Functional and Structural Studies* 

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Plasmamembrane P-glycoprotein is known as an ATP-dependent drug efflux pump that confers multidrug resistance to tumor cells. None of the reported purification procedures worked properly for our P-glycoprotein-over-producing cell lines, i.e., murine lymphoid leukemia P388/ADR25, rat hepatoma AS30-D/COL10, and human lymphoblastic leukemia CEM/VLB3 cells. We have thus developed a general procedure for efficient purification of P-glycoprotein by combining solubilization with sodium dodecyl sulfate and chromatography on ceramic hydroxyapatite. This procedure was successful for the three cell lines and yielded 70% of the P-glycoprotein present in the starting plasma membranes with more than 99% purity. After exchanging sodium dodecyl sulfate into dodecyl maltoside and reconstitution into liposomes, purified P-glycoprotein exhibited a specific ATPase activity of about 200 nmol/min/mg, which was very similar to that obtained for P-glycoprotein solubilized and purified with 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid. This ATPase activity was sensitive to orthovanadate inhibition and stimulated by verapamil and other drugs. More importantly, drug transport properties of the reconstituted P-glycoprotein were comparable with those of P-glycoprotein embedded in plasma membranes. Since it is virtually devoid of lipids, this preparation is suitable for both functional and structural investigations.

Multidrug resistance (MDR) is a phenomenon encountered in cancer treatments in which the tumors become resistant to a variety of cytotoxic chemotherapeutic agents. The molecular basis for one major type of MDR is the overexpression of the P-glycoprotein (Pgp), a plasma membrane glycoprotein with molecular masses ranging from 130 to 170 kDa, depending on its glycosylation state in a specific drug-resistant cell line. There are two classes of genes in human (mdr1 and mdr2) and three in rodents (mdr1a (or mdr3), mdr1b (or mdr1), and mdr2), but only mdr1 genes encode Pgp that is involved in the MDR phenotype (1, 2). Pgp has been described as an ATP-dependent drug efflux pump that extrudes drugs out of cells and thus confers cell resistance by lowering intracellular drug concentration (1). However, the mechanism by which this phenomenon occurs remains unclear and is of particular interest because of its broad specificity for structurally dissimilar substrates. Two models have been proposed (3). The first one states that Pgp would be organized as a hydrophilic pore through which the drug is effluxed. In the second model, Pgp would act as a flipase to export the drug, which interacts first with the membrane lipid bilayer before it binds to Pgp. Pgp has also been supposed to function as a volume-regulated chloride channel (4). It is clear today that Pgp exhibits an ATPase activity, but little is known about how this activity is coupled to the drug transport process.

One of the most important factors limiting our understanding of Pgp function is the lack of structural information. A prerequisite for Pgp structural and functional studies is the availability of a large amount of highly purified and active Pgp. Although several efforts have been made to purify Pgp from multidrug-resistant cells (5–9), none of the described procedures has been designed for preparative scale purification, due to poor separation of contaminants from Pgp, to rather low ATPase activity of the resultant Pgp, or to low Pgp yield. Recently, by using dye-ligand chromatography, Urbatsch et al. (10) have purified active Pgp in the presence of lipids from an octylglucoside extract of membranes from multidrug-resistant Chinese hamster ovary (CHO) cells. However, this procedure has not been successful for the purification of Pgp from SF9 insect cells infected by baculovirus carrying the mdr1 gene, due either to the difference of membrane composition or to the alteration in posttranslational modification of recombinant Pgp (11). A combination of DEAE-cellulose anion exchange with immunoaffinity chromatography has also been used for the isolation of hamster Pgp from CHO cells after solubilization by Zwittergent 3–12 (12). However, this procedure requires the use of an expensive monoclonal antibody immunoaffinity column. During the completion of this study, Sharom et al. (13) reported the purification of Pgp from multidrug-resistant CHO cells by combining selective Chaps extraction and lentil lectin affinity chromatography. The resultant purified hamster Pgp displayed a high basal ATPase activity.

