Detection of Oligomeric and Monomeric Forms of P-glycoprotein in Multidrug Resistant Cells†

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ABSTRACT: P-glycoprotein (P-gp) is thought to function as a drug efflux pump in multidrug resistant (MDR) cells. The functional form of P-gp in its native state is not known. Previous results from radiation target size analysis have suggested that P-gp occurs as dimers in MDR cell plasma membranes [Boscoboinik *et al.* (1990) *Biochim. Biophys. Acta 1027*, 225–228]. In this study, we used sucrose gradient velocity sedimentation to determine if P-gp oligomers could be retrieved from detergent extracts of hamster and human MDR cell lines. The proportion of P-gp recovered as higher order oligomers was dependent on the detergents used for solubilization of the cells. When a detergent such as CHAPS was used, 50% or more of the P-gp sedimented as higher order oligomers. In contrast, in the presence of SDS, only monomers were retrieved, but naturally occurring oligomers could be preserved if the cells were treated with a cross-linker prior to detergent solubilization. The oligomers and monomers were both able to bind the photoactive analog of ATP (8-azido[α-^{32}P]ATP) or the drug [^{3}H]azidopine in membrane preparations. P-gp is a phosphoprotein, and its phosphorylated state is thought to be important for function. When MDR cells were labeled with [^{32}P]orthophosphate *in vivo*, we observed that the monomer and dimer were more highly phosphorylated than the larger oligomers, suggesting that these different forms of P-gp may be functionally distinct. The assembly of oligomers appears to occur in an early biosynthetic compartment, and asparagine-linked glycosylation is not required for their formation. Our findings indicate that oligomers of P-gp exist in MDR cells and raise the possibility that the dynamics of oligomer formation and dissociation may be important in the mechanism of action of P-gp.

Malignant tumor cells which continue to proliferate when subjected to a variety of cytotoxic drugs are said to express multidrug resistance (MDR),[1] and this phenotype may be a major impediment limiting successful chemotherapy. The basis of this resistance appears to be the reduced net intracellular accumulation of anticancer drugs stemming from an energy-dependent increase in drug efflux (Gottesman & Pastan, 1993; Bradley *et al.*, 1988). A correlation has been made between the MDR phenotype and the presence of P-glycoprotein (P-gp) in tumor cells of diverse tissue origin (Gerlach *et al.*, 1987; Goldstein *et al.*, 1989; Dalton *et al.*, 1989; Chan *et al.*, 1990) and in cells where the gene sequence encoding P-gp has been exogenously introduced by transfection (Deuchars *et al.*, 1987; Gros *et al.*, 1986; Ueda *et al.*, 1987). P-gp also occurs in a variety of normal tissues (Fojo *et al.*, 1987; Thiebaut *et al.*, 1987), consistent with its postulated role for the transport of cell metabolites. Following its synthesis on membrane-bound polysomes P-gp is vectorially transported, acquiring modification(s) during transit (Greenberger *et al.*, 1987, 1988), and is targeted to the plasma membrane (Kartner *et al.*, 1985), a location compatible with its proposed role in transport. The analysis of P-gp cDNAs indicates that it is a highly conserved protein. It consists of a tandem duplication of six predicted hydrophobic membrane-spanning segments followed by a hydrophilic cytoplasmic domain containing a nucleotide-binding consensus site. This motif, comprising the juxtaposition of a multispanning hydrophobic membrane domain and a hydrophilic nucleotide-binding region, is homologous with bacterial proteins known to have transport function (Juranka *et al.*, 1989) and has therefore warranted its inclusion in the family of *ABC* (ATP binding cassette) transporters.

Efforts to elucidate the mechanism of action of P-gp as a drug transporter have demonstrated that transport is an energy-dependent process (Endicott & Ling, 1989) and that P-gp can bind photoactive analogs of ATP and drugs (Cornwell *et al.*, 1986; Georges *et al.*, 1991). In addition, P-gp is a phosphoprotein where transport function may be regulated by its phosphorylation state (Carlsen *et al.*, 1977; Hamada *et al.*, 1987). However, the functional unit of P-glycoprotein in the native membrane has not been established. It is not known if P-gp acts alone or requires the formation of a complex with other proteins to function as a plasma membrane drug efflux pump. Data concerning the binding of the P-gp antibody, MRK-16, to a discontinuous epitope (Georges *et al.*, 1993), results from experiments using radiation inactivation analysis of membranes from MDR cells (Boscoboinik *et al.*, 1990) and results from initial chemical cross-linking experiments (Naito & Tsuruo, 1992), are consistent with the occurrence of at least a dimer structure of P-gp. Such putative oligomers have not been isolated for characterization. The purpose of the present study was to determine if oligomers of P-gp can be isolated biochemically using detergent solubilization and if such complexes are functionally active.

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[1] Abbreviations: MDR, multidrug resistance; Fr. #, fraction number; P-gp, P-glycoprotein; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BFA, Brefeldin A; ER, endoplasmic reticulum; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DSP, dithiobis(succinimidyl propionate).

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MATERIALS AND METHODS

Reagents. Vincristine, colchicine, bovine serum albumin, hexokinase, alcohol dehydrogenase, immunoglobulin G, and catalase were purchased from Sigma Chemical Co. 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and tunicamycin were purchased from Calbiochem. Nonidet P40 (NP40), Triton X-100 (TX100), and sucrose of AnalaR grade were purchased from BDH Laboratories. SDS was of ultrapure molecular biology grade, and was obtained from International Biotechnologies Inc. The cross-linker dithiobis(succinimidyl propionate) (DSP) was obtained from Pierce. Tran^{35}S label (a mixture of [^{35}S]methionine and [^{35}S]-cysteine) and 8-azido[α-^{32}P]ATP were purchased from ICN Biochemicals, Inc. [^{35}S]Methionine, [^{3}H]azidopine, and Amplify were purchased from Amersham. All other chemicals were purchased from Fisher Scientific and were of reagent grade.

Cell Lines and Tissue Culture Conditions. AuxB1 is a drug-sensitive Chinese hamster ovary (CHO) cell line. CH^{R}-C5 and CH^{R}B30 are drug-resistant cell lines derived from AuxB1 cells by selection with colchicine (Kartner *et al.*, 1985). The cell lines were grown as monolayers on 100-mm dishes (Nunc) in α-MEM supplemented with 10% fetal calf serum. SKOV3, a drug-sensitive human ovarian carcinoma cell line (Bradley *et al.*, 1989), and the drug-resistant derivative selected with vincristine, SKVCR2.0, were grown as monolayers in α-MEM supplemented with 15% fetal calf serum. Drug-resistant cell lines were maintained in medium containing the drug used for selection (i.e., CH^{R}B30 in 30 μg of colchicine/mL and SKVCR2.0 in 2 μg of vincristine/mL). Cells were kept at 37 °C in a humidified atmosphere containing 5% CO_2.

Metabolic Radiolabeling of P-glycoprotein. For pulse/chase labeling followed by immunoprecipitation, cell monolayers, which were approximately 80% confluent in 100-mm dishes, were incubated for 15 min in α-MEM lacking methionine and serum, followed by incubation for 10 min in the same medium with 100–250 μCi/mL [^{35}S]methionine, and then chased for 0, 5, 20, and 45 min or for 18 h in complete medium containing serum and unlabeled methionine. For pulse/chase labeling followed by velocity sedimentation analysis, cells were labeled for 20 min and either harvested immediately or chased overnight in complete medium as described. For metabolic labeling, cells underwent the same preincubation in methionine and serum-free medium, prior to labeling with 100–250 μCi/mL [^{35}S]methionine for 3–8 h. Cells treated with tunicamycin were incubated in complete medium containing a final concentration of 4 μg/mL tunicamycin for 4 h prior to a brief preincubation in methionine- and serum-free medium. They were then pulse-labeled with [^{35}S]methionine and chased as described above, except that the media contained tunicamycin.

