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Large-scale purification of functional human P-glycoprotein (ABCB1)

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Abstract

Human P-glycoprotein (P-gp) is an ATP-binding cassette transporter that has been implicated in altering the pharmacokinetics of anticancer drugs in normal tissues and development of multidrug resistance in tumor cells via drug efflux. There is still no definitive explanation of the mechanism by which P-gp effluxes drugs. One of the challenges of large-scale purification of membrane transporters is the selection of a suitable detergent for its optimal extraction from cell membranes. In addition, further steps of purification can often lead to inactivation and aggregation, decreasing the yield of purified protein. Here we report the large-scale purification of human P-gp expressed in High-Five insect cells using recombinant baculovirus. The purification strategies we present yield homogeneous functionally active wild type P-gp and its E556Q/E1201Q mutant, which is defective in carrying out ATP hydrolysis. Three detergents (1,2-diheptanoyol-*sn*-glycero-3-phosphocholine, dodecyl maltoside and *n*-octyl- β -D-glucopyranoside) were used to solubilize and purify P-gp from insect cell membranes. P-gp purification was performed first using immobilized metal affinity chromatography, then followed by a second step of either anion exchange chromatography or size exclusion chromatography to yield protein in concentrations of 2 to 12 mg/mL. Size exclusion chromatography was the preferred method, as it allows separation of monomeric transporters from aggregates. We show that the purified protein, when reconstituted in proteoliposomes and nanodiscs, exhibits both basal and substrate or inhibitor-modulated ATPase activity. This report thus provides a convenient and robust method to obtain large amounts of active homogeneously purified human P-gp that is suitable for biochemical, biophysical and structural characterization.

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Author contributions

Formation of purification strategy: KN, SL, SS, SVA

Contributions to experimental concept and design: KN, SL, SS, SVA

Analysis and interpretation of data: KN, SL, SS, SVA

Writing and revision of the manuscript: SL, SS, KN, SVA

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Declarations of interest

The authors declare no conflicts of interest.

Graphical Abstract

Keywords

ABC transporter; ATP hydrolysis; multidrug resistance; P-glycoprotein; ABCB1; nanodiscs; reconstitution

1. Introduction

P-glycoprotein (P-gp), encoded by the *mdr1* or *ABCB1* gene, is a 140-170 kDa integral plasma membrane phospho-glycoprotein (also known as a permeability glycoprotein) that belongs to the ATP-binding cassette (ABC) superfamily of transporters. Exposure of certain tumors and cultured cell lines to anticancer drugs and xenobiotics results in overexpression of this transporter, resulting in the phenomenon of multidrug resistance (MDR) [1, 2]. Human P-gp effluxes a range of hydrophobic or amphipathic anticancer drugs such as vinblastine, doxorubicin, paclitaxel, colchicine and actinomycin-D from cells against a concentration gradient. Human P-gp is a single polypeptide comprising two symmetric halves, each including a transmembrane domain (TMD) with six transmembrane α -helices and a nucleotide-binding domain (NBD) with hydrophilic regions [2–5]. The cytoplasmic NBDs bind and hydrolyze ATP, which is essential for drug transport. Various studies have demonstrated that the drug-stimulated ATPase activity and drug transport by this transporter are linked [6, 7]. Yet there is still no comprehensive understanding of the mechanism of drug efflux by P-gp. We and other researchers have extensively characterized the catalytic cycle of ATP hydrolysis and the molecular basis of drug-transporter interactions [8–12]. Mutation of the glutamate residues to glutamine in the NBDs (E556Q/E1201Q) results in a P-gp mutant that can bind ATP but has lost the ability to hydrolyze it efficiently. It has been shown that this mutant can be used to trap P-gp in an ATP-bound pre-hydrolysis conformation, with dimerization of NBDs [13, 14]. The EQ mutant has been used in several studies for biochemical and structural characterization of the ATP hydrolysis cycle of ABC transporters [16], and recently for MD simulations [17].

In order to investigate these basic questions, large quantities of pure and functionally active human P-gp or its EQ mutant, which has played a central role in elucidating the mechanism of ATP hydrolysis, are needed to determine protein structure by X-ray crystallography or by cryo-electron microscopy (Cryo-EM). Preparing a large amount of homogeneously purified functional human P-gp reconstituted in proteoliposomes or nanodiscs is a challenge [18]. Therefore, we have developed two highly reproducible methods to obtain large quantities of pure human P-gp and catalytically inactive EQ mutant P-gp. The purification strategies presented here allow purification of relatively large amounts of monomeric functional P-gp at high concentrations, which is desirable for structural and functional studies [18–21]. We

show that the WT protein upon its reconstitution in a lipid membrane environment exhibits robust basal ATPase activity, which is modulated by substrates such as verapamil and inhibitors including tariquidar.