Nevertheless, all of these procedures that have been designed for the multidrug-resistant CHO cell line do not work properly on our Pgp-overproducing lines, i.e. P388 murine lymphoid leukemia (P388/ADR25), AS30-D rat hepatoma (AS30-D/COL10), and CEM human lymphoblastic leukemia (CEM/VLB5) cells. This is mainly due to the poor efficiency of Pgp solubilization obtained with the reported mild detergents (such as octylglucoside, Zwittergent 3–12, Chaps, etc.), which varies from cell line to cell line. Also, in order to preserve its...
ATPase activity, no attempt has been made to delipidate Pgp in the reported purification procedures. These preparations are thus difficult to use for structural studies and crystalization, which require a monodisperse detergent-protein complex. Therefore, the main purpose of the present study is to find a way for efficient solubilization of Pgp and to set up a reliable protocol for its purification that can be applied to every resistant cell line and that will yield a Pgp preparation suitable for structural and functional investigations.

Mild nonionic and some Zwittergent detergents are known to be able to preserve the biological activities of membrane proteins and thus are generally used for their solubilization and purification. However, the solubilization efficiency of these detergents is sometimes low, and it depends on membrane species and varies from protein to protein, although it can be increased by the addition of high concentrations of salt (14, 15). Furthermore, the ability of mild detergents to disrupt aggregates of membrane proteins is limited (16). On the contrary, SDS is a powerful solubilizing and dissociating detergent that is also very effective at preventing a protein of interest from aggregating and nonspecifically associating with other proteins into mixed micelles, especially during chromatographic purification steps. The drawback of using SDS is the loss of biological activity of solubilized proteins. However, successful reactivation of SDS-solubilized proteins has been repeatedly reported (17–21) even after SDS-PAGE (22). In fact, the main point for reactivation is the removal of SDS that can be achieved by exchanging it with a mild detergent (21).

Because of the poor efficiency of mild detergents, as stated above, we have chosen SDS for solubilization of Pgp from plasma membranes and for its subsequent purification. By comparison, a combination of Chaps with NaCl has also been evaluated for Pgp purification. To purify Pgp in the presence of detergent, we have used ceramic hydroxyapatite chromatography (HAP-HPLC), which has been successfully used for purification of both soluble and membrane proteins (15, 23–25). Hydroxyapatite often resolves components that other chromatographic media fail to separate and is especially very useful for separating protein-detergent complexes (15). By combining SDS solubilization and HAP-HPLC, we have designed a general protocol for effective purification of Pgp. We report here this protocol that has been successfully and reproducibly applied to purify Pgp from our three cell lines and yields large amounts of highly purified Pgp. After SDS exchange into decyl maltoside and reconstitution into liposomes, Pgp displayed a similar drug-sensitive ATPase activity to that obtained for Pgp solubilized and purified with Chaps. Moreover, the reconstituted SDS-purified Pgp exhibited drug transport properties comparable with that of Pgp embedded in plasma membranes prepared from the same multidrug-resistant cancer cells.

**Experimental Procedures**

**Materials**—Cell culture media (DMEM and RPMI 1640) were from BioWhittaker, and fetal calf serum was from Sigma. Adriamycin (ADR), 28876

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RESULTS

Pgp Overproduction by Different Drug-resistant Cell Lines

We have established several resistant cell lines (i.e. murine lymphoid leukemia P388/ADR25, rat hepatoma AS30-D/COL10, and human lymphoblastic leukemia CEM/VLB5), which were, respectively, 7,830, 103, and 18,990 times more resistant to the respective drug than their corresponding sensitive parental cell lines (these data will be reported elsewhere). Electrophoresis presented in Fig. 1A shows that plasma membranes of all the resistant cell lines contain a large amount of Pgp when compared with their parental cells. Pgp was revealed by Western blotting using anti-Pgp monoclonal antibody C219 (Fig. 1B), while no Pgp could be detected in sensitive lines. The comparison of the electrophoretic patterns among the resistant and their sensitive parental lines shows that Pgp was the only overproduced protein in plasma membranes (Fig. 1A). Pgp overproduction in each of these resistant lines represents an average of 30, 27, and 25% (w/w) of total detected membrane proteins for P388/ADR25, AS30-D/COL10, and CEM/VLB5, respectively, as estimated by laser densitometry of electrophoresis gels. The smears of high molecular weight revealed by anti-Pgp monoclonal antibody C219 are among the resistant and their sensitive parental lines shows.