Antibodies and Immunoprecipitation. After the cell monolayers were radiolabeled, the labeling medium was removed, and the cells were rinsed twice in phosphate-buffered saline (PBS) at 4 °C. Cells were scraped with a rubber policeman into 1 mL of PBS and were pelleted for 2 min in an Eppendorf centrifuge ($\sim$14000g). The cell pellet was resuspended in PBS, and an equal volume of a solution containing 2% NP40 and 1% sodium deoxycholate (DOC) in PBS was added to solubilize the cells. A cocktail of protease inhibitors (Sigma Chemical Co.) was added to yield aprotinin (200 units/mL), soybean trypsin inhibitor (100 μg/mL), benzamidine (1 mM), ϵ-aminocaproic acid (5 mM), and PMSF (2 mM). The cells were then vortexed vigorously, nuclear debris and unbroken cells were pelleted by Eppendorf centrifugation, and P-glycoprotein was immunoprecipitated from the supernatant by addition of 1–3 μg of one of the monoclonal antibodies C219 or C494 (Kartner *et al.*, 1985; Georges *et al.*, 1991), followed by agitation for several hours on a nutator (Adams) at 4 °C. Each antibody has been shown to recognize a specific highly conserved epitope in P-glycoprotein (Georges *et al.*, 1991). P-gp/antibody complexes were precipitated using protein A–Sepharose CL4B (Prot-A)-(Pharmacia) at a concentration of >2 mg dry weight per sample, for several hours or overnight, and then washed twice in a solution of 1% NP40, 0.5% DOC, 0.1% SDS, 0.5 M NaCl, and 50 mM Tris, pH 7.5 (Poruchynsky & Atkinson, 1988), followed by one wash in PBS. For immunoprecipitation of P-gp from fractions of the sucrose gradients, either the entire volume or half-volumes of each fraction were diluted with an equal volume of a solution containing 2% NP40 and 1% DOC in PBS. P-gp was immunoprecipitated by adding 1–3 μg of either C219 of C494 monoclonal antibody to each solubilized fraction, and samples were processed as described above. Immobilized immune complexes were solubilized with 1% SDS/50 mM Tris, pH 6.7, and diluted with 3× gel sample preparation buffer, which contained the reducing agent β-mercaptoethanol.

For cells which were radiolabeled, solubilized with detergent, and then analyzed by velocity sedimentation, the labeling medium was removed, and the cells were rinsed once with PBS and then with 90 mM KCl/50 mM Hepes buffer, pH 7.5 (KCl/Hepes). Cells were scraped from the dishes into KCl/Hepes, pelleted, resuspended in 500 μL of the same buffer, and then solubilized with either SDS, TX100, NP40, or CHAPS, added from stock solutions, to a final concentration of either 1% or 0.1%. The samples were vortexed vigorously and the insoluble and nuclear debris pelleted by centrifugation for 5 min in the Eppendorf centrifuge.

Chemical Cross-Linking. Radiolabeled cells to be treated with the homobifunctional, membrane-permeable, thiol-cleavable cross-linking reagent DSP (Lomant & Fairbanks, 1976) were harvested and resuspended in the KCl/Hepes buffer as described above. This was followed by the addition of DSP from a stock solution of DSP in dimethyl sulfoxide to a final concentration of 1–2 mg/mL (Poruchynsky *et al.*, 1991). Samples were placed on a nutator for 45 min at 22 °C, and then glycine was added to a final concentration of 2 mM to bind unreacted groups. SDS was added to a final concentration of 1%, and the insoluble cellular debris was pelleted by Eppendorf centrifugation for 3 min.

Sucrose Gradient Velocity Sedimentation. Detergent-solubilized postnuclear supernatants of radiolabeled cells from a single 100-mm dish, with or without cross-linker treatment, were layered atop 11-mL continuous 8–36% w/w sucrose gradients, made up in 20 mM MOPS, 100 mM NaCl, and 30 mM Tris, pH 7.5 (MNT), containing 0.1% of either CHAPS, TX100, NP40, or SDS. In order to standardize the conditions of centrifugation and assure consistency from one experimental run to another, the gradients were centrifuged at either 4 or 18 °C in an SW41 rotor such that $\omega^2 t$ was equal to 1.10×10^{12} rad^2/s (i.e., 38 000 rpm for 19.25 h, or 39 000 rpm for 18.25 h). The ultraclear tubes were punctured from the bottom using an 18-gauge needle, and fractions of equal volume were collected. In earlier experiments, 12 fractions were collected, and in later experiments, up to 22 fractions were collected to achieve better resolution. Marker proteins were run on identical gradients, in order to characterize the sedimentation and to determine the location of proteins of

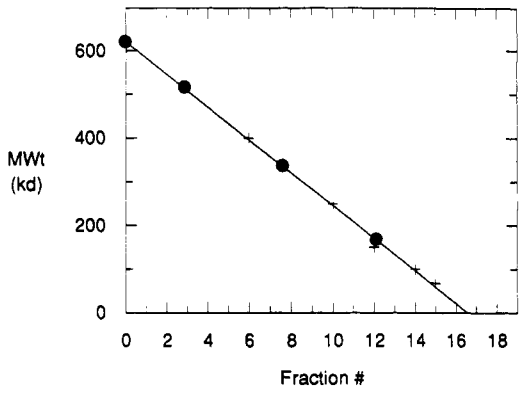

FIGURE 1: Velocity sedimentation of marker proteins of known molecular mass on 8–36% w/w continuous sucrose gradients. Tubes were centrifuged in an SW41 rotor with an $\omega^2 t = 1.10 \times 10^{12}$ rad^2/s. For an 11-mL gradient collected across 18 fractions, the positions of the peaks for sedimentation of chloroplast coupling factor 1 (400 kDa) (Fr. #6), catalase (250 kDa) (Fr. #10), alcohol dehydrogenase (150 kDa) (Fr. #12), hexokinase (100 kDa) (Fr. #14), and BSA (68 kDa) (Fr. #15) are each indicated (+). The predicted positions of P-gp monomer (170 kDa), dimer (340 kDa), trimer (510 kDa), and tetramer (780 kDa) are indicated (●). Fraction 0 corresponds to the pellet.

known molecular mass across the gradient under these conditions of centrifugation and collection. Figure 1 illustrates the peaks of sedimentation of each of the proteins for a gradient collected over 18 fractions: bovine serum albumin (68 kDa) (Fr. #15), hexokinase (100 kDa) (Fr. #14), yeast alcohol dehydrogenase or IgG (150 kDa) (Fr. #12), catalase (250 kDa) (Fr. #10), and chloroplast coupling factor 1 (CF1) (400 kDa) (a generous gift from Richard E. McCarty, Johns Hopkins University, Baltimore, MD) (Fr. #6). The predicted positions of P-gp monomer (170 kDa), dimer (340 kDa), trimer (510 kDa), and tetramer (780 kDa) were estimated on the basis of the positions of the marker proteins in the gradients (Figure 1).

Treatment of MDR Cells with Brefeldin A. CH^{R}B30 cells were incubated in complete medium containing Brefeldin A (BFA) (Epicentre Technologies, Cedarlane Laboratories, Hornby, Ontario) at a final concentration of 10 μg/mL for 20 min prior to a 10-min incubation in methionine- and serum-free labeling medium which also contained BFA. Cells were then labeled for 2 h in the same medium with [^{35}S]methionine at a final concentration of 100 μCi/mL. Either cells were harvested immediately after the labeling period or the medium was removed and replaced with complete medium with or without BFA for an overnight chase period. The control cells were treated identically except that no BFA was present during any part of the preincubation, labeling or chase periods. After being harvested, the cells were solubilized with CHAPS and treated as described above for analysis by velocity sedimentation and immunoprecipitation.

