Analysis of ATP hydrolysis activities of ABC transporters involved in multidrug resistance and K_{ATP} channel regulation

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ABBREVIATIONS

ABC	ATP binding cassette
ADP	adenosine 5’-diphosphate
AMP	adenosine 5’-monophosphate
ATP	adenosine 5’-triphosphate
CFTR	cystic fibrosis transmembrane conductance regulator
DDM	*n*-dodecyl-β-D-maltoside
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylene glycol bis(beta-aminoethylether)-N,N,N,N-tetraacetic acid
FBS	fetal bovine serum
HPLC	high performance liquid chromatography
K_{ATP}	ATP sensitive potassium
Kir	inward rectifier potassium
KDa	kilodalton
MDR	multidrug resistance
MOI	multiplicity of infection
NBF	nucleotide binding fold
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PS	phosphatidylserine
SDS	sodium dodecyl sulfate
SUR	sulfonylurea receptor
TM	transmembrane domain
Tris	tris(hydoroxymethyl) aminomethane

CONTENTS

INTRODUCTION

ATP-binding cassette (ABC) transporter superfamily consists of huge membrane anchored proteins that translocate a wide variety of substrates across the membrane lipid bilayer. ABC transporters are found in all prokaryotic and eukaryotic cells and play important roles in cell physiology. To date, 49 ABC transporters have been identified in human genome and numerous studies revealed their important functions in various cells[1, 2]. Overexpression of drug transport ABC transporters in cancer cells transform them simultaneously resistant to structurally unrelated anticancer drugs[3-5]. This phenomenon is generally referred as multidrug resistance (MDR). The dysfunction of ABC transporters often causes severe genetic diseases including cystic fibrosis, various sterol-homeostasis disorders, impairments of bile formation and insulin disorder. ABC transporters in eukaryote are classified into three categories based on their functions. The majority of ABC transporters are active ATP-driven transporters which transport various compounds across the lipid bilayer. Many compounds, including steroids, lipids, hormones and various hydrophobic chemotherapeutic drugs, are transported by ABC transporters. Cystic fibrosis transmembrane conductance regulator (CFTR) was identified as a responsible gene of cystic fibrosis and revealed to function as a channel[6, 7]. The targets of sulfonylurea agent, sulfonylurea receptors (SUR) function as a regulatory subunit of ATP sensitive potassium

channel and convert cellular metabolic states to electric potentials of plasma membrane[8-11].

ABC transporters are usually composed of two symmetrical units. Each half contains transmembrane domain which forms the substrate binding pocket and highly conserved nucleotide binding fold (NBF) where the ATP hydrolysis occurs to energize the active transport cycle. Since the ATP hydrolysis tightly couples to the function of ABC transporters, it is difficult to understand their roles in physiological systems without characterization of their specific ATP hydrolysis activity in details. However, because of difficulties in construction of effective expression and purification systems of such gigantic membrane proteins, there are few reports published previously which succeeded in purifying and examining the specific ATP hydrolysis activities of ABC transporters.

In the research presented in this thesis, the author focused on methodological development of functional purification of ABC transporters and sensitive assay system for ATPase activity in chapters 1 and 2. Using the large scale expression and purification system described in chapter 1, the author succeeded in purifying MDR1 and SUR1 in a functional three-dimensional structure. The ATPase assay system described in chapter 2 was sensitive more than twenty-times than conventional colorimetric inorganic phosphate detection and applicable to any protein. In chapter 3, the author discussed the influence of cholesterol on drug-MDR1 interactions. By examining the ATPase activity of MDR1 in detail, the author demonstrated that cholesterol is utilized for the constituent of fully active transporter. The results described in chapter 3 will be valuable not only for understanding of the transport mechanisms *in vivo* but also for the development of a complete model of MDR1-substrate interactions.