2. Materials and methods

2.1 Chemicals

TALON Metal Affinity Resin was purchased from Takara formerly Clontech (Mountain View, CA), Ni-Nitrilotriacetic acid (Ni-NTA) agarose was purchased from Qiagen (Germantown, MD). DE-52 (Whatman anion exchange cellulose) was obtained from Sigma-Aldrich (St. Louis, MO). Amicon Ultracel -100K centrifugal devices with regenerated cellulose with a molecular weight cut-off (MWCO) of 100,000 Daltons were purchased from Millipore (Bedford, MA). *n*-Dodecyl- β -D-Maltoside (DDM) and cholesteryl hemisuccinate (CHS) were obtained from Anatrace (Maumee, OH). *n*-Octyl- β -D-Glucopyranoside (OG), and cyclosporine-A (CsA) were obtained from Calbiochem (Biosciences, Inc., La Jolla, CA). DHPC (1, 2-Diheptanoyl-*sn*-Glycero-3-Phosphocholine), Cholesterol, L- α -Phosphatidylcholine (Egg), L- α -Phosphatidylserine (Brain, 90%), *E. coli* polar lipid extract were purchased from Avanti Polar Lipids, Inc (Alabaster, AL). Bio-Beads SM-2 Resin, size exclusion column protein standards and empty glass columns were purchased from Bio-Rad (Hercules, CA). Dialysis bags (Float-A-Lyzer, MWCO 100,000 Daltons) were from Spectrum Laboratories, Inc. (Rancho Dominguez, CA); dialysis cassettes (Slide-A-Lyzer, MWCO 10,000) were from Thermo Fisher Scientific (Waltham, MA). The P-gp-specific monoclonal antibody C219 was obtained from Fujirebio Diagnostics Inc. (Malvern, PA). Gel filtration chromatography standards were obtained from Bio-Rad (Hercules, CA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO), Research Products International Corp. (Mt. Prospect, IL), or Thermo Fisher Scientific (Waltham, MA).

2.2 Preparation of the lipid mixture

The lipid mixture for proteoliposomes was prepared by combining *E. coli* bulk phospholipids, phosphatidylcholine, phosphatidylserine, and cholesterol dissolved in chloroform in a ratio of 60:17.5:10:12.5 by percent weight. The *E. coli* polar lipid mixture was used for the preparation of nanodiscs. Chloroform was evaporated to dryness under N₂ gas, and lipids were washed extensively with ethyl ether (anhydrous) and evaporated to dryness under N₂ gas. The lipid mixture was resuspended in ethyl ether containing 2 mM β -ME and dried as described above. The lipid mixture was lyophilized overnight to remove traces of ethyl ether and stored at -70 °C under N₂ gas until resuspended at 50 mg/mL in a 2 mM β -ME solution [22].

2.3 Total membrane vesicle preparation

Human wild type (WT) P-gp and its double mutant E556Q/E1201Q (EQ mutant P-gp) were expressed as previously described with minor changes [23, 24]. Briefly, High-Five insect cells (Thermo Fisher Scientific, Waltham, MA) were infected in 3 L Fernbatch flasks with recombinant baculovirus carrying either the human MDR1 cDNA with a 6-histidine tag at the C-terminal end [BV-MDR1(H₆)], WT, or the human double mutant MDR1-E556Q/

E1201Q with a 6-histidine tag at the C-terminal end [BV-MDR1-EQ(H₆)], both at a multiplicity of infection of 10. A typical batch of 4 L (at total viable cells 1-2×10⁹ cells/L) of cells, was harvested at 54-60 h after post infection, washed once with PBS containing 1% aprotinin and cell pellets were stored at -70 °C. To start the total membrane vesicle preparation, the cell pellets were thawed and resuspended in cold hypotonic homogenization buffer (50 mM Tris HCl pH 7.5, 50 mM mannitol, 2 mM EGTA, 2 mM DTT, 0.5 mM AEBSF and 0.5 % aprotinin) and then centrifuged at 3500 rpm (2,000 × g) for 10 min. The turbid supernatant was discarded, and the pellets were resuspended in the same buffer (90 mL/pellet) followed by incubation for 30-45 min on ice to allow swelling of the cells. Then each resuspended pellet was homogenized (35 to 40 strokes with each pestle) in a Dounce homogenizer and subjected to low speed centrifugation at 2000 × g for 10 min to remove unbroken cells. The supernatant was then centrifuged for 1 h at 150,000 × g (Beckman rotor T45). After high-speed centrifugation, the membrane pellets were resuspended in resuspension buffer (50 mM Tris-HCl pH 8.0, 300 mM mannitol, 1 mM EGTA, 2 mM DTT, 2.5 mM AEBSF and 1% aprotinin), after which the ultracentrifugation step was repeated. The final pellets were resuspended in 50-60 mL of resuspension buffer containing 10% glycerol (v/v) and stored at -80°C until further use. A small aliquot was saved for characterization of the total membranes including total protein quantification, gel electrophoresis to determine the level of expression of WT and EQ mutant P-gp (colloidal blue stain or silver stain), and ATPase activity. A typical cell pellet from a 4 L culture yielded a preparation with 650-900 mg total membrane protein.

2.4 Solubilization of membrane vesicles with detergent

WT or EQ mutant P-gp expressing membranes were solubilized with buffers containing one of three detergents: 1,2-diheptanoyol-sn-glycero-3-phosphocholine (DHPC), dodecyl maltoside (DDM) or *n*-octyl-β-D-glucopyranoside (OG) (Table 1). Briefly, membrane vesicles (350-750 mg total protein) were mixed with freshly prepared solubilization buffer (the concentration of detergent and other solubilization buffer components are given in Table 1) to a 4.5-5.5 mg/mL final protein concentration. The solution was gently stirred for 20-30 min at 4 °C. The solubilized protein was then separated from insolubilized membranes by centrifugation for 1 h at 150,000 × g. The solubilized protein extracts were quickly frozen in dry ice and stored at -80 °C until further use.