SDS–PAGE Analysis. SDS–PAGE was performed as described by Laemmli on 7.5% acrylamide gels and run at constant current. Gels were fixed in 50% methanol/7% acetic acid and then in 10% methanol/7% acetic acid prior to fluorography with Amplify reagent. Gels were dried and exposed to X-omat autoradiograph film (Eastman Kodak). Molecular masses were estimated using standard protein markers of 200, 110 or 92.5, 68, 46, 30, and 24 kDa (Amersham and Bethesda Research Laboratories), and are indicated in each figure.

Preparation of Total Cell Membranes. Six to eight 100-mm dishes of confluent monolayers of CH^{R}B30 cells were rinsed once with PBS and then in hypotonic lysis buffer consisting of 10 mM KCl, 1.5 mM $MgCl_2$, and 10 mM Tris, pH 7.4 (Georges *et al.*, 1991). Additional buffer was added to the cells, and the dishes were incubated on ice, for 20 min. The cells were scraped into a smaller volume of the same buffer and homogenized batchwise using 25 strokes of a glass Dounce homogenizer. Nuclei and unbroken cells were pelleted by centrifugation for 10 min at 2000 rpm. The supernatants were centrifuged in an SW55 rotor at 35 000 rpm for 45 min at 4 °C, to pellet total membranes. The membrane pellets were resuspended in 700–800 μL of 250 mM sucrose/10 mM Tris, pH 7.4, and stored frozen at −70 °C. Protein concentration was determined using the Markwell modification (Markwell *et al.*, 1978) of the Lowry method.

UV Cross-Linking of a Photoactive ATP Analog to P-gp. To each of three 40-μL aliquots of a CH^{R}B30 membrane preparation was added 2.5 μL of 8-azidoadenosine 5′-[α-^{32}P]-triphosphate, 5 μCi, 9.15 Ci/mmol. The aliquots were incubated in the dark, on ice, for 20 min. While on ice, the samples were exposed for 10 min to a UV light source of 254 nm, 12 J, using a Stratalinker UV apparatus (Stratagene, LaJolla, CA) (Georges *et al.*, 1991). The reaction mixes were pooled following cross-linking, and the membranes were pelleted at 4 °C in the Eppendorf centrifuge for 10 min. The supernatant was discarded and the membrane pellet solubilized in 450 μL of 90 mM KCl/50 mM Hepes, pH 7.5, with CHAPS added to a final concentration of 1%. The sample was vortexed vigorously and the particulate insoluble material pelleted by spinning 3 min in the Eppendorf centrifuge. The supernatant was layered on an 8–36% w/w sucrose gradient containing CHAPS and centrifuged under the conditions described above, and P-gp was immunoprecipitated from each fraction using C219 antibody. An equal aliquot of the immune complexes from each fraction was solubilized with 1% SDS/50 mM Tris, pH 6.7, and counted after the addition of ACSII scintillation fluid. The remainder of the sample was analyzed by SDS–PAGE.

UV Cross-Linking of [^{3}H]Azidopine to P-gp. Aliquots of [^{3}H]azidopine, 50 Ci/mmol, were dried, and CH^{R}B30 membrane preparations were added so that the concentration of label was 62.5 μCi/125 μg of protein in a total of 125 μL. Samples were incubated in the dark at room temperature, for 1 h. The total volume was divided between several tubes as 25-μL aliquots, and the membrane/azidopine mixtures were exposed to UV light as described above. The volume was then adjusted to 500 μL with 90 mM KCl, 50 mM Hepes, pH 7.5, and CHAPS added to a final concentration of 1%. After vigorous vortexing and pelleting of insoluble material, the supernatant was subjected to velocity sedimentation as described above. Fractions of the gradient were immunoprecipitated to detect P-gp, a portion of the precipitated material was counted, and the remainder was analyzed on SDS–PAGE as described. Total membrane samples were also prepared from CH^{R}B30 cells which had been previously labeled with [^{35}S]methionine and processed in the same way, with or without addition of [^{3}H]azidopine, so that the overall sedimentation pattern of [^{35}S]methionine-labeled P-gp could be compared with the sedimentation pattern of P-gp cross-linked to [^{3}H]azidopine.

The conditions for competition of azidopine binding with vinblastine were established using 10-μg membrane samples incubated with various concentrations of vinblastine ranging from 0.1 to 1000 μg/mL, for 20 min in the dark at room temperature, and these samples were then added to dried aliquots of [^{3}H]azidopine (5 μCi) and cross-linked with UV as described above. These samples were solubilized with 1%

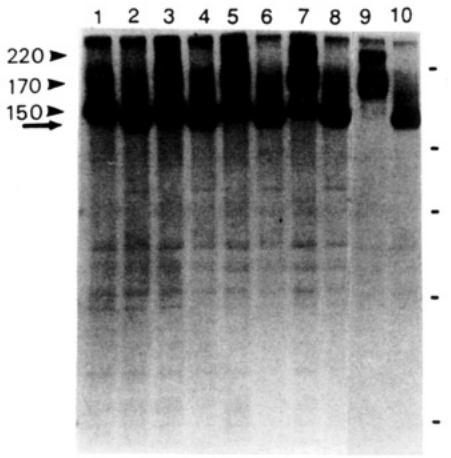

FIGURE 2: Pulse–chase labeling of P-glycoprotein in CH^{R}C5 cells. Cells, which were either treated (lanes 2, 4, 6, 8, 10) or not treated (lanes 1, 3, 5, 7, 9) with 4 μg/mL tunicamycin, were pulsed for 10 min with [^{35}S]methionine and chased in excess unlabeled methionine for 0, 5, 20, or 45 min or for 18 h, respectively. P-glycoprotein was immunoprecipitated from each sample using the monoclonal antibody C219, and separated by 7.5% SDS–PAGE. The positions of ~150-, 170-, and 220-kDa forms are indicated. The nonglycosylated ~140-kDa form of P-gp made in the presence of tunicamycin is indicated by the arrow.

NP40 and 0.5% DOC in PBS and immunoprecipitated using the antibody C219, and the amount of [^{3}H]azidopine bound to P-gp was determined following the addition of ACSII scintillation fluid. For the samples analyzed by sucrose velocity sedimenation and immunoprecipitation as described above, 125-μg membrane samples were incubated with 0, 100, or 1000 μg/mL vinblastine.

In Vivo Phosphorylation. Growth medium was removed from a T75 flask of semiconfluent CH^{R}B30 cells and rinsed with citrate saline before a 30-min incubation at 37 °C in phsphate-free medium, and the cells were then labeled in the same medium with 750 μCi of [^{32}P]orthophosphoric acid for 5 h at 37 °C. After the labeling medium was removed, the cells were rinsed in citrate saline, followed by 90 mM KCl/50 mM Hepes, pH 7.4, and harvested in the same buffer. The cells were pelleted and resuspended in 500 μL of this buffer, and CHAPS was added to a final concentration of 1%. The postnuclear supernatant was processed as described above and phosphorylated P-gp analyzed by velocity sedimentation, immunoprecipitation, and SDS–PAGE, as described. The bands on the autoradiograph were quantitated by scanning using a Computing Densitometer/Image Quant (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Detection of Radiolabeled P-glycoprotein. P-glycoprotein, under denaturing and reducing conditions, migrates as several high molecular mass bands of approximately 150, 170, and 220 kDa in SDS–PAGE using the Laemmli gel system. Newly synthesized P-gp is observed as a 150-kDa component and is processed over time to the 170- and 220-kDa forms. This is shown in Figure 2. When the colchicine-resistant Chinese hamster ovary CH^{R}C5 cells are pulse-labeled with [^{35}S]-methionine for 10 min, P-gp is observed as the early processed form at 150 kDa (Figure 2, lane 1). With increasing times of chase, this form decreases in intensity as the 170- and 220-kDa species increase in intensity (Figure 2, lanes 3, 5, 7, and 9). All three molecular mass forms of P-gp have been demonstrated by Western blot analysis when monoclonal antibody C219 specific for P-gp is used to probe the blot (data not shown). All forms apparently differ from each other only in their N-linked oligosaccharide moieties, since in the presence of tunicamycin, an inhibitor of asparagine-linked glycosylation, only a single form of P-gp migrating at 140 kDa is observed (Figure 2, arrow, lanes 2, 4, 6, 8, and 10). When [^{35}S]-methionine-pulsed cells are chased for 18 h in complete culture medium containing unlabeled methionine, only the 170- and 220-kDa forms are observed (Figure 2, lane 9). Under this long chase/labeling regimen or that of continuous labeling for several hours, these mature glycosylated forms of P-gp predominate and are likely to reside in the plasma membrane. Similar results were obtained for other multidrug resistant cell lines, for example, the colchicine-resistant CHO line CH^R-B30 and the human ovarian carcinoma cell line SKVCR2.0, which express high levels of P-gp (data not shown).