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Chapter 1
Expression and purification of ABC transporters

ABC transporters are found in most cells of all species from prokaryotes to humans, and hence make up one of the largest protein superfamilies. A typical ABC transporter consists of symmetrical two halves. Each half contains six transmembrane helices and well-conserved nucleotide binding fold (NBF). The vast majority of ABC transporters are ATP-driven transporters which transport various compounds across the lipid bilayer. However, despite their similar secondary structures, some proteins act as channel or channel regulator. Currently, it is not clear how ABC transporters exert different functions as a transporter, a channel, or a channel regulator despite their containing well-conserved NBFs.

Functions of ABC transporters are supposed to be coupled to the conformational changes induced by ATP binding and hydrolysis. Moreover, the ATPase activities of ABC transporters are stimulated by transport substrates. Thus, the characterization of the intrinsic ATPase activity is expected to give us important information about physiological roles of ABC transporters.

It usually requires relatively large amounts of purified protein to characterize ATPase activity of ABC transporters in detail. To meet this need, it is necessary to develop large

scale expression and purification systems. There are several methods that are commonly utilized for expressing foreign proteins. Bacterial expression systems often satisfy these needs, however these systems are not suitable for expressing large membrane proteins of eukaryote because such proteins are usually incorrectly folded, aggregated in inclusion bodies, and lost their functional activities. While the expression systems using mammalian cells are suitable methods to obtain functional proteins, it is usually difficult to produce the recombinant proteins in a large scale. In baculovirus expression system, the foreign proteins are expressed in insect cells by infecting the recombinant viruses. Insect cells grow well in a suspension culture that allow the protein expression in a large scale.

The aim of this study is to establish large scale expression and purification systems for ABC transporters. For this purpose, the author tried to express MDR1 in insect cells. MDR1 is a plasma membrane located ABC transporter which excludes a wide variety of hydrophobic compounds [1, 2]. At present, the biochemical properties of this enzyme are best studied among ABC transporters both in native membrane and a detergent-solubilized purified form [3-5]. Thereby, MDR1 would be the best model protein to confirm the functional expression and purification of ABC transporters in insect cells. In contrast to MDR1, studies of SUR1, a regulatory subunit of ATP sensitive K^+ channel (K_{ATP} channel), have been mainly performed by electrophysiological techniques, and the biochemical properties of SUR1 are still poorly characterized.

K_{ATP} channels are heterooctameric complexes, composed of two types of subunits; an inwardly rectified potassium channel pore subunit (Kir6.2) and a regulatory subunit (sulfonylurea receptor: SUR1) in a 4 : 4 stoichiometry[6, 7]. K_{ATP} channel couples intracellular metabolic state to a plasma membrane electrical activity by sensing the changes of the intracellular ATP and ADP concentrations. The sensing mechanism of metabolic state

is achieved by opposite effects of ATP and ADP on channel current via different subunits. Binding of ATP to Kir6.2 subunit causes a channel closure, whereas ADP acts as a channel opener by binding to SUR1 subunit. Although the electrophysiological studies have provided numerous information about channel regulation by nucleotides, it is difficult to understand the precise mechanism of channel regulation by nucleotides. Biochemical characterization of interaction of SUR1 and nucleotides is required to understand it. It has been demonstrated that the NBF2 of SUR1 is photoaffinity labeled by 8-azido-[α-^{32}P]-ATP, but not by 8-azido-[γ-^{32}P]-ATP [8]. Gribble *et al*. reported when reconstituted with ATP-insensitive mutant of Kir6.2, ATP can activate channel current only in the presence of Mg^{2+} [9]. These reports suggested that the active state of K_{ATP} channel (MgADP binds to the NBF2 of SUR1) could result from MgATP hydrolysis at NBF2 of SUR1. However, biochemical analysis of ATPase activity of SUR1 has not been demonstrated yet.

