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Improved expression and purification of human multidrug resistance protein MDR1 from baculovirus-infected insect cells

Atsushi Kodan [a,c,d], Hiroyuki Shibata [a,1], Takashi Matsumoto [a,2], Kanako Terakado [a,b], Keita Sakiyama [a,b], Michinori Matsuo [d], Kazumitsu Ueda [c,d], Hiroaki Kato [a,b,*]

[a] Membrane Dynamics Research Group, RIKEN Harima Institute at SPring-8, Sayo, Hyogo 679-5148, Japan
[b] Department of Structural Biology, Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29 Yoshida-Shimo-Adachi-cho, Sakyo-ku, Kyoto 606-8501, Japan
[c] Institute for Integrated Cell-Material Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan
[d] Laboratory of Cellular Biochemistry, Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan

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ABSTRACT

Multidrug resistance protein MDR1 (P-glycoprotein/ABCB1) is an ATP-dependent efflux pump for various cytotoxic agents, and is implicated in the resistance of human tumors to chemotherapeutic drugs. To achieve the three-dimensional structural analysis for its mechanistic implications, large amounts of high-quality and homogeneous MDR1 protein are essential. Here we report a cost-effective method for large-scale expression of human MDR1 using a baculovirus/insect *express*SF+ cell system and an alterative purification method to maintain MDR1 in a monodispersed state. After extensively optimizing the detergent, pH, and additives, a high yield (2.8 mg/L) of purified MDR1 was obtained by immobilized metal chelate affinity and size-exclusion chromatographies with 49% recovery. The purified MDR1 exhibited specific ATP hydrolase activity (1.7 μmol/min/mg) in the presence of a substrate, verapamil. This value was 14-fold greater than the basal activity without the drug. Size-exclusion chromatography analysis of purified MDR1 showed a monodispersed elution profile. The present purification method provides suitable material for structural and functional studies on human MDR1.

Multidrug resistance protein MDR1 (P-glycoprotein/ABCB1) is a transmembrane (TM)[3] efflux pump that is a member of the ATP binding cassette (ABC) transporter superfamily [1,2]. Human MDR1 (hMDR1) confers multidrug resistance to tumor cells. It removes a variety of chemically dissimilar antitumor drugs or hydrophobic compounds [3,4] from cells by transporting them across the plasma membrane to the outside of the cell in a process powered by ATP hydrolysis [5,6]. Therefore, hMDR1 is of considerable clinical and pharmacological interest. hMDR1 consists of a single polypeptide chain of 1280 amino acids with two TM domains and two nucleotide-binding domains (NBD). hMDR1 contains three *N*-linked (Asn-X-Ser/Thr) glycosylation sites in the first extracellular loop at residues Asn-91, -94, and -99 [7]. Thus, although the estimated size of hMDR1 based on the amino acid sequence is 140 kDa, the molecular weight of hMDR1 purified from mammalian cells is 170 kDa [8] due to glycosylation of the polypeptide. However, because the *N*-glycosylation-deficient mutant conferred a typical MDR phenotype on human culture cells [9] and unglycosylated hMDR1 expressed in yeast and insect cells is functional [10,11], glycosylation is not essential for the functional activity of hMDR1. The characteristic ATP hydrolase

* Corresponding author. Address: Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29 Yoshida-Shimo-Adachi-cho, Sakyo-ku, Kyoto 606-8501, Japan. Fax: +81 75 753 9272.
E-mail address: katohiro@pharm.kyoto-u.ac.jp (H. Kato).
[1] Present address: Department of Cardiac Physiology, National Cardiovascular Center, 5-7-1 Fujishiro-dai, Suita, Osaka 565-8565, Japan.
[2] Present address: PharmAxess Inc., Saito Bio-Innovation Center 101, 7-7-20 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan.