Solubilization of Pgp from Plasma Membranes

Various detergents were evaluated for their efficiency to solubilize Pgp. Prior to detergent addition, peripheral membrane proteins and adsorbed soluble ones were removed as follows. 1 ml of thawed plasma membranes were diluted with 1 ml of 10 mM sodium phosphate buffer (pH 7.0) containing 1 mM NaCl and a protease inhibitor mixture (2 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, and 4 mM pepstatin A). After homogenization by brief sonication and incubation at 4 °C for 15 min, the membranes were ultracentrifuged (100,000 × g, 30 min, 4 °C). The pellet was homogenized by brief sonication in 1 ml of 10 mM sodium phosphate buffer (pH 7.0) containing 1 mM DTT and the selected detergent at the concentration indicated in Table I. Detergent concentration for maximal Pgp solubilization was determined as described in the legend of Table I. This table shows that nearly all of the mild detergents tested poorly solubilized Pgp from P388/ADR25 plasma membranes. However, in the presence of 1 mM NaCl, Pgp solubilization was 3–4 times more efficient depending on the detergent used. Among mild detergents, the most efficient Pgp solubilization was achieved with Chaps in the presence of 1 mM NaCl, but it never exceeded 60%. In contrast, the strong dissociating agent SDS could almost completely solubilize Pgp and did not require

| Detergent                  | Solubilization efficiency |
|----------------------------|----------------------------|
|                            | No NaCl | With NaCl (1x) |
| Octylglucoside (1.4%)      |          |               |
| Dodecyl maltoside (0.4%)   |          |               |
| Chaps (1%)                 |          |               |
| Zwittergent 3–12 (0.4%)    |          |               |
| Sodium deoxycholate (2%)   |          |               |
| SDS (2%)                   |          |               |

* Not soluble in the presence of 1 mM NaCl.
* Not determined.
Pgp Purification from Overproducing Cells

A number of chromatographic methods were tested for the purification of P388/ADR25 Pgp. Ion exchange on DEAE-cellulose used by others (7, 12) could not be applied for the purification of Pgp or SDS-solubilized Pgp because of the strong binding of these detergents to the column. Gel filtration could poorly separate Pgp from the contaminants due to the existence of mixed detergent-protein complexes. Among several affinity chromatography matrix screened, only Cibacron Blue F3-GA could be used to remove limited contaminant proteins from Chaps-solubilized preparation without binding Pgp. Tentative experiments using Bio-Gel hydroxyapatite revealed that Pgp could be separated from most of the membrane proteins in the presence of high concentrations of detergent, in particular SDS or Chaps in the presence of salts. However, the chromatographic resolution of Bio-Gel hydroxyapatite was poor. Further experiments with ceramic hydroxyapatite HPLC showed that efficient separation of Pgp from all other membrane proteins has been obtained. Thus, a completely new procedure for purification of Pgp has been set up and is described here.

Purification of SDS-solubilized Pgp—After removal of peripheral membrane proteins and adsorbed soluble ones, the membranes were dissolved with 2% SDS and ultracentrifuged for 30 min at 100,000 × g at 20 °C. The supernatant, i.e. Pgp crude extract, was diluted to 1% SDS with an equal volume of 10 mM phosphate buffer (pH 7.0) containing 1 mM DTT and then applied onto a prepacked HAP-HPLC column equilibrated with 50 mM phosphate buffer (pH 7.0), 1% SDS, and 1 mM DTT. After washing with the equilibration buffer, the chromatography was developed with a linear gradient of sodium phosphate in the same buffer. The buffers and the column were maintained at 28 °C to prevent SDS precipitation. A typical separation profile for P388/ADR25 Pgp is shown in Fig. 2A, and the electrophoretic analysis of eluted fractions is shown in Fig. 2B. Pgp interacts with hydroxyapatite more strongly than all other proteins and was eluted as a single peak at about 0.45 M phosphate. Since Pgp was the last protein eluted under the indicated phosphate gradient. The chromatography was performed at a flow rate of 1 ml/min and monitored continuously at both 280 (data not shown) and 220 nm. Pgp was eluted as a broad single peak at about 0.45 M phosphate. B, electrophoretic analysis of eluted fractions from HAP-HPLC in A. Samples were resolved on an 8% SDS-PAGE gel followed by silver staining. Lane 1, SDS-solubilized membrane protein crude extract, 8 μg; lanes 2–6, aliquot fractions collected at different elution times during the phosphate gradient; lane 2, 15.5–16 min (18 μl, 0.2 μg); lane 3, 17.2–17.7 min (18 μl, 1 μg); lane 4, 18.7–19.2 min (15 μl, 3 μg); lane 5, 22.5–23 min (18 μl, 2 μg); lane 6, 26–26.5 min (24 μl, 2 μg). Most of the compounds eluted between 10 and 15 min were not proteins. C, silver-stained 5–10% gradient SDS-PAGE of purified Pgp from three cell lines. Lanes 1–3, purified Pgp from P388/ADR25, AS30-D/COL10, and CEM/VLB5 cells, respectively (2 μg of protein for each lane). Note that silver-staining was overdeveloped to reveal as many proteins as possible. A larger number of bands were thus revealed in Fig. 2B, lane 1, when compared with lane 2 in Fig. 1A.