2.5 Purification of human P-gp

Two different stepwise purification methods of WT P-gp or EQ mutant P-gp were standardized to generate a bulk quantity of biologically active pure protein (flow chart in Figure 1). All the purification experiments were carried out at 4 °C unless otherwise mentioned. Briefly, solubilized protein extracts were subjected to metal affinity chromatography on TALON resin or Ni-NTA resin followed by DE-52 anion exchange chromatography or Superdex 200 size exclusion chromatography.

Purification Method 1.—First, TALON resin was pre-equilibrated extensively (25-30 column volumes) with immobilized metal affinity chromatography (IMAC) buffer containing DHPC, an OG/lipid mixture or DDM (Table 1). The solubilized protein extract of WT P-gp or EQ mutant P-gp was then mixed with pre-washed TALON resin (1 mL of resin

per 150-180 mg of total membrane protein) and stirred for 1 h. Resin containing bound P-gp was centrifuged at $3000 \times g$ for 3 min; supernatant containing unbound protein was discarded and the resin was resuspended in the corresponding IMAC binding buffer (Table 1) to be transferred to a polypropylene gravity-flow column and washed with 10 column volumes of IMAC binding buffer. P-gp was eluted from the resin with 5-6 column volumes of the corresponding IMAC elution column buffer. Fractions containing P-gp in 200 mM imidazole were concentrated to 3-4 mL, which contained 12-15 mg of protein. A 5-to 10-mL Float-A-Lyzer (100 kDa) was loaded with protein sample and floated in a 1 L beaker with 600-700 mL buffer with gentle stirring; the buffer was replaced with fresh buffer after 30 minutes. The protein solution was carefully withdrawn from the Float-A-Lyzer after 1 h. The dialyzed protein solution was subjected to DE-52 column chromatography. The purpose of this step was to bind non-specific proteins to the DE-52, whereas WT P-gp and EQ mutant P-gp flow through the column. The DE-52 resin was washed several times with sterile water and then was loaded onto a column. The binding capacity of the DE-52 resin used was always 10 times higher than the total protein binding capacity. The DE-52 column was equilibrated with at least 10 volumes of appropriate buffer without NaCl (Table 1). The protein solution was carefully layered on top of the column without distorting the surface of the bed. The protein solution was allowed to flow through the column at about 15-20 mL/h flow rate. The flow-through fraction (2-3 column volumes) of WT P-gp or EQ mutant P-gp was collected in a tube containing 150 mM NaCl. This fraction, containing pure WT or EQ mutant P-gp protein, was supplemented with 150 mM NaCl, concentrated using an Amicon concentration device (MWCO 100 kDa), aliquoted and stored at -80°C .

Purification Method 2.—In this method, an Ni-NTA resin was used instead of a TALON resin. (Both resins are capable of binding to the hexa-histidine tag at the C-terminus of the protein.) First, the Ni-NTA resin was prewashed (1 mL of 50% resin slurry for 150 mg total solubilized protein) with the corresponding IMAC binding buffer (Table 1). In this case, we used either a buffer containing DDM alone or DDM and CHS. Next, we combined the resin with the solubilized protein extract and incubated the mixture overnight at 4°C with gentle stirring. After 16-18 h incubation, the suspension was centrifuged for 5 min at $5000 \times g$ and the supernatant with unbound protein was saved separately at -70°C . Resin with bound protein was resuspended in the corresponding IMAC binding buffer (Table 1), and transferred to a polypropylene gravity-flow column (Bio Rad 25 mL capacity), where it was washed with 5-6 column volumes of IMAC binding buffer. Next, P-gp was eluted from the column using the 300 mM imidazole containing IMAC elution buffer (Table 1). Typically, 4-7 mg of total protein was eluted at 0.4-1 mg/mL concentration. For size exclusion chromatography, first the solution containing WT P-gp or EQ mutant P-gp protein was concentrated to 400-500 μL (10-15 mg/mL) using an Amicon concentration device (MWCO 100 kDa) and centrifuged at $160,000 \times g$ for 30 min using a Sorvall WX Ultra series centrifuge with a Fiberlite F37L-8 $\times$ 100 rotor (Thermo Fisher Scientific, Waltham, MA). This ultracentrifugation step is necessary in order to remove multimers of P-gp and aggregates of detergent micelles. The concentrated protein fraction was loaded onto a Superdex 200 10/300 GL column preequilibrated with the gel filtration buffer using an ÄKTA Pure chromatography system (GE Healthcare Life Sciences, Chicago, IL), kept at 4°C following the manufacturer's recommended limits for flow rate and column pressure.

Fractions containing monomeric WT or EQ mutant P-gp were then combined and concentrated using an Amicon concentration device (MWCO 100 kDa) to 0.75-1.5 mL (1-3 mg/mL). DTT was added to produce a 10 mM final concentration. The concentrated fraction was aliquoted into small volumes, frozen quickly in dry ice and stored at -80°C .

2.6 Reconstitution of P-gp in proteoliposomes

Human P-gp was reconstituted into proteoliposomes (PLs) by the dialysis exchange method, as previously described, with a few modifications [22, 25]. The P-gp protein samples from different purification stages (detergent extract, eluate of TALON metal affinity chromatography and flow through from ion exchange chromatography), were combined with a freshly sonicated lipid mixture at a ratio of 1:50 (protein: lipid w/w) and incubated on ice for 20 min. These reconstituted mixtures were extensively dialyzed against detergent-free buffer (50 mM Tris-HCl, pH 7.5, 2 mM DTT and 0.1% aprotinin) (16-18 h, 3-4 buffer changes at 4°C) using dialysis cassettes. P-gp-containing PLs of each fraction were collected by centrifugation at 75,000 rpm ($200,000 \times g$) in a Sorvall RC-M120EX for 35 min. PLs were resuspended in ATPase buffer (see composition below) and ATPase activity was determined immediately. The PLs stored at 4°C retain 80-90 % ATPase activity for at least 10 days.

