaternary structure in the membrane. It has been suggested that the P-glycoprotein is an ATP-dependent pump. Other active transport systems such as Ca^{2+}-ATPase from the sarcoplasmic reticulum [24] and H^+,K^+-ATPase [25] are oligomeric in membranes. Radiation inactivation is one of the few methods that can be used to determine the state of oligomerization of proteins in membranes. It has been extensively applied to many membrane systems [14,15,24,25]. No single method can conclusively prove the state of association in situ of a protein in a complex biological specimen. However, our results make it highly likely that the P-glycoprotein is dimeric in the membrane of resistant

cells. Recent freeze fracture electron microscopy studies are also consistent with the P-glycoprotein being present as a dimer in the membranes [26]. This possibility should be taken into account in explaining new experimental results.

Acknowledgements

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