Western blotting of purified Pgp from the three resistant cell lines. SDS-PAGE was done as in C, and blots were revealed with anti-Pgp monoclonal antibody C219. Lanes 1–3, purified Pgp from P388/ADR25, AS30-D/COL10, and CEM/VLB5 cells, respectively (1 μg of protein for each lane). MW, molecular weight marker, 1 μg of protein for each band.

detected in P388/ADR25 and AS30-D/COL10. Failure of anti-Pgp monoclonal antibody C219 to detect any band lower than the monomeric Pgp band indicates the absence of any proteolytic Pgp fragment in the Pgp preparation. Fig. 2C also shows...
that, apart from monomeric Pgp and some Pgp oligomers, no other protein was detected by silver staining. Other SDS-PAGE experiments designed to show low molecular weight proteins (but that could not resolve Pgp oligomers) did not reveal any other bands except Pgp, as shown in Fig. 2B, lane 6 (data not shown for AS30-D/COL10 and CEM/VLB5 cell lines). Since 2 μg of Pgp were loaded for each lane of Fig. 2C and taking into account that less than 0.01 μg can be detected by silver staining, one can estimate that the purity of Pgp exceeds 99% of detected proteins. By comparison, the molecular mass of P388/ADR25 Pgp (140 kDa) is lower than those estimated for both AS30-D/COL10 and CEM/VLB5 Pgp (170 kDa), likely due to the lower glycosylation level in resistant P388 cells (36).

Table II summarizes the recovery of Pgp during the purification procedure described above for the three cell lines. The yield of purified plasma membranes (16/31% sucrose gradient interface fraction) from each of these cell lines is about the same, about 2.6 mg/10⁶ cells. The yield of purified Pgp from plasma membranes is very high for the three cell lines, about 70% of overall Pgp present in starting plasma membranes (i.e. 0.2 mg of Pgp/mg of membrane proteins). This results from the high efficiency of SDS solubilization and the excellent recovery of purified Pgp from HAP-HPLC (75% of solubilized Pgp).

**Purification of Chaps-solubilized Pgp**—The conditions of Pgp solubilization with Chaps were the same as with SDS, except that SDS was replaced by 1% Chaps and 1 M NaCl according to Table I. The subsequent HAP-HPLC was performed in the presence of 1% Chaps and 0.5 M NaCl instead of SDS in all buffers used. Highly purified Pgp was also obtained with only one-step HAP-HPLC. However, the yield of purified Pgp was 10 times lower (about 20 μg/mg of starting plasma membrane proteins) than that obtained with SDS. Apart from the relatively lower solubilization efficiency of Chaps, the important loss of Pgp during HAP-HPLC step is attributed to aggregation of Chaps-solubilized Pgp on the column matrix. However, contrary to the SDS-Pgp preparation, this Chaps-Pgp preparation does not need any exchange of detergent prior to reconstitution into liposomes in order to restore Pgp ATPase activity.

**Detergent Exchange and Pgp Concentration**

Since Pgp strongly interacts with hydroxyapatite, Pgp concentration and detergent exchange can easily be done with that, apart from monomeric Pgp and some Pgp oligomers, no other protein was detected by silver staining. Other SDS-PAGE experiments designed to show low molecular weight proteins (but that could not resolve Pgp oligomers) did not reveal any other bands except Pgp, as shown in Fig. 2B, lane 6 (data not shown for AS30-D/COL10 and CEM/VLB5 cell lines). Since 2 μg of Pgp were loaded for each lane of Fig. 2C and taking into account that less than 0.01 μg can be detected by silver staining, one can estimate that the purity of Pgp exceeds 99% of detected proteins. By comparison, the molecular mass of P388/ADR25 Pgp (140 kDa) is lower than those estimated for both AS30-D/COL10 and CEM/VLB5 Pgp (170 kDa), likely due to the lower glycosylation level in resistant P388 cells (36).