2.7 Reconstitution of P-gp in nanodiscs

Human P-gp was reconstituted into nanodiscs (NDs) as previously described [26–28]. First, a 5 mM lipid solution was prepared containing 3.3 mM DDM, 30 mM sodium cholate with or without 1.25 mM CHS. Next, P-gp purified in DDM micelles (or DDM/CHS micelles) was combined with MSP1D1 protein, *E. coli* lipid in a 1:4:200 ratio. The mixture was incubated in the presence of pre-washed bio-beads (0.8 mg/mL) with continuous shaking for at least 3 h at 4°C . The ND solution was collected with a 22-gauge needle and injected into the Superdex 200 10/300 GL column pre-incubated with a buffer containing 40 mM Hepes pH 7.5, 150 mM NaCl and 5 mM DTT. Fractions containing P-gp NDs were combined, concentrated using an Amicon concentration device (MWCO 100,000 kDa) to 50-100 μL and stored at 4°C . The P-gp in NDs stored at 4°C retained >50% ATPase activity for at least 10 days.

2.8 ATPase assays

The ATPase activity of P-gp in membrane vesicles, detergent micelles and in various reconstituted fractions during purification was measured by an end point phosphate release assay, as described previously [22, 23, 27, 29, 30]. The P-gp-specific activity was determined as vanadate (Vi)-sensitive ATPase activity. Briefly, the assay in a final volume of 0.1 mL of ATPase buffer (50 mM MES pH 6.8, 50 mM KCl, 5 mM Sodium azide, 1 mM EGTA, 1 mM Ouabain, 2 mM DTT, 10 mM MgCl_2), was carried out under basal conditions with DMSO solvent alone or in the presence of 30-50 μM verapamil or 10 μM tariquidar at 37°C . The reaction was initiated by adding 5 mM ATP and it was terminated after 20 min by addition of SDS at 2.5% final concentration. The amount of inorganic phosphate (Pi) released was quantified by a colorimetric method, as described previously [23, 31].

2.9 Gel electrophoresis

Proteins in membranes, solubilized extract, purified fractions, proteoliposomes and NDs were quantified by the Sherman and Weismann method using Amido Black B dye [32]. Denaturing SDS gel electrophoresis was conducted using a precast 7% Tris-Acetate gel (NuPage) at constant voltage according to the manufacturer's recommendations. Gels were directly stained with Instant Blue or silver staining.

2.10 Gel filtration of purified human P-gp in the presence or absence of cyclosporine A and ATP

A Superdex 200 column 10/300 GL (GE Healthcare Life Sciences, Chicago, IL) was thoroughly washed (4-5 column volumes) with DDM gel filtration buffer (Table 1) until a steady baseline was established. A gel filtration chromatography standard kit containing Thyroglobulin (MW 670 kDa), Bovine gamma globulin (MW 158 kDa), Chicken ovalbumin (MW 44 kDa), Equine myoglobin (MW 17 kDa) and Vitamin B-12 (MW 1.35 kDa) was dissolved in DDM gel filtration buffer (Table 1), as suggested by the supplier. Before running the P-gp sample, the standard sample was subjected to gel filtration under identical conditions and then the column was washed thoroughly. A sample of purified human WT P-gp (245-250 µg per 0.5 mL) was incubated with and without 0.075 mM CsA, 5 mM ATP and 5 mM MgCl₂ for 5 min on ice before loading onto the Superdex 200 column. The protein sample was eluted at the flow rate of 0.3 mL/min and at 0-1.5 mPa pressure limits. The peak fractions were then collected and analyzed.

3. Results

3.1 P-gp expression in High-Five insect cells

Structural characterization of membrane proteins requires large amounts of purified functional protein. Here, we describe a comprehensive protocol with two different purification approaches of wild type P-glycoprotein (WT P-gp) and its double mutant MDR1-E556Q/E1201Q (EQ mutant P-gp). A detailed description of the protocols is presented in Figure 1. We used a baculovirus High-Five insect cell expression system for overexpression of WT and EQ mutant P-gp as previously described [23], with some modifications. For maximum expression of functional P-gp, High-Five cells were infected with baculovirus carrying either the WT or EQ mutant MDR1 gene at a multiplicity of infection (MOI) of 10. Cells were harvested between 54-58 h post infection at 60 % viability. The harvested cells were washed once with ice-cold PBS containing 1% aprotinin quickly frozen in dry ice and stored at -80 °C for at least 18-24 months without any loss of activity or structural integrity of P-gp.