Velocity Sedimentation Analysis of P-glycoprotein Complexes. A number of detergents, both nonionic and zwitterionic, were used for the extraction of P-gp from the membranes of MDR cells to determine if P-gp can be isolated as oligomeric complexes. Cellular proteins in detergent lysates were examined by sucrose gradient velocity sedimentation analysis. Fractions from each gradient contained multiple proteins which presumably formed complexes that sedimented in the gradient according to their apparent sizes (Figure 3A–C; Fr. #1–12). The faster sedimenting, or larger, species appeared in fractions of lower number. A virtually identical distribution of radiolabeled total cell protein across the gradient was observed for each detergent (Figure 3A–C), indicating that the detergents used solubilize the cells to a similar extent and do not have different effects on the hydrodynamic properties of the bulk cellular proteins. However, when the distribution of P-gp was revealed by immunoprecipitation of each sucrose gradient fraction, a different sedimentation pattern was observed in cells solubilized with CHAPS when compared with those solubilized with TX100 or NP40 (Figure 3D–F). For TX100- and NP40-solubilized material, P-gp appeared mainly in fractions 8 and 9, sedimenting at a position corresponding to monomers (Figure 3D,E). There was a small amount of P-gp sedimenting lower in the gradient, corresponding to larger complexes. Extraction using the zwitterionic detergent CHAPS (Figure 3F) resulted in the retrieval of P-gp in fractions which corresponded to monomers (Fr. #8,9) and substantial amounts (>50% of total) of P-gp in fractions where dimers (Fr. #5,6), trimers (Fr. #2,3), and tetramers (Fr. #1) would be expected to sediment. Both the 170- and 220-kDa mature glycosylated forms of P-gp were found in all regions of the sucrose gradients. Thus, these components are observed as complexes in the sucrose gradient but are dissociated during the SDS–PAGE procedure.

Retrieval of P-gp Monomers in the Sucrose Gradient following Solubilization with SDS. The above results suggest that the efficiency of recovery of oligomers of P-gp is dependent on the detergent used. To determine if using the strongly denaturing detergent SDS would result in the complete retrieval of P-gp monomers, CH^{R}B30 cells were solubilized with SDS, and the material was separated on a gradient containing CHAPS. The monomeric form of P-gp was exclusively retrieved and peaked in tube 12 (Figure 4B). In a parallel control experiment, when cells were solubilized with 1% CHAPS, the sedimentation profile for P-gp revealed only a small amount of monomers in fractions 11 and 12, and the presence of significant amounts of oligomeric forms of P-gp in fractions 6 and 7 (dimers), 3 and 4 (trimers), and 1 (tetramers) (Figure 4A). Also notable is that the least processed form of P-gp (150 kDa), as well as the more mature

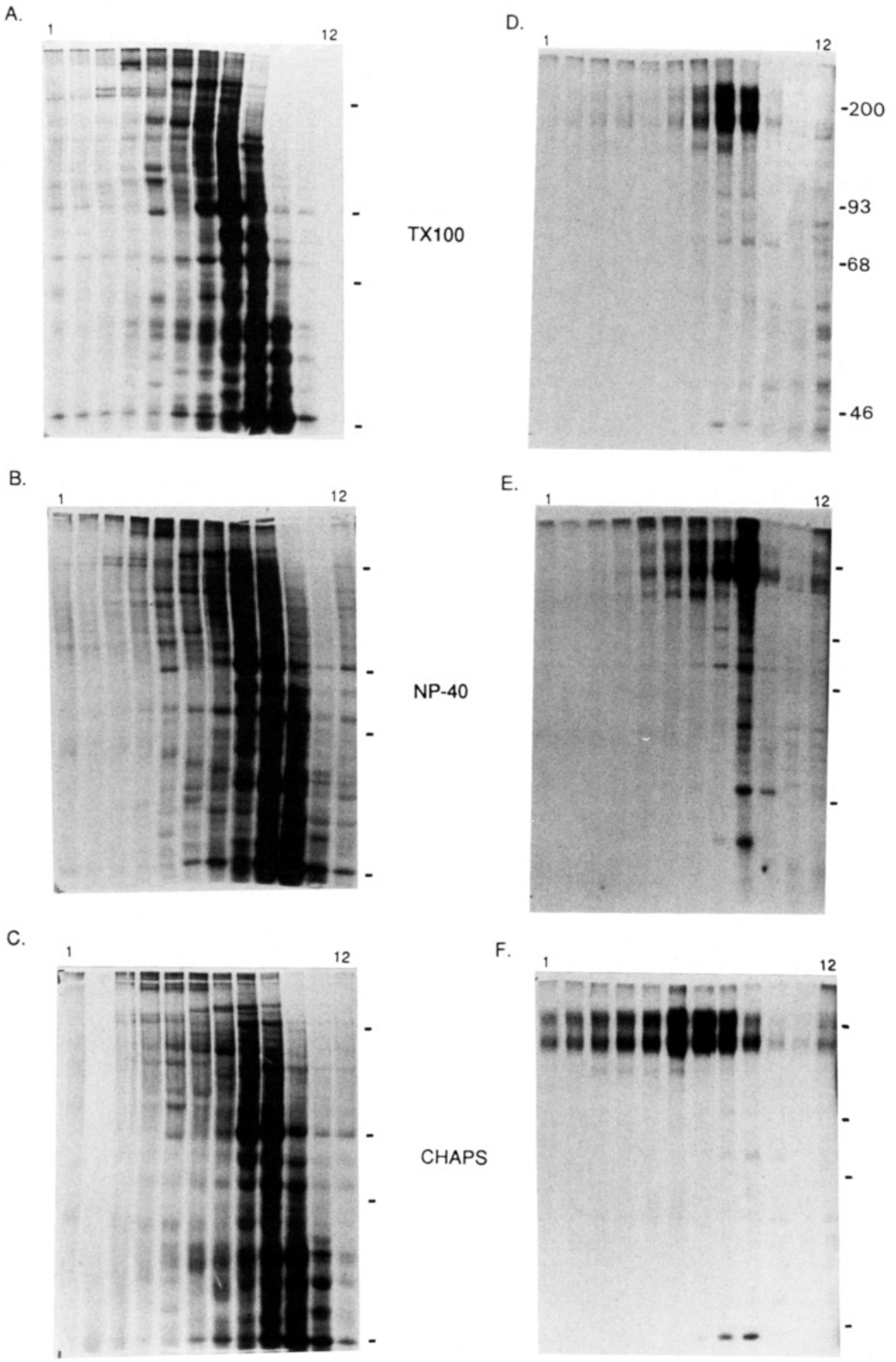

FIGURE 3: Sucrose gradient velocity sedimentation analysis of P-glycoprotein from CH^{R}C5 cells extracted with different detergents. Cells were labeled with [^{35}S]methionine for 4.5 h and solubilized with either 1% TX100, NP40, or CHAPS. Equal volume fractions were collected by puncturing the tubes from the bottom. Fraction 1 corresponds to the bottom of each gradient, and fraction 12 is the last fraction collected. Aliquots of each fraction derived from either TX100 (A), NP40 (B), or CHAPS (C) solubilized cells were analyzed by SDS–PAGE. Other aliquots from each gradient (panels D–F) were immunoprecipitated with monoclonal antibody C219 and were run alongside SDS–PAGE molecular mass markers of 200, 92.5, 68, and 46 kDa. The positions of monomer (Fr. #8,9), dimer (Fr. #5,6), trimer (Fr. #2,3), and tetramer (Fr. #1) on the sucrose gradient were determined from the sedimentation of proteins of known molecular mass (see Figure 1).

glycosylated forms (170 and 220 kDa), was able to form oligomers and was found in virtually all fractions of the gradient (Figure 4A).