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Pgp from P388/ADR25, AS30-D/COL10, and CEM/VLB5 cell lines was purified in the presence of SDS and reconstituted into liposomes after exchanging SDS into dodecyl maltoside. Plasma membranes were prepared as described under “Experimental Procedures” from P388/ADR25, AS30-D/COL10, and CEM/VLB5 cell lines in which Pgp represents about 30, 27, and 25% (w/w) of total plasma membrane proteins, respectively, as estimated by laser densitometry of SDS-PAGE. The hydrolysis of ATP by Pgp was measured by NADH fluorimetric assay as detailed under “Experimental Procedures.” The specific ATPase activity of membrane Pgp was calculated by taking into account the Pgp percentage of total membrane proteins after subtraction of residual ATPase activity insensitive to NaO4, ouabain, and EGTA in the corresponding parental sensitive cell lines (P388, AS30-D, and CEM). Data are presented as the means of triplicate determinations (mean ± S.D.). Pgp-EYL, Pgp reconstituted in egg yolk lipids; Pgp-SBL, Pgp reconstituted in sheep brain lipids; Pgp-PC:PA, Pgp reconstituted in PC:PA (10:1, w/w) liposomes; PM, plasma membranes; ND, not determined.

| Cell line       | Specific ATPase activity | Sample | Basal | Vanadate (50 μM) |
|-----------------|--------------------------|--------|-------|------------------|
|                 |                          | nmol/min/mg Pgp |       |                  |
| P388/ADR25      | Pgp-EYL                  | 109 ± 6 | 21 ± 1 |                  |
|                 | Pgp-SBL                  | 171 ± 9 | 53 ± 3 |                  |
|                 | Pgp-PC:PA                | 158 ± 20 | ND     |                  |
|                 | PM                       | 1450    | 350    |                  |
| AS30-D/COL10    | Pgp-EYL                  | 121 ± 6 | 24 ± 1 |                  |
|                 | Pgp-SBL                  | 181 ± 9 | 56 ± 3 |                  |
|                 | Pgp-PC:PA                | 209 ± 22 | ND     |                  |
|                 | PM                       | 2430    | 580    |                  |
| CEM/VLB5        | Pgp-EYL                  | 132 ± 7 | 26 ± 1 |                  |
|                 | Pgp-SBL                  | 199 ± 10 | 61 ± 3 |                  |
|                 | Pgp-PC:PA                | 219 ± 11 | ND     |                  |
|                 | PM                       | 2450    | 580    |                  |

TABLE III

Comparison of the ATPase activities of reconstituted purified Pgp with Pgp embedded in plasma membranes from murine, rat, and human drug-resistant cell lines

Verapamil could stimulate the ATPase activity of reconstituted Pgp (either Pgp-egg yolk lipids or Pgp-sheep brain lipids) up to 2-fold, while colchicine and vinblastine were less effective, and both gave a 1.5-fold maximal stimulation. In the case of Pgp in plasma membranes, all of the drugs used were less effective than in the case of reconstituted Pgp. Furthermore, higher concentrations of verapamil (10 instead of 5 μM) and vinblastine (1.5 instead of 1 μM) were needed to give maximal stimulation. Taken together, all of the above data indicate that either SDS- or Chaps-purified Pgp, after reconstitution into liposomes, displayed a substantial ATPase activity that was lipid-dependent and could be modulated by a number of drugs.

TABLE IV

Stimulatory effects of selected drugs on the Pgp ATPase activity

Verapamil was the most effective activator among the drugs tested. Verapamil could stimulate the ATPase activity of reconstituted Pgp (either Pgp-egg yolk lipids or Pgp-sheep brain lipids) up to 2-fold, while colchicine and vinblastine were less effective, and both gave a 1.5-fold maximal stimulation. In the case of Pgp in plasma membranes, all of the drugs used were less effective than in the case of reconstituted Pgp. Furthermore, higher concentrations of verapamil (10 instead of 5 μM) and vinblastine (1.5 instead of 1 μM) were needed to give maximal stimulation.