3.2 Membrane isolation and solubilization of P-gp

The total membrane vesicles containing P-gp as well as other membrane proteins were isolated using hypotonic lysis. Swelling and homogenization steps allow isolation of not only the plasma membrane but also membranes of intracellular organelles. This is important to maximize the yield, since overexpression of P-gp may result in protein present in intracellular vesicles [33, 34]. Quantification of the total protein in the isolated membrane

vesicles using the Amido black B method [32] yielded about 0.5-1.0 g of total protein. To maximize the solubilization of P-gp from the membrane vesicles, we decided to compare three different detergents: DHPC, OG and DDM. A description of the solubilization buffer composition used for each detergent is given in Table 1. The concentration of DHPC, OG or DDM detergent used for membrane protein solubilization was significantly higher than the critical micelle concentration (CMC) for each detergent (1.25 % DHPC = $20 \times$ CMC, 1.5 % OG = $3 \times$ CMC and 2% DDM = $230 \times$ CMC). Interestingly, we found that irrespective of the choice of detergents, approximately 50 to 75% of total membrane proteins can be solubilized by this method.

3.3 Protein purification

Once total membrane protein was solubilized with detergent, the next step was the purification of P-gp. We optimized two purification methods, which both yield comparable levels of ATPase activity of purified protein. However, only the second method, using size exclusion chromatography, ensured that the purified P-gp was in a monomeric state. The first step in both approaches was to separate the proteins according to their affinity to specific metal ions by immobilized metal affinity chromatography (IMAC), using either a TALON or a Ni-NTA resin. The TALON resin, which is charged with cobalt, binds his-tagged protein with weaker affinity compared to Ni-NTA [35]. However, with High-Five insect cell membranes and the conditions described here, we observed that recovery of WT and EQ mutant P-gp with both resins was comparable. The IMAC purification of WT P-gp and/or EQ mutant P-gp was carried out in buffers containing either DHPC, OG, or DDM (Table 1). After the partial purification of P-gp from the total solubilized proteins using IMAC, a second step was necessary to ensure the maximum purity needed for further studies. We tested two different purification approaches for this step, either ion exchange chromatography or size exclusion chromatography. Before proceeding to the second step, those samples that were in DHPC or OG buffers were first dialyzed against DDM-containing buffer. DDM was the detergent of choice in this step, since buffer containing DDM at $5 \times$ CMC and 150-200 mM sodium chloride has been shown to facilitate the concentration of the protein at concentrations optimal for crystallographic studies, while minimizing the aggregation despite the presence of 15-20 % glycerol [36]. Hence, we ensured that after the ion exchange chromatography or the size exclusion chromatography, the protein was stored in such conditions. Table 2 depicts the variation in yield for both WT P-gp and EQ mutant P-gp purified using different detergents after ion exchange chromatography or size exclusion chromatography. Clearly, the recovery of WT P-gp or EQ mutant P-gp is influenced by the choice of detergents for the purification of these transporters. As shown in Table 2, using Method 1 (IMAC using TALON resin followed by ion exchange chromatography using a DE-52 ion exchanger), we found that WT P-gp or EQ mutant P-gp was effectively purified in the presence of either DHPC or DDM, with a recovery of about 1% from the total solubilized protein, although only 0.6% P-gp WT was recovered using OG in combination with a lipid mix (OG/Lipid). We observed that when using DHPC and DDM, the recovery of the EQ mutant P-gp was better when compared with the WT P-gp (about 50% more protein was recovered). Addition of NaCl to the fractions collected from the ion exchange chromatography was essential to prevent protein aggregation and precipitation as reported previously [36].

In addition to the ion exchange chromatography, we decided to test if size exclusion would produce protein at a quality comparable to or better than that purified by ion exchange chromatography. Another advantage of using size exclusion chromatography as a second purification step is that fractions containing aggregated protein can be separated from those containing the monomeric P-gp. As shown in Table 2, using this method, the recovery of WT P-gp as well as EQ mutant P-gp was less than what was obtained by Method 1, due to separation of monomeric P-gp from aggregated forms. Addition of CHS to the DDM buffer slightly improved the purification of WT P-gp when compared to using DDM alone. Using this method, we observed that the yield of EQ mutant P-gp was comparable to WT P-gp.

3.4 Characterization of purity and activity of P-gp

A typical denaturing gel profile of the different fractions collected during the purification of WT P-gp using Method 2 is presented in Figure 2 and Supplementary Figure 1. InstantBlue stained protein profiles show a homogeneous preparation, with a single band corresponding to P-gp. Western analysis (data not shown) using polyclonal antibodies raised against peptides derived from human P-gp suggested that the transporter is cleaved under the experimental conditions, as reflected in the very low intensity, fast moving—lower molecular weight—protein band. In order to compare the quality of the protein during the different steps of purification using Method 1, we first incorporated the protein into proteoliposomes (PLs). A detailed protocol for this reconstitution is presented in the Methods section. Proteoliposomes are useful because they stabilize the protein more effectively than detergent micelles [22, 30]. Figure 3A shows the SDS denaturing gel of the proteoliposomes during the purification steps using DHPC detergent according to Method 1. The flow-through fraction of TALON chromatography containing ~80-90% P-gp was eluted with DHPC detergent. P-gp was eluted with DDM detergent instead of DHPC before subjecting it to DE-52 column chromatography. It was observed that DHPC, DDM or OG detergents can be used at this step without compromising P-gp's ATPase activity.