Oligomers of P-gp can also be converted to monomers by treatment with SDS. Presumptive trimers and dimers of P-gp, isolated from CH^{R}B30 cells in the presence of CHAPS, could be dissociated into monomers when treated with the denaturing detergent SDS. This was shown when sucrose gradient fractions taken from the position in the gradient corresponding to the location of P-gp trimers or dimers were each pooled and

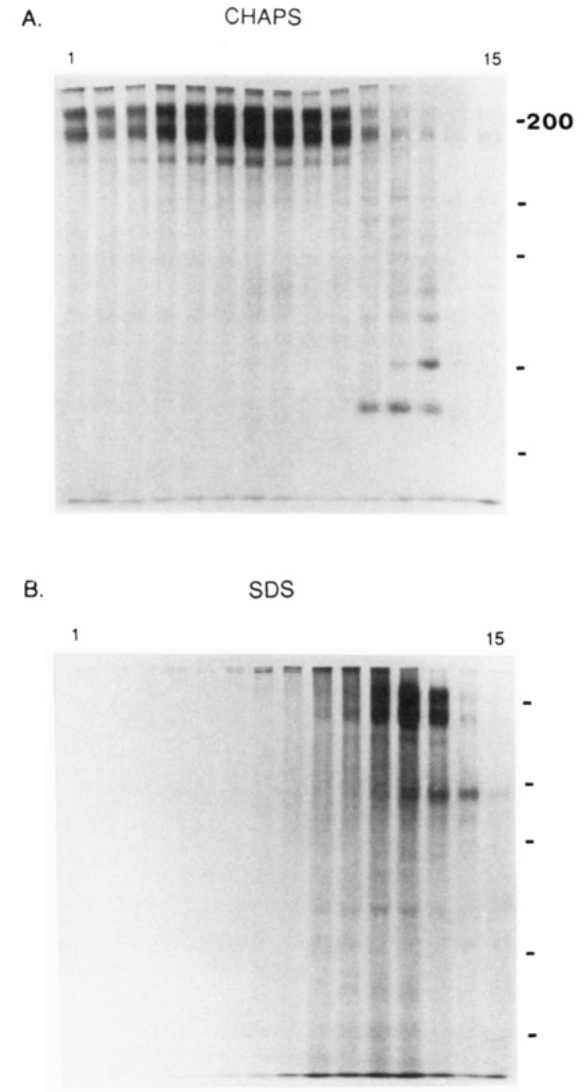

FIGURE 4: Velocity sedimentation analysis of P-glycoprotein from CH^{R}B30 cells extracted either with CHAPS or with SDS. Cells were labeled for 3 h with [^{35}S]methionine and solubilized with 1% CHAPS (A) or with 1% SDS (B). Each fraction was immunoprecipitated with C219 antibody. A total of 17 or 19 fractions were collected for each gradient, respectively. Monomers of P-gp sedimented in peak fractions 11 and 12, dimers in fraction 7, and trimers in fraction 3.

dialyzed against a solution containing SDS, as well as adding SDS prior to loading onto the sucrose gradient. Resedimentation on gradients containing SDS resulted only in the detection of monomers. In contrast, when the identical starting material was treated in the same manner except that CHAPS was substituted for SDS, P-gp was retrieved as oligomers in the fractions corresponding to the original position in the gradient (data not shown). Moreover, monomers formed when cells were solubilized in SDS did not artifactually associate into oligomers when separated on a gradient containing CHAPS (Figure 4B).

Taken together, all these findings suggest that large complexes of P-gp identified by sucrose gradient analysis result from the noncovalent interaction of monomers. Although the P-gp complexes fractionate in the sucrose gradient at positions corresponding to homooligomers, we cannot eliminate the possibility that other proteins may be associated with the P-gp complexes, accounting for the broadening of some peaks (Figure 4A). Such proteins might be lost from a complex during immunoprecipitation under denaturing conditions and would not be revealed in SDS–PAGE.

Stabilization of P-gp Oligomers in Cell Membranes by a Cross-Linking Reagent. To determine further if oligomers of P-gp identified by detergent extraction occur in the membranes of MDR cells, an independent method using a cross-linking reagent to preserve oligomers *in situ* was used. Homobifunctional, thiol-cleavable, membrane-permeable, cross-linking reagents, such as dithiobis(succinimidyl propionate) (DSP), are useful for stabilizing naturally occurring protein complexes (Lomant & Fairbanks, 1976; Doms, 1991; Poruchynsky *et al.*, 1991). In the presence of such a reagent, proteins in close proximity to one another have the potential to be chemically cross-linked to one another. If P-gp occurs in cell membranes as oligomers, it should be possible to cross-link such complexes, and they would remain in the oligomeric form even in the presence of a strong denaturing detergent such as SDS. Thus, CH^{R}B30 cells were treated with the thio-cleavable cross-linker DSP, prior to solubilization with SDS. Since no detergent was present during cross-linking, DSP presumably stabilizes native protein–protein interactions. Analysis of P-gp by velocity sedimentation, immunoprecipitation, and reduction of the thiol-cleavable cross-linker reveals the presence of some oligomeric forms of P-gp in fractions 7–10 where dimers would sediment, in addition to the monomeric form in fractions 12 and 13 (Figure 5B). Reduction of the sample allows the retrieval of the monomeric P-gp forms of 170 and 220 kDa, which make up the oligomer (Figure 5B). As a control, in cells solubilized with SDS with no prior treatment with cross-linker, P-gp sediments exclusively as monomers in fractions 12 and 13 (Figure 5A).

The above data are consistent with the interpretation that monomers and larger oligomers of P-gp occur naturally in the plasma membranes of multidrug resistant cells. Although the cross-linking reaction tends to be inefficient, oligomers of P-gp corresponding to at least the dimers were captured. We cannot determine if the oligomers captured using DSP are identical to those retrieved when cells are solubilized without pretreatment with a cross-linker using detergents such as NP40 and CHAPS (Figure 3E,F). The DSP cross-linking reagent has an arm length of 12 Å and results in the joining of molecules which are in close proximity to one another. Treatment with DSP did not result in the nonspecific association of many proteins, further confirming that complexes observed are likely composed of oligomers of P-gp.