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incubation time during 10 min and reached a steady state at 20 min. The [3H]vinblastine uptake slowly decreased after 20 min, suggesting that [3H]vinblastine diffused out of the proteoliposomes and that a continuous supply of ATP is needed for vinblastine transport (31). The maximal [3H]vinblastine uptake at 20 min was 111 ± 36 pmol/mg of Pgp. Fig. 4B shows that the [3H]vinblastine uptake was dependent on ATP concentration and that 1.5 mM was observed as the optimum ATP concentration. Higher concentrations of ATP inhibited drug transport, a phenomenon also observed for the ATPase activity of Pgp. It should be mentioned that the optimum ATP concentration for Pgp ATPase activity was also 1.5 mM, indicating the close coupling between ATP hydrolysis and ATP-dependent drug uptake.

These features of [3H]vinblastine uptake by reconstituted Pgp are very similar to those observed for plasma membranes (Fig. 5). However, the [3H]vinblastine uptake by CEM/VLB5 plasma membranes linearly increased with time up to 20 min, but the steady-state uptake was also obtained at 20 min as for reconstituted Pgp. Moreover, the maximum [3H]vinblastine uptake was also obtained for 1.5 mM ATP (23.4 ± 0.9 pmol/mg of protein per 20 min). Although the nature of the vesicles is quite different in terms of lipid and protein composition, the reconstituted Pgp transport activity (111 pmol/mg Pgp per 20 min) is very close to that obtained for plasma membrane Pgp (117 pmol/mg Pgp per 20 min) calculated when taking into account the percentage of Pgp in plasma membranes (about 20% of total proteins). Similarly, the [3H]vinblastine uptake by reconstituted purified P388/ADR25 and AS30-D/COL10 Pgp (119 ± 10 and 97 ± 25 pmol/mg Pgp per 20 min, respectively) are close to that calculated for both [3H]vinblastine uptake by P388/ADR25 and AS30-D/COL10 plasma membrane Pgp (13.5 ± 2.6 and 13.5 ± 1.0 pmol/mg protein per 20 min, respectively, i.e. 68 pmol/mg Pgp per 20 min for both). These results suggest that the ATP-dependent drug transportation catalyzed by reconstituted SDS-purified Pgp appears as efficient as that by Pgp embedded in plasma membranes.

**DISCUSSION**

We have established three highly multidrug-resistant cell lines, i.e. murine lymphoid leukemia P388/ADR25, rat hepatoma AS30-D/COL10, and human lymphoblastic leukemia CEM/VLB5 cells. These cells constitutively overproduce Pgp up to 25–30% by weight of total plasma membrane proteins. Unfortunately, the reported methods for purification of Pgp did not work properly with these cell lines. Thus, by combining SDS solubilization with ceramic hydroxyapatite HPLC, we developed a new, simple, reliable and high yielding procedure for efficient purification of Pgp that was effective for the three tumor lines. The ATPase activity of highly SDS-purified Pgp was restored after exchange of SDS into dodecyl maltoside and reconstitution into liposomes. This activity was close to that of Pgp purified with Chaps, indicating that SDS did not irreversibly denature the Pgp. Also, this reconstituted ATPase activity could be modulated by a number of drugs, especially those used for selection of cell resistance, and was shown to be more sensitive to drug stimulation than Pgp in plasma membranes. Furthermore, the reconstituted Pgp was shown to transport [3H]vinblastine with an efficiency comparable with plasma membrane-embedded Pgp. The drug transport features reported in this work constitute the first detailed demonstration of ATP concentration and time dependences for highly purified reconstituted Pgp.
Purification of membrane proteins by chromatographic methods unavoidably begins with solubilization, during which proteins are dispersed into solution from the anchoring lipid bilayer. Effective solubilization of membrane proteins is accompanied by the appropriate choice of detergent and buffer systems (16). Because SDS was able to solubilize Pgp almost completely (Table I), we used it to set up the Pgp purification procedure, although SDS is well known to inactivate proteins.