Next, we used the PLs to compare the ability of P-gp to hydrolyze ATP under different detergent solubilization/purification conditions. We found that irrespective of the detergent used for solubilization and purification, 25-40% of the WT P-gp was recovered in the resuspended PLs after collection by ultracentrifugation. Similarly, the ATPase activity of the PLs was independent of the detergent used during purification. Figure 3B presents the average of two ATPase hydrolysis experiments for each of the reconstituted PLs from fractions obtained using DHPC-dependent soluble and purified fractions by Method 1. Clearly, PLs prepared from total membrane vesicles contain a number of other proteins. It is possible that such proteins could contribute to the observed ATPase activity. However, only the vanadate-sensitive activity (total activity minus activity in the presence of vanadate) is reported here as basal activity. Additionally, we know that stimulation of the ATPase activity by verapamil is related to P-gp activity, as other ATPases are not stimulated by verapamil. The verapamil-stimulated ATPase activity was approximately 4-fold greater than basal activity in the native membranes. The ATPase activity increased 1.8 to 3.6-fold during the process of purification of human WT P-gp present in the PLs (Figure 3B). The presence of osmolytes such as glycerol at higher concentrations during solubilization and purification preserves the specific activity in PLs [36].

The ATPase activity of P-gp in membrane vesicles, detergent micelles and nanodiscs was also compared using the protein purified by Method 2. As presented in Figure 4, the basal activity of P-gp in detergent micelles was observed to be generally lower than the activity in nanodiscs. Interestingly, addition of CHS increased the activity of P-gp in both detergent micelles and nanodiscs. Reconstitution of P-gp in nanodiscs increased the basal activity of P-gp. In addition, using P-gp purified in a DDM/CHS mixture resulted in nanodiscs with 10-fold greater activity when compared with those made with P-gp purified in only DDM. Consistent with our previously published data, we observed that tariquidar, a well-known P-gp inhibitor, stimulated the activity of P-gp in detergent micelles, while it inhibited the activity of P-gp in membrane vesicles [28]. This effect was observed whether or not CHS was used for purification and preparation of nanodiscs. As expected, the inhibition properties of tariquidar are recovered upon reconstituting P-gp in nanodiscs, as previously reported [28].

Cyclosporine A (CsA), a transport substrate of P-gp, has been shown to partially inhibit its ATP hydrolysis [30]. In addition, it has been shown that in the presence of CsA the binding of UIC2 antibody to the extracellular region of P-gp is enhanced [37]. We compared the effect of CsA and ATP on the separation of P-gp by size exclusion chromatography. Figure 5 shows that the relative mobility of P-gp in gel filtration on a Superdex 200 column was not affected by pretreatment with ATP and CsA. Interestingly, the presence of two peaks in the size exclusion chromatogram indicates that P-gp has separated into two parts, a slower fraction consistent with monomeric P-gp that corresponds to approximately 85% of the total protein, and a second faster moving peak (~15%) that contains an aggregate form of P-gp. Collection of the fraction with the monomeric species of the transporter and re-injection into the same column yields a profile identical to that of the original injection, indicating an equilibrium between the monomeric species and multimeric species, which is a minor component.

4. Discussion

Although large-scale purification of homogeneous and functional membrane proteins is challenging, it is essential for biochemical, biophysical and structural characterization. This is particularly true for human P-gp, as its atomic structure has only been recently solved [14, 38]. We present here a highly reproducible large-scale purification method to generate large quantities of human WT P-gp protein and its double EQ mutant from High-Five insect cells. P-gp from humans, mice, and *C. elegans* had been purified by earlier methods with yields ranging from 0.006 mg to 0.7 mg protein [39–43]. Reports of large-scale purification of human P-gp are still rare. In one report, mouse P-gp (product of the *mdr1a* gene) was purified using *Pichia pastoris* as an expression system, yielding approximately 6 mg of protein at 1–2 mg/L concentration from 564 mg of total protein, using DDM [44]. Others have obtained enough protein for biophysical [45] and crystallization studies even though purification yields were not reported [43, 46–48]. Recombinant baculovirus has been shown to provide a high expression of P-gp in Sf9 or High-Five insect cells [8, 23]. Insect cells yield unglycosylated P-gp. Although the lipid composition of insect cells differs from the mammalian cell composition [49], the function of human P-gp is very well preserved [23, 24, 28, 30].