Binding and Cross-Linking of Monomers and Oligomers to Azido-ATP. In order to determine if the monomeric and oligomeric forms of P-gp are both functionally active with respect to ATP binding, preparations of membranes were isolated from CH^{R}B30 cells and incubated with the photoactive ATP analog, [α-^{32}P]-8-azido-ATP. P-gp contains two nucleotide-binding consensus sites, and it is thought that the binding of ATP may be required for P-gp function. It has been shown previously, using membrane preparations from MDR cells, that both halves of the P-gp molecule can bind to a photoactive form of ATP, 8-azido-ATP (Georges *et al.*, 1991). Following exposure of the membrane/azido-ATP mixture to UV light, solubilization with CHAPS, and velocity sedimentation, it was observed that both the oligomeric and monomeric forms of P-gp bind ATP (Figure 6). The labeling corresponded to the various oligomeric forms of P-gp across the gradient, with apparent peaks in fractions 1,2 (tetramer); 4,5 (trimer); 7,8 (dimer); and 12 (monomer) (Figure 6). It was also observed that the 170- and 220-kDa forms and the highest mass form of P-gp at the very top of the resolving gel, all immunoprecipitable with antibody to P-gp, were each well-labeled with azido-ATP (Figure 6). The distribution across the gradient

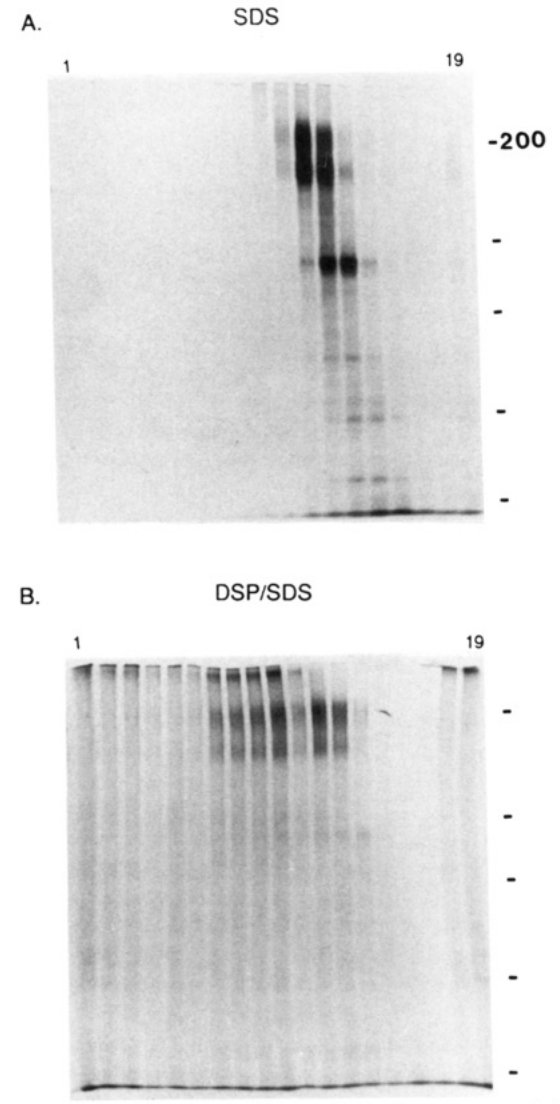

FIGURE 5: Velocity sedimentation analysis of P-glycoprotein from CH^{R}B30 cells either treated or not treated with the cross-linker DSP before extraction with SDS. CH^{R}B30 cells were pulse-labeled with [^{35}S]methionine for 20 min and then chased overnight with excess unlabeled methionine. Cells were solubilized in 1% SDS immediately after the chase period (A) or treated with the membrane-permeable cross-linker dithiobis(succinimidyl propionate) (DSP) (1–2 mg/mL) prior to solubilization with SDS (B). P-glycoprotein was immunoprecipitated from each sample using the antibody C219. Nineteen fractions were collected in each case. P-gp monomers peak in fraction 12 and dimers in fractions 8,9.

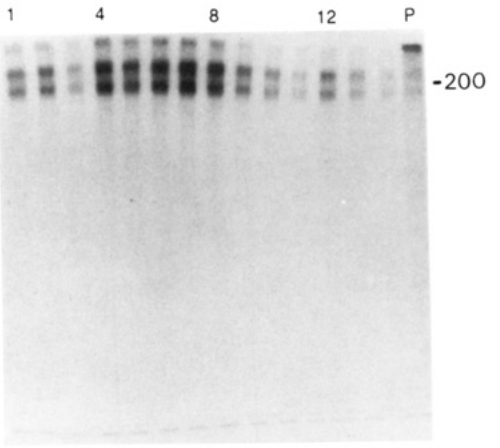

FIGURE 6: Velocity sedimentation analysis of CH^{R}B30 cell membranes incubated with [α-^{32}P]-8-azido-ATP. The membranes and photoactive ATP analog were exposed to 254-nm UV light for 10 min and then solubilized with CHAPS. Nineteen fractions were collected in total. The immunoprecipitation of labeled P-gp from the first 14 fractions is displayed. The monomer sedimentation peak is in Fr. #12, dimer in Fr. #7,8, trimer in Fr. #4,5, and tetramer at the bottom of the gradient. The (–) denotes the 200-kDa marker. (P) denotes immunoprecipitation of the pellet.

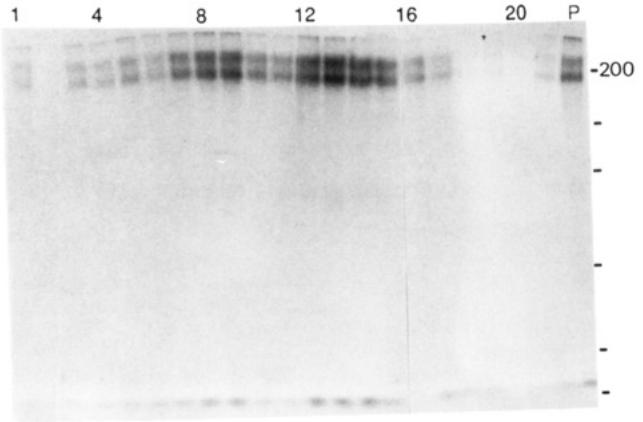

FIGURE 7: Velocity sedimentation of CH^{R}B30 cell membranes incubated with [^{3}H]azidopine. The membranes and photoactive substrate were exposed to 254-nm UV light for 10 min and then solubilized with CHAPS. The immunoprecipitation of P-gp from the 21 fractions collected is displayed alongside that immunoprecipitated from the pellet (P). Monomers appear in Fr. #15,16, dimers in Fr. #12–14, trimers in Fr. #7–9, and tetramers in Fr. #3–5. Fr. #2 was lost. Molecular mass markers are denoted in the margin, and (P) denotes immunoprecipitation of the pellet.

of the labeled ATP bound to P-gp is similar to the pattern seen when cells were labeled with [^{35}S]methionine and extracted with CHAPS. Thus, it appears that all forms of P-gp were able to bind ATP.

Azidopine Binding to Oligomers and Monomers of P-gp. Photoactive labeled azidopine is a compound which has frequently been used to investigate the binding of P-gp with a substrate in crude membrane preparations (Safa *et al.*, 1987; Yang *et al.*, 1988; Georges *et al.*, 1990; Bruggemann *et al.*, 1992; Zordan-Nudo *et al.*, 1993). As can be seen in Figure 7, [^{3}H]azidopine bound to both monomeric and oligomeric forms of P-gp although there was some variation between experiments in the relative amount of azidopine bound to oligomers as compared with monomers. Azidopine binding could be competed by vinblastine in a concentration-dependent manner in the cross-linking reaction mixture. Concentrations of vinblastine ranging from 0.1 to 1000 μg/mL were used to establish the region of the curve where competition is observed (data not shown). From this determination, the competition of [^{3}H]azidopine binding was examined using either 0, 100 or 1000 μg/mL vinblastine (10 or 100 mM molar excess vinblastine), followed by sucrose gradient velocity sedimentation analysis and immunoprecipitation. There was an overall decrease in azidopine binding for all forms of P-gp in the presence of vinblastine, and the inhibition was more pronounced for the higher concentration of the competitive substrate (Figure 8). These results indicate that both the monomeric and oligomeric forms of P-gp are functional with respect to *in vitro* drug binding.