In order to compare the activity of purified Pgp after removal of SDS, we also purified Pgp solubilized with Chaps because (i) it was the most effective mild detergent in the presence of high concentrations of salt (Table I) and (ii) it was reported to allow the preparation of active Pgp (13, 43). The key point of the Pgp separation was the use of ceramic hydroxyapatite HPLC column, which allowed the complete purification of Pgp in only one step in the presence of 1% SDS or 1% Chaps after pH and phosphate gradient optimization. This one-step chromatographic purification was possible because Pgp bound strongly to hydroxyapatite and it was the last protein eluted from the column. The presence of DTT was required to prevent protein aggregation during HAP-HPLC. Although Chaps was able to solubilize up to 60% Pgp in the presence of 1 M NaCl, the recovery of purified Pgp from HAP-HPLC in the presence of Chaps was much lower (10%) than in the presence of SDS (75%). Obviously, this is due to the poor efficiency of Chaps in disrupting nonspecific protein association, resulting in Pgp aggregation once the Chaps-mixed micelles interact with HAP-HPLC matrix. Indeed, it has been shown by sucrose gradient velocity sedimentation that Chaps solubilized Pgp essentially as oligomers, while SDS yielded essentially monomers (44). Moreover, SDS is known to overcome the formation of aggregates during chromatographic separations (24). One main advantage for the use of SDS solubilization and HAP-HPLC of Pgp is its general applicability, whatever the cell line used, with only a minor adjustment of phosphate gradient to optimize the separation of Pgp on HAP-HPLC. The yield of SDS-purified Pgp is considerably high for all three cell lines (i.e., about 70% of overall Pgp in plasma membranes; in other words, 0.2 mg of purified Pgp/mg of plasma membrane proteins). This is the highest yield of purified Pgp ever reported. In contrast, the yield of Chaps-purified Pgp is 10 times lower. This discrepancy between Pgp yields justifies the development of the SDS purification procedure.

Apart from the fundamental value of hydroxyapatite for Pgp purification, another very interesting application is its use for detergent exchange (45) as described here for SDS exchange into dodecyl maltoside. SDS present in Pgp preparation was easily exchanged into a mild detergent by extensively washing the column with almost no loss of Pgp. In addition, hydroxyapatite can also be used to concentrate diluted Pgp solution without concentrating the detergent micelles, which is the major problem when using ultrafiltration. The detergent exchange procedure would be applicable to other membrane proteins, and other detergents such as Chaps, Zwittergent 3–12, octylglucoside, deoxycholate, octaethyleneglycol mono-octyl ether, etc. could also be efficiently exchanged by this method.2

Both SDS- and Chaps-purified Pgp were obtained with purity exceeding 99% of detected protein (Fig. 2). In addition to monomeric Pgp, some oligomerized Pgp was also detected, especially in the CEM/VLB5 cell line in comparison with the other two lines. Although it is a common tendency for membrane proteins to aggregate in the detergent-solubilized state, several reports have suggested that Pgp oligomers exist in plasma membranes and that Pgp functioning may be modulated by its state of oligomerization (44, 46). Furthermore, our previous work also indicated the tendency of the murine Pgp C-terminal nucleotide-binding domain to form oligomers (47), as confirmed recently (48). Besides this oligomerization state of membrane Pgp, we assume that the high level of overexpression reached would favor an aggregation state of the protein into the membrane, perhaps yielding the formation of Pgp clusters. This may explain the difficulties that we encountered in solubilizing Pgp.

Chaps-solubilized hamster Pgp was shown to retain partial or complete ATPase activity during the reported purification procedures (13, 43). However, in our case, Chaps-purified Pgp in the presence of salt, like SDS-purified Pgp, did not display any ATPase activity. This is likely a consequence of the phospholipid depletion in Pgp-detergent mixed micelles during solubilization and, probably more importantly, during the HAP-HPLC in the presence of a high concentration of detergent. Indeed, detergent delipidation was shown to inactivate Pgp ATPase activity, which could subsequently be restored by the addition of phospholipids (43, 49). Accordingly, restoration of Pgp ATPase activity and its drug sensitivity, as well as drug transport activity, were readily achieved in the present work by reconstitution into liposomes. Although SDS-purified Pgp needed the exchange of SDS into mild detergent prior to reconstitution, there was no significant difference in reconstituted activity between Chaps- and SDS-purified Pgp, showing that SDS does not irreversibly denature Pgp although some denaturation could not be completely ruled out. These results clearly demonstrate that SDS can be successfully used to purify Pgp without loss of Pgp function provided that SDS is efficiently removed. In fact, the detergent exchange procedure we report here completely fulfills this requirement.