A major hurdle in purifying a membrane protein is finding a detergent that can preserve the protein, after releasing it from a given membrane, in a stable and non-aggregated state during purification steps [18, 50]. The ability of various detergents used to solubilize and purify membrane proteins has been reviewed [42], and in particular, the efficacy of different detergents to solubilize P-gp has been compared in previous publications [10, 29, 51–53]. Generally during the solubilization step, the detergent is used at $>20 \times \text{CMC}$ to ensure spontaneous micelle formation. However, during purification, the concentration of detergent employed is usually just above the CMC. High concentrations of detergent can affect the integrity of the protein. Efforts to develop detergent-free isolation of membrane proteins are still under development [54]. Unfortunately, there is no single universal detergent that will work for all membrane proteins. For example, P-gps from murine lymphoid leukemia P388/ADR25, rat hepatoma AS30-D/COL10 and human lymphoblastic leukemia CEM/VLB5 are highly adaptable to extreme denaturing conditions in which solubilization and purification are carried out in the presence of SDS [19]. However, their function was significantly diminished when the protein was reconstituted in lipid vesicles. Lauryl maltose neopetyl glycol/CHS (LMNG/CHS) has recently been used for purification of a mouse/human P-gp chimera and for resolution of its structure by cryo-EM [47]. However, we found that with this detergent, the yield of solubilized human P-gp from insect cell membranes was considerably lower compared to the yield with DDM (data not shown). As noted in our earlier reports [23, 30], DHPC, DDM and OG in combination with added lipids were each found to be effective detergents with reference to their ability to solubilize P-gp. They can be used interchangeably for column chromatography and can be removed during reconstitution in proteoliposomes or nanodiscs. These detergents also preserve the ATPase activity of P-gp. Here, we show that for preparation of homogeneous and biologically functional proteins, stability can be achieved by solubilizing the insect-cell membranes in either DHPC, DDM or OG. However, the choice of detergent in the second step of purification is critical for applications such as crystallization and functional assessment. Generally, detergents with lower CMCs are preferred for structural studies. Particularly, DDM has been widely proven to be a good detergent for P-gp structural characterization [14, 46, 48, 55]. For these reasons, DDM was our detergent of choice for the second purification step. This detergent belongs to the class of alkyl maltosides and contains a hydrophilic maltose headgroup and a hydrophobic alkyl chain. DDM has a CMC of 0.17 mM (0.0087%), which is several orders of magnitude lower than OG or DHPC [56]. Apparently, the inhibition of ATPase activity of P-gp purified from Chinese hamster ovary (CH^RB30 cells) by DDM detergent above CMC level could be prevented when used in combination with phospholipid mixtures. Even though the CMC and aggregation number (i.e., the number of detergent molecules per micelle) are specific to each detergent in a given buffer condition [57], the DDM present in the concentrated final column fraction containing human P-gp is much higher than CMC, which helps to prevent the aggregation of homogeneous preparations of human P-gp at higher concentrations. We have previously shown that the ATP hydrolysis behavior of P-gp is affected by DDM micelles [28]. The yield of P-gp or its mutant EQ protein and specific activity of ATPase activity were dependent on the type of detergent used in the final step. Though most non-ionic detergents readily solubilize P-gp, they significantly inhibit ATPase activity [39]. In addition to DDM or DHPC detergents, we found that the presence of 150

mM NaCl and 20 % glycerol [36], an osmolyte, increases the stability during solubilization and throughout the process of purification and storage at -80°C .

Incorporation of tags at the C-terminus of P-gp has proven to be useful for the purification, including hexahistidine tags and GFP [14]. We chose to use the hexahistidine tag because it provides a rapid and easy purification through IMAC. When proteins are subjected to IMAC, metal ions are immobilized by chelation to an insoluble matrix, and then amino acids, particularly histidine, binds to the chelated ions. Changes in pH or addition of a competitive inhibitor allows elution of the bound proteins. Our results showed that resins containing either cobalt or nickel can be used for purification of P-gp, with comparable results. Others have obtained mouse P-gp suitable for high-resolution structures using different purification strategies. For example, *Pichia pastoris* membranes containing mouse ABCB1 with a hexahistidine tag at the C-terminus were solubilized using 4.5% Triton® X-100, and an Ni-NTA step using 0.04% sodium cholate and 0.0675% β -DDM for elution, followed by size exclusion chromatography [46]. Amphiphiles or β -sheet peptide assemblies in complex with lipids have also been used [58] to obtain structural information by electron microscopy. Previously, Pollock et al. [59] showed that addition of CHS improves the thermal stability of P-gp and reduces the fraction that corresponds to aggregated forms, likely due to reduction of the fluidity of the micelle. In our experience, the yield of recovery was comparable to that achieved without CHS. However, we found that protein purified in DDM/CHS had a higher basal ATP hydrolysis rate when compared to protein purified in DDM alone. This is most likely due to increased stability in the presence of mixed micelles of DDM and CHS.

Even though detergent micelles can provide a good environment for P-gp structure characterization [14, 46, 48, 55] it has been shown that they may affect the stability of P-gp and its ability to bind modulators in the transmembrane region [28]. For these reasons, incorporation of P-gp in proteoliposomes or nanodiscs is preferable to study its properties in a membrane environment [21]. Membrane proteins can be reconstituted into lipid bilayers by detergent removal. P-gp has been reconstituted in both proteoliposomes and giant liposomes [30, 52]. Here we demonstrate that purified P-gp can be easily reconstituted in proteoliposomes and nanodiscs in a functional form.

5. Conclusions

We report here a highly reproducible large-scale purification method for purifying human WT P-gp and its double EQ mutant expressed using a baculovirus High-Five insect cell expression system. The homogeneous preparation of P-gp after its reconstitution in nanodiscs or proteoliposomes is currently being used for biochemical, biophysical and structural studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

P-gp	P-glycoprotein
ABC	ATP-binding cassette
MDR	multidrug resistance
DHPC	1,2-Diheptanoyol-sn-glycero-3-phosphocholine
DDM	Dodecyl maltoside
OG	<i>n</i> -Octyl- β -D-glucopyranoside
CHS	Cholesteryl hemisuccinate
TMD	transmembrane domain
NBD	nucleotide-binding domain
Cryo-EM	cryo-electron microscopy
WT	wild-type
IMAC	immobilized metal affinity chromatography
Ni-NTA	Ni-Nitrilotriacetic acid
MOI	multiplication of infection
CsA	Cyclosporine A
AEBSF	4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride
β-ME	β -mercaptoethanol
CMC	critical micelle concentration
MWCO	molecular weight cut-off

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Highlights

- Human P-glycoprotein is expressed at high levels in High-Five insect cells
- Active P-glycoprotein can be purified and solubilized using different detergents
- Functional P-glycoprotein can be purified in large quantities

Fig. 1.
Flow chart outlining the total membrane preparation, detergent solubilization, and purification of WT or E556Q/E1201Q mutant P-gp.