[3] *Abbreviations used:* MDR, multidrug resistance; TM, transmembrane; ABC, ATP-binding cassette; hMDR1, human MDR1; NBD, nucleotide-binding domain; ATPase, ATP hydrolase; High Five, *Trichoplusia ni*; β-DDM, *n*-dodecyl-β-D-maltoside; IMAC, immobilized metal chelate affinity chromatography; SEC, size-exclusion chromatography; SF+, *express*SF+; Sf, *Spodoptera frugiperda*; FBS, fetal bovine serum; E-64, (L-trans-3-carboxyoxirane-2-carbonyl)-L-luecine-4-guanidinobutylamide hemihydrate; CHS, cholesteryl hemisuccinate; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate; CHAPSO, 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxypropane sulfonate; BigCHAP, *N*, *N*-bis(3-D-gluconamidopropyl) cholamide; deoxy-BigCHAP, *N*, *N*-bis(3-D-gluconamidopropyl) deoxycholamide; OG, *n*-octyl-β-D-glucoside; DG, *n*-decyl-β-D-glucoside; OM, *n*-octyl-β-D-maltoside; NM, *n*-nonyl-β-D-maltoside; DM, *n*-decyl-β-D-maltoside; UDM, *n*-undecyl-β-D-maltoside; α-DDM, *n*-dodecyl-α-D-maltoside; LDAO, *n*-dodecyl-*N*,*N*-dimethylamine-*N*-oxide; CYFOS 6, 6-cyclohexyl-1-hexylphosphocholine; Fos-Choline 12, *n*-dodecylphosphocholine; C12E9, polyoxyethylene(9)dodecyl ether; C10E6 polyoxyethylene(6)decyl ether; HECAMEG, methyl-6-*O*-(*N*-heptylcarbamoyl)-α-D-glucopyranoside; C8E4, tetraethylene glycol monooctyl ether; OTG, *n*-octyl-β-D-thioglucoside; HTG, *n*-heptyl-β-D-thioglucoside; MOI, multiplicity of infection; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); DTT, dithiothreitol; EGTA, [ethylenebis(oxyethylenenitrilo)] tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

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(ATPase) activity of hMDR1 is coupled to drug transport. Therefore, in the presence of the substrate drug, the ATPase activity of hMDR1 is higher than the basal level in the absence of the substrate [12]. This modest basal activity could be due to the activation by endogenous lipids or uncoupled activity [13,14]. The mechanism by which the energy of the ATPase is used to drive drug extrusion is of great interest because inhibiting the catalytic activity could develop methods that circumvent or disable hMDR1. However, the detailed molecular mechanism is still unknown.

Understanding this mechanism and developing new therapeutic strategies that target hMDR1 would be considerably accelerated with the three-dimensional structure of hMDR1. Previous results from electron microscopy [15–17] and single particle image analysis [18] have provided low-resolution structures of MDR1. However, except for bacterial homologs, notably Sav1866 and MsbA [19,20], the crystal structures of hMDR1 and its related homologs from other species have not been solved. These bacterial homologs are half-size ABC transporters that exist as homodimers composed of two sets of six TM α-helices and an NBD. Their overall architecture is thought to be similar to the hMDR1 structure; however, Sav1866 and MsbA have substrate specificities that are distinct from hMDR1 [19–21]. To characterize the substrate specificity and transport mechanism of hMDR1, the structure of hMDR1 needs to be determined.

To generate an hMDR1 preparation for crystal structure analysis, large amounts of properly folded hMDR1 protein are needed. For this reason, yeast and baculovirus/insect systems have been used for large-scale purification of hMDR1 as reviewed previously [22,23]. Using budding yeast species, such as *Saccharomyces cerevisiae* [24] and *Pichia pastoris* [25] as an expression host, hMDR1 was purified with yields of 0.4 mg/L and 0.75 mg/L, respectively. The baculovirus/insect system has also been shown to express hMDR1 [26] with yields of ~1 mg of purified hMDR1 per 100 mg of crude membranes from *Trichoplusia ni* (High Five). In the presence of *n*-dodecyl-β-D-maltoside (β-DDM), the membrane fractions containing hMDR1 were solubilized, and then purified by immobilized metal chelate affinity chromatography (IMAC). However, size-exclusion chromatography (SEC) analysis of purified hMDR1 revealed aggregates or functionally unrelated oligomeric species in solution [27,28]. In general, structural studies require a purified protein preparation that is stable and monodispersed in a detergent solution. Therefore, the MDR1 purification conditions must be further optimized, especially the selection of appropriate detergents. In addition, the phospholipid content of the purified preparation is an important determinant of the crystallizability of membrane proteins [29–32] and should be assessed experimentally.

In the present study, we report improved methods for the expression and purification of hMDR1 that are suitable for structural and functional studies. For expression, a new insect cell line, *express*SF+ (SF+), derived from *Spodoptera frugiperda* (Sf9) cells [33] was used. Unlike Sf9 cells, SF+ cells grow in suspension to a high-density (10^7 cells/mL) with doubling times of 18–20 h in serum-free media, which facilitates scaling up to large-scale bioreactors. In addition, SF+ cells sometimes require lower baculovirus titers for protein expression than Sf9 cells. For these reasons, the SF+ expression system enabled us to conduct a cost-effective, large-scale expression of hMDR1. Furthermore, after extensively optimizing the purification conditions, including detergents, the purified hMDR1 preparation showed a monodispersed elution profile by SEC analysis.