The basal specific ATPase activity of lipid-depleted Chaps- and SDS-purified Pgp obtained after reconstitution (about 200 nmol/min/mg Pgp for each cell line) is in the range of that reported by Shapiro and Ling (12) for 90% pure Pgp, but it remains about 8 times lower than that for Pgp preparations for which no attempt was made to delipidate the protein (11, 13). This activity is also 8–12 times lower than that of plasma membrane-embedded Pgp, although it is dependent on the sources of lipids used (Table III). Pgp ATPase activity was indeed reported to be modulated by phospholipids (43, 49), some of which allowed a great stimulation of catalytic activity whereas others produced inhibition (13). Furthermore, Sharom and co-workers (13) reported that Pgp selectively associates with only certain phospholipid species, and their Pgp preparation was associated with an unidentified phospholipid. Specific lipids are possibly required for Pgp catalytic activity, and this unidentified species might play an important role. Several other factors may account for the observed different level of the ATPase activity between reconstituted and plasma membrane Pgp: (i) the real amount of Pgp functionally reconstituted into liposomes, (ii) the orientation of Pgp in the proteoliposomes (inside-out/right side-out Pgp distribution), (iii) the size of reconstituted vesicles, (iv) the oligomerization state of Pgp, (v) the protein:phospholipid ratio, (vi) the presence of traces of detergent in reconstituted membranes, (vii) the lack of possible modulation of Pgp activity by other plasma membrane proteins, etc. In addition, the enzymatic activity of membrane proteins is often difficult to estimate because it varies greatly with the conditions of preparation and measurement used. This is particularly obvious for the Pgp ATPase activity, which is activated by several compounds. For example, in the case of P388/ADR25, if the comparison between reconstituted and plasma membrane Pgp is made on the basis of the ATPase

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activity obtained under maximal stimulation by verapamil, and assuming that only 50% of reconstituted Pgp was right side-out, one can calculate that the reconstituted Pgp activity is only about 3-fold lower than that observed for plasma membrane Pgp.

Nevertheless, the reconstitution conditions used here allowed us to analyze the modulation of the restored ATPase activity by known Pgp inhibitors and activators. There was almost no difference in the inhibition by vanadate between plasma membrane-embedded and reconstituted Pgp. Moreover, the stimulatory effects of verapamil, colchicin, and vinblastine were higher on reconstituted Pgp than on plasma membrane-embedded Pgp, and in the latter case, the drug concentrations of verapamil and vinblastine needed for the maximal stimulation were higher (Table IV). The lower sensitivity of plasma membrane-embedded Pgp to activators might be explained by a constant stimulation due to the presence of residual drug used for the selection of cell resistance. Another reason might be the presence of plasma membrane ATPases that are neither inhibited by the inhibitor mixture used nor stimulated by the drugs (50).

The functional integrity of SDS-purified Pgp is well demonstrated by its great ability to direct drug transport after reconstitution into PC:PA liposomes as shown by the [3H]vinblastine uptake experiment (Fig. 4). The strength of the large differences in lipid and protein composition of vesicles and the difficulties of transport rate measurements, the [3H]vinblastine uptake catalyzed by reconstituted Pgp and by plasma membrane-embedded Pgp could only be compared in a qualitative point of view. Nevertheless, identical experimental measurement conditions gave a similar steady state of [3H]vinblastine uptake for both types of vesicles. This indicates that reconstituted Pgp behaves qualitatively like in vivo Pgp and is suitable to use in functional assays in order to address the questions remaining that concern the function of Pgp and its regulation. In particular, the fact that SDS-purified Pgp is free from phospholipid should allow its use in the analysis of specific lipids required for Pgp functioning.

The best preparations of Pgp reported were obtained by maintaining large amounts of phospholipids during purification (11, 12, 49). However, these preparations are difficult to use for structural analysis of Pgp due to the presence of phospholipids. On the contrary, the present SDS-purified Pgp is virtually devoid of lipids, and after exchanging ATP into a mild detergent, such a preparation is suitable for crystalllogenesis attempts and for structural investigations. This is illustrated by the very first Pgp circular dichroism analysis to be published elsewhere.5

In conclusion, the SDS-purified Pgp obtained here fulfills all the criteria that should be expected for a purified protein, i.e., high yield and purity, biological activity, and structural integrity. Moreover, our SDS preparation procedure can very likely be successfully applied on any other Pgp-producing cell line. The present work opens the way to structural investigations of Pgp that are indispensable to understanding the functional mechanism of drug transport.

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