Fig. 2. Purification of WT P-gp using Method 2. (A) An InstantBlueTM stained 7% NuPAGE-SDS profile of fractions at the different stages of purification. Lane 1: molecular weight marker; Lane 2: DDM/CHS extract (20 μ g); Lane 3: eluate from the Ni-NTA resin (4 μ g); Lane 4: combined fractions containing monomeric WT P-gp from size exclusion chromatography (4 μ g) (B) Size exclusion chromatogram of a typical purification of WT P-gp using DDM/CHS buffer. Highlighted in gray are fractions collected for final concentration that correspond to lane 4 in the denaturing gel presented in (A).

Fig. 3. Reconstitution of proteins into proteoliposomes from fractions during purification using Method 1. (A) Silver stained profile of fractions collected using Method 1 purification after reconstitution into proteoliposomes. Lane 1: molecular weight marker; Lane 2: DHPC extract reconstituted in PLs (3.5 μ g); Lane 3: PLs containing P-gp eluted from TALON resin (1.0 μ g); Lane 4: PLs containing flow through sample from ion exchange chromatography (1.0 μ g). (B) ATPase activity of total membrane vesicles compared to proteoliposomes made with the DHPC extract, the TALON 200 mM imidazole elute or the flow through from the ion exchange column. Data presented are the average values of two independent experiments.

Fig. 4.

ATPase activity of P-gp in membrane vesicles, detergent micelles and nanodiscs in the absence or presence of tariquidar at 10 μ M unless specified. Columns 1 and 2: total membranes with and without 1 μ M tariquidar respectively; columns 3 and 4: P-gp in DDM micelles; columns 5 and 6: P-gp in DDM/CHS micelles; columns 7 and 8: P-gp in NDs reconstituted from protein in DDM micelles; and columns 9 and 10: P-gp in NDs reconstituted from protein in DDM/CHS micelles. Data presented are the mean $\pm$ SD of three or more experiments.

Fig. 5. Sephadex-200 gel filtration of purified human P-gp in the presence (■) and absence of ATP/cyclosporine A (◆).

Table 1

Buffer composition for the three steps of purification as described in the Materials and Methods section

Core buffers	Composition
Core buffer 1	20 mM Tris-HCl pH 8.0, 200 mM NaCl, 20% Glycerol, 2 mM β -ME, 1 mM AEBSF, 0.5% aprotinin, 2 μ g/ml of pepstatin, 2 μ g/mL leupeptin
Core buffer 2	50 mM Tris-HCl pH 7.5, 200 mM NaCl, 15% glycerol, 5 mM β -ME, 20 mM imidazole
Solubilization buffer	
DHPC	Core buffer 1, and 1.25% DHPC (20 $\times$ CMC)
OG	Core buffer 1, 1.5% OG (3 $\times$ CMC), and 0.1% lipid mix
DDM	Core buffer 2, 2% DDM (230 $\times$ CMC), protease inhibitor cocktail tablets (1 tablet/25 mL)
IMAC (TALON or Nickel-NTA resin) binding buffer	
DHPC	Core buffer 1, 20 mM imidazole, and 0.25 % DHPC.
OG	Core buffer 1, 20 mM imidazole, 1.25 % OG, 0.1 % lipid mix.
DDM	Core buffer 2, 0.0675 % DDM, 0.04 % Sodium cholate, protease inhibitor cocktail tablets (1 tablet/25 mL)
DDM/CHS	Core buffer 2, 0.0675 % DDM, 0.00675% CHS, 0.04 % Sodium cholate, protease inhibitor cocktail tablets (1 tablet/25 mL)
IMAC elution buffer	
DHPC	Core buffer 1 (pH 6.8), 200 mM imidazole, and 0.25 % DHPC.
OG	Core buffer 1 (pH 6.8), 200 mM imidazole, 1.25 % OG, 0.1 % lipid mix.
DDM	Core buffer 2 (with 200 mM imidazole), 0.0675 % DDM, 0.04 % sodium cholate, protease inhibitor cocktail tablets (1 tablet/25 mL)
DDM/CHS	Core buffer 2 (with 200 mM imidazole), 0.0675 % DDM, 0.00675% CHS, 0.04 % sodium cholate, protease inhibitor cocktail tablets (1 tablet/25 mL)
Ion exchange buffer	
DDM	10 mM Tris-HCl pH 8.0, 20% glycerol, 10 mM DTT, 0.25 mM AEBSF, and 0.02% DDM
Gel filtration buffer	
DDM	Core buffer 2 (no imidazole), 0.0675 % DDM, 0.04 % sodium cholate.
DDM/CHS	Core buffer 2 (no imidazole), 0.0675 % DDM, 0.00675% CHS, 0.04 % sodium cholate.

Table 2

Percentage yield of purified WT and EQ mutant P-gp with detergents using Method 1 or Method 2, as described in the Materials and Methods section

Detergent	Method 1		Method 2	
	WT	E556Q/E1201Q	WT	E556Q/E1201Q
OG	0.6	0.4	ND	ND
DHPC	0.9	1.4	ND	ND
DDM	1.1	1.6	0.58	ND
DDM/CHS	ND	ND	0.76	0.64

Starting amount of total membrane protein (350-750 mg)

Percentage relative to the total soluble protein.

Variation factor: $\pm 20\%$ depending on the batch of cells and protein preparation (n =3-5). ND, not determined.