In Vivo Phosphorylation of P-gp. It has been demonstrated previously that P-gp is a phosphoprotein and the phosphorylation state may be important for function (Carlsen *et al.*, 1977; Hamada *et al.*, 1987; Gottesman & Pastan, 1993). Therefore, it was of interest to determine if oligomers and monomers of P-gp exhibit a difference in their phosphorylation state. When CH^{R}B30 cells were labeled with [^{32}P]orthophosphoric acid *in vivo*, solublized with CHAPS, and then

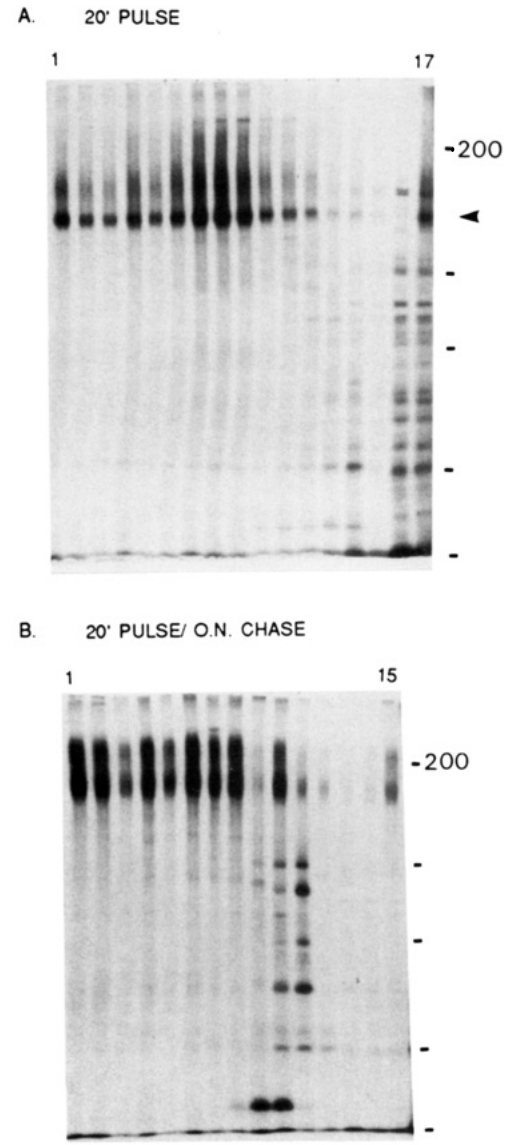

FIGURE 10: Velocity sedimentation analysis of P-glycoprotein from CH^{R}B30 cells pulse-labeled or pulsed and then chased before extraction with 1% CHAPS. Detergent lysates of cells which were pulse-labeled for 20 min with [^{35}S]methionine (A) or those which were pulsed for 20 min and then followed by an overnight chase in the presence of unlabeled methionine (B) were applied to sucrose gradients. A total of 17 or 15 fractions were collected, respectively, each was immunoprecipitated with the antibody to P-gp, C219, and the samples were analyzed on SDS–PAGE alongside molecular mass standards. The arrowhead indicates the position of an early processed form of P-gp at ~150 kDa. Monomers occur in Fr. #9–11, dimers in Fr. #6–8, trimers in Fr. #3–5, and tetramers in Fr. #1,2. The last fraction contains residual material which is released from the pellet during collection.

~140-kDa form of P-gp (data not shown).

These results suggest that oligomerization can occur early in the biosynthetic pathway of P-gp and that further processing and maturation of the P-gp molecule do not appear to play a critical role in the oligomerization process. Factors which may modulate the dynamics of monomers and oligomers have not been elucidated. For example, it is not known if the P-gp oligomers, once formed, dissociate readily into monomers, nor is it known if monomers associate to form oligomers in the plasma membrane.

DISCUSSION

The functional unit of P-glycoprotein in the plasma membrane of multidrug resistant cells and the factors mediating its maintenance and modulation are currently unknown. Previously, several lines of indirect evidence have suggested that P-gp occurs in the plasma membrane as dimers (Boscoboinik *et al.*, 1990; Naito & Tsuruo, 1992; Georges *et al.*, 1993) although such putative higher order complexes have not been isolated for characterization. There is presently no single method to estimate unequivocally the subunit association of membrane proteins. The corroboration of complementary lines of evidence for protein oligomerization, especially stability in velocity sedimentation and cross-linking experiments, has often been used to ascertain the quaternary structure of proteins (Doms, 1991). Taking such an approach, we determined in the present study whether or not oligomeric complexes of P-gp can be isolated from multidrug resistant cells using detergent extractions. By using the zwitterionic detergent CHAPS, we were able to identify presumptive P-gp oligomers and monomers by velocity sedimentation. Moreover, we could preserve putative native oligomers by treatment of cells with a chemical cross-linker prior to any detergent solubilization. The oligomers of P-gp were shown to be derived from a noncovalent association of monomers since they could be dissociated and retrieved on sucrose gradients following harsh treatment with the denaturing detergent SDS. The separation of oligomers by velocity sedimentation enabled us to discriminate a difference in the degree of phosphorylation between monomers and dimers of P-gp, as compared to higher order oligomers. We thus provide independent lines of evidence to show that P-gp oligomers do exist in the membranes of MDR cells and that such complexes can be isolated and characterized.

It may be significant that among the detergents used for extraction CHAPS yielded the highest percentage of P-gp oligomers. Although it is possible that the P-gp oligomers may be due to CHAPS-induced aggregation, it should be noted that CHAPS does not appear to induce aggregation of bulk cellular proteins (Figure 3) nor does it appear to aggregate P-gp monomers extracted with SDS. Moreover, recent reports (Georges *et al.*, 1991; Arceci *et al.*, 1993) using two different monoclonal antibodies, each having specificity against an external epitope of P-gp, have demonstrated that immunoprecipitation is best achieved using CHAPS detergent. While the antibody MRK16 inhibits P-gp transport function, the antibody 4E3 can bind to live cells, each result implying the recognition of a native P-gp structure preserved in the presence of CHAPS. This detergent has also been used for the purification of P-gp and demonstration of its *in vitro* ATPase activity (Hamada & Tsuruo, 1988). Recently, Zordan-Nudo *et al.*, (1993) showed that P-gp can be photolabeled with azidopine in intact cells in the presence of high concentrations of CHAPS even under conditions where P-gp is solubilized from the cell membranes. Taken together, all these findings are consistent with the hypothesis that CHAPS extraction results in the preservation of naturally occurring functional oligomers of P-gp. In other systems, CHAPS extraction of membrane proteins has been used successfully for the isolation of oligomers of the murine colony stimulating factor receptor with retention of its receptor activity (Fukunuaga *et al.*, 1990) and the purification of the microsomal signal peptidase complex (Evans *et al.*, 1986) and active prolactin (Liscia *et al.*, 1982), opiate (Simonds *et al.*, 1980), muscarinic (Kuno *et al.*, 1983), and adenosine receptors (Bitonti *et al.*, 1982).

For many plasma membrane-targeted proteins which function in either a transport, fusogenic, receptor, or other capacity, the functional activity is modulated via their incorporation into homooligomeric complexes (Amster-Choder & Wright, 1992; Jacobs & Moxham, 1991; Kuppuswamy &