Materials and methods

Materials

SF+ insect cells were obtained from Protein Sciences (Meriden, CT, USA). Sf900II SFM, fetal bovine serum (FBS), and gentamicin were from Invitrogen (Carlsbad, CA, USA). Acetyl-L-leucyl-L-leucyl-L-argininal hemisulfate monohydrate (luepeptin) and (L-trans-3-carboxyoxirane-2-carbonyl)-L-luecine-4-guanidinobutylamide hemihydrate (E-64) were from Peptide Institute, Inc. (Osaka, Japan). Complete® (protease inhibitor cocktail tablets) were from Roche Molecular Biochemicals (Mannheim, Germany). Cholesteryl hemisuccinate (CHS) was purchased from Sigma (St. Louis, MO, USA). Sucrose monolaurate, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxypropane sulfonate (CHAPSO), *N*, *N*-bis(3-D-gluconamidopropyl) cholamide (BigCHAP), *N*, *N*-bis(3-D-gluconamidopropyl) deoxycholamide (deoxy-BigCHAP), and sucrose monocarprate were from Dojindo Co. Ltd. (Kumamoto, Japan). Digitonin was from Wako Pure Chemical Industries (Osaka, Japan). Sodium cholate was from Nakarai Tesque, Inc. (Kyoto, Japan). All other detergents were purchased from Anatrace (Maumee, OH, USA), including *n*-octyl-β-D-glucoside (OG), *n*-decyl-β-D-glucoside (DG), *n*-octyl-β-D-maltoside (OM), *n*-nonyl-β-D-maltoside (NM), *n*-decyl-β-D-maltoside (DM), *n*-undecyl-β-D-maltoside (UDM), *n*-dodecyl-α-D-maltoside (α-DDM), *n*-dodecyl-β-D-maltoside (β-DDM), *n*-dodecyl-*N*,*N*-dimethylamine-*N*-oxide (LDAO), 6-cyclohexyl-1-hexylphosphocholine (CYFOS 6), *n*-dodecylphosphocholine (Fos-Choline 12), polyoxyethylene(9)dodecyl ether (C12E9), polyoxyethylene(6)decyl ether (C10E6), methyl-6-*O*-(*N*-heptylcarbamoyl)-α-D-glucopyranoside (HECAMEG), tetraethylene glycol monooctyl ether (C8E4), *n*-octyl-β-D-thioglucoside (OTG), and *n*-heptyl-β-D-thioglucoside (HTG). Verapamil and sodium orthovanadate were from Wako Pure Chemical Industries.

Preparation of recombinant baculoviruses

A thrombin-specific cleavage motif and a His_{10} tag were introduced into the transfer vector pVL1392 (Pharmingen, San Diego, CA, USA) by inserting an oligonucleotide cassette (forward sequence, 5′-AATTCCTCTAGAGCTGGTTCCGCGTGGTTCC(CACCAT)$_5$TGAG; reverse sequence, 5′- GATCCTCA(ATGGTG)$_5$GGAACCACGCGGAACCAG CTCTAGAGG) at EcoRI and BamHI restriction sites. This construct was termed pVL1392-TH-His_{10}. The entire coding region of hMDR1 cDNA was subcloned into pVL1392-TH-His_{10}, using EcoRI and XhoI restriction enzymes. The DNA sequence of the insert was confirmed in both orientations. Recombinant virus was prepared using the BaculoGold starter kit (Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions. The resulting virus was titrated using the BacPAK Rapid Titer kit (BD Bioscience, Palo Alto, CA, USA).

Insect cell culture

SF+ insect cells were grown in suspension at 27 °C. Cells were routinely cultured in disposable sterile 250 mL Erlenmeyer flasks (Corning, New York, NY, USA) containing 100 mL of Sf900 II SFM supplemented with 50 μg/mL gentamicin. The flasks were seeded at a density of 0.8–1.0 × 10^6 cells/mL and incubated with shaking at 100 rpm on an orbital shaker. After 48 h, the culture reached a density of 4.0–5.0 × 10^6 cells/mL, and the cells were subcultured at the same seeding density as above.

Large-scale expression with recombinant baculoviruses

Large-scale SF+ cultures (10 L) were grown in the Wave Bioreactor System 20/50 EH (Wave Biotech LLC, Bridgewater, NJ, USA) under an air/oxygen mixture to maintain the atmosphere at 50% O_2 saturation. When SF+ cells reached a density of 1.5 × 10^6 cells/mL, the cells were infected with high-titer recombinant baculovirus at a multiplicity of infection (MOI) of 2 plaque-forming units per cell. At this point, E-64 and leupeptine were added at a final concentration of 10 and 2 μM, respectively. After a 72-h