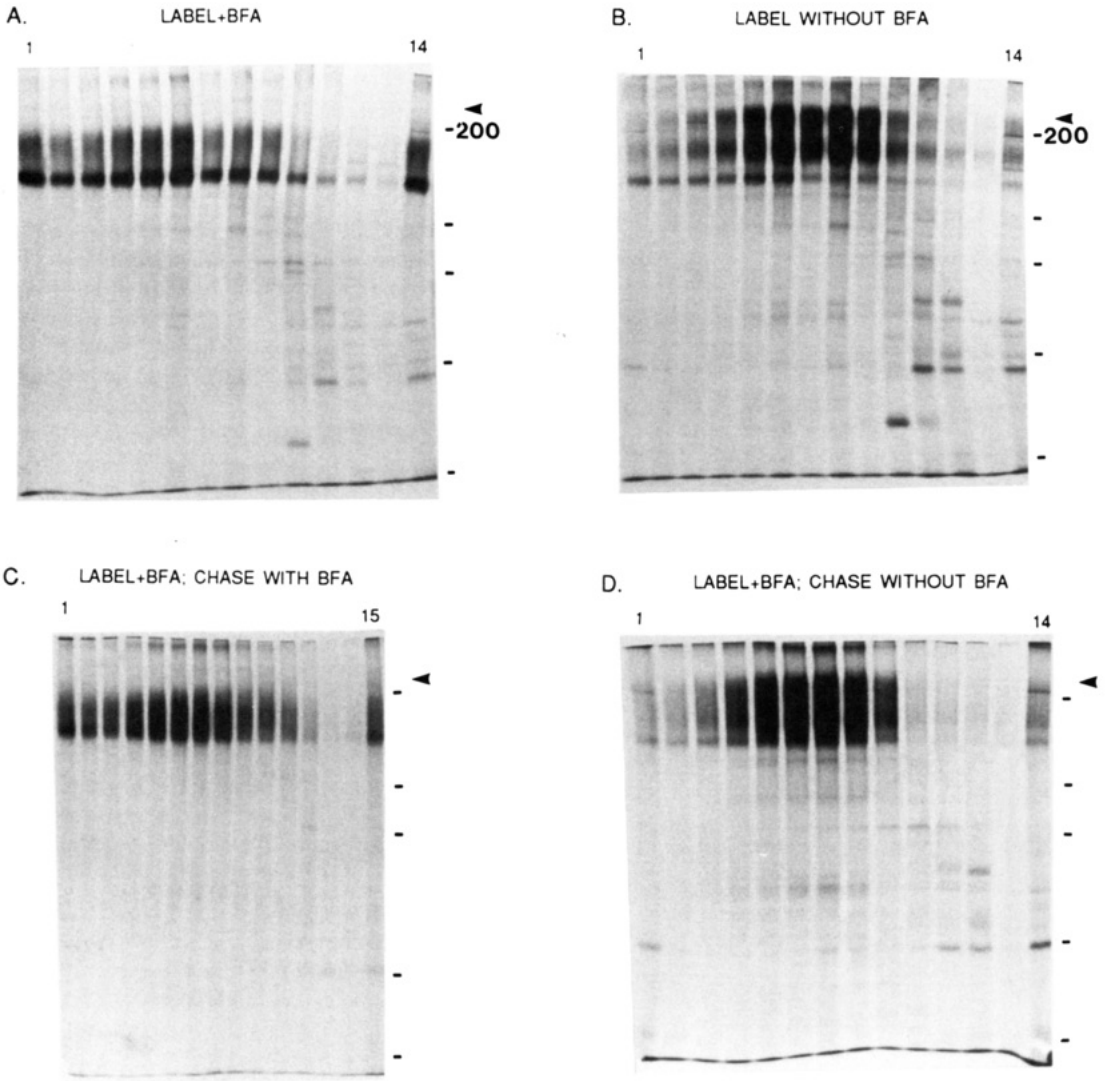

FIGURE 11: Velocity sedimentation analysis of CHAPS-solubilized CH^RB30 cells either treated or not treated with BFA. (A) Cells treated with 10 μg/mL BFA for 30 min prior to and during labeling with [^{35}S]methionine for 2 h. (B) Control cells not treated with any BFA prior to or during the 2-h label. (C) Cells treated with BFA and labeled as in (A) except that they were chased overnight in medium containing BFA as well as unlabeled methionine. (D) Cells treated with BFA as in (A) but chased overnight in medium without BFA containing unlabeled methionine (D). The arrowhead indicates the position of the 220-kDa form of P-gp.

Pike, 1991; Donaldson *et al.*, 1991; Doms, 1991) or into heterooligomers (Cosson & Bonifacino, 1992; Oka *et al.*, 1990; Stow *et al.*, 1991; Jahangeer & Rodbell, 1993). Complex formation can be mediated or accompanined by a modification which precedes or occurs only on the assembled unit. Processing events may modify the protein in such a way as to result in a conformational change in the complex, the transduction of a signal, or the movement of molecules across a membrane. Thus, it was of interest to determine if monomers and oligomers of P-gp have measurable functional differences such as a differential capacity to become phosphorylated or to bind ATP or a substrate, azidopine. Our observation that the oligomeric and also the monomeric forms of P-gp have the ability to bind to a photoactive analog of ATP illustrates that all species of P-gp are functional to the extent that they are able to bind nucleotide triphosphate. This binding may potentially mediate the action of P-gp to function as an energy-dependent drug efflux pump. In an investigation of the drug-binding capacity of oligomers and monomers of P-gp, we observed that each form of P-gp was able to bind azidopine. The binding of azidopine to P-gp could be competed with vinblastine. Study of the binding properties of other P-gp substrates may be useful to determine if there are differences in substrate specificity among the different oligomeric forms of P-gp.

Since P-gp is a phosphoprotein, MDR cells were labeled *in vivo* with [^{32}P]orthophosphate to determine if the phosphorylation state of P-gp differed for the various forms of P-gp. P-gp characterized from cells labeled in this manner showed that the monomer and dimer of P-gp appeared to be phosphorylated to a greater degree than the higher order oligomers; however, we do not know if the phosphorylation state has an effect on the P-gp transport function. Dephosphorylation may accompany the formation of oligomers from monomers, and this modification may reflect a change in the conformation, structure, or transport function of P-gp. An example of this is found for the transcriptional antiterminator protein (Amster-Choder & Wright, 1992), whose dimeric dephosphorylated form is active and whose monomeric phosphorylated form is inactive. Also notable in the present study is that the 170-kDa form of P-gp was more extensively phosphorylated than the 220-kDa species. This may indicate that dephosphorylation occurs more readily on the more mature glycosylated species of P-gp.

Similar to a number of other proteins, the oligomerization of P-gp occurs in an early biosynthetic compartment, i.e., the

endoplasmic reticulum (Doms, 1991; Cosson & Bonifacino, 1992; Stow *et al.*, 1991; Lax *et al.*, 1990), as determined by analysis of P-gp synthesized during a pulse-label or when Brefeldin A is present prior to and during the labeling period. Under these two sets of conditions, the vast majority of P-gp being examined is likely to reside in the ER (Doms *et al.*, 1989, Pelham, 1991). The least processed species of P-gp detected were able to form oligomers. Thus, the modification or lack of addition of asparagine-linked oligosaccharides, which is known to have no effect on P-gp function (Endicott & Ling, 1989), also plays no role in the formation of oligomers. In fact, P-gp synthesized in cells treated with the glycosylation inhibitor tunicamycin was also able to oligomerize to the same extent as modified P-gp. The dynamics of P-gp monomer/oligomer formation are not understood. It is not known if an accessible pool of monomers exists, if the pool size can be regulated and monomers recruited given a particular stimulus, or if there is a monomer population which is degraded if not incorporated. The modulation of the population of P-gp at the plasma membrane by mechanisms such as endocytosis and transit to intracellular compartments for degradation, dissociation, or recycling to the plasma membrane has also not been examined.

In summary, the results of the present study are consistent with the concept that P-gp is present in the plasma membranes of MDR cells, not only as monomers but also as oligomeric complexes derived from the noncovalent interaction of monomers. From studies of MDR cells, P-gp is thought to function as an energy-dependent drug efflux pump, as a chloride channel, as an ATP channel, and as a peptide transporter (Gottesman & Pastan, 1993). Current efforts to purify P-glycoprotein to homogeneity (Shapiro & Ling, 1994; Gottesman & Pastan, 1993) should allow for future experiments to reconstitute pure P-gp into a lipid bilayer to determine if all the above properties are inherent to P-gp. In such experiments, it would be important to determine if functional differences can be demonstrated for the monomeric and oligomeric forms of P-gp. It is possible that such differences may be the basis for the different functions attributed to P-gp *in vivo*. It is possible also that different functional oligomeric forms of P-gp may be expressed differentially in normal tissues.

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