In this chapter, the author showed that the baculovirus expression system is efficient to purify ABC transporters in a large scale. Furthermore, the author demonstrated that the ATPase activity of SUR1 is significantly lower than that of MDR1. This suggests that the turnover rate of ATP hydrolysis maybe a key feature to distinguish the functions of ABC transporters. The author also revealed that K_{ATP} channel blockers promote the ATPase activity of SUR1. From this result the author proposed a new model for the action of channel blockers.

EXPERIMENTAL PROCEDURES

Materials- Sf9 cells were purchased from Pharmingen. L-α-lecithin and ATP were purchased from Sigma-Aldrich. Media, pluronic F-68, and gentamycin were obtained from Invitrogen. *N*-Dodecyl-β-D-maltoside (DDM) was purchased from Anatrace. Sodium ortho-vanadate and verapamil hydrochloride were purchased from Wako. Sf9 cells were purchased from pharmingen.

General techniques of cell culture- For suspension cultures, cells were cultivated in Grace's insect medium (GIBCO) supplemented with 10% of fatal bovine serum and 0.1 % pluronic F68. Cells were cultivated at a cell density from 0.2 to 2.0 x 10^6 cells/ml in a spinner flask. For monolayer cultures, cells were cultivated in Grace's insect medium supplemented with 10% of fatal bovine serum.

Generation of recombinant baculovirus encoding ABC transporters- The sequence encoding thrombin cleavage site, ten histidine codons, and a termination codon were inserted at the 3' end of human *MDR1* cDNA [10] and hamster *SUR1* cDNA subsequently subcloned into the transfer vector pVL1392 (Pharmingen). Recombinant baculovirus was generated by co-transfection of Sf9 cells with transfer vector and Baculogold DNA (Pharmingen). Recombinant viruses were purified by plaque assay and amplified according to the manufacturer's protocol. Virus titer was determined by plaque assay.

Protein expression and preparation of microsomes- Sf9 cells were infected with baculovirus encoding MDR1 or SUR1 at a multiplicity of infection 5. After 48 h incubation, cells were harvested and washed with 5-volume of ice cold PBS. The cell pellet was stored at –80 ^{o}C. Frozen Sf9 cells were resuspended in 10-fold volume of sonication buffer (20 mM Tris-Cl pH7.5, 10% v/v glycerol, 1 mM EDTA). All further steps were done at 4 ^{o}C or on ice.

Cells were disrupted by Ultrasonic processor XL (Misonix) for 30 sec at output level 5, then returned to the ice for 2 min. These steps were repeated ten times. The suspension was centrifuged at 650 x *g* for 10 min. The pellet (unbroken cells) was sonicated again as above and centrifuged, and the pellet was discarded. The homogenate was centrifuged at 2,000 x *g* for 30 min to remove nuclei. The supernatant was centrifuged at 40,000 x *g* for 60 min. The pellet containing the microsomes was resuspended in ice cold buffer A (50 mM Tris-Cl pH8.0, 50 mM NaCl, 30% v/v glycerol, 1mM mercaptoethanol) containing 15 mM imidazole and protease inhibitors as follows: 100 μg/ml pAPMSF, 10 μg/ml leupeptin, 2 μg/ml aprotinin. The microsomes were stored at –80 ^{o}C.

Protein purification- To remove the peripherally anchored protein, microsomes were thawed on ice and diluted to 4 mg protein/ml with ice cold high salt wash buffer (50 mM Tris-Cl pH8.0, 0.5 M NaCl) plus protease inhibitors and kept on ice for 10 min. Integral membrane protein was recovered by centrifugation (60,000 x *g*, 30 min) and resuspended in ice-cold buffer A. An equal volume of detergent in buffer A plus protease inhibitor was added to the solution, and the suspension was mixed by inversion and kept on ice for 10 min. Detergent insoluble proteins were removed by centrifugation (60,000 x *g*, 30 min). Then, Ni-NTA agarose resin (Qiagen) was added to the supernatant at a ratio of 0.5 ml of resin per 100 mg of microsomal protein. The slurry was incubated at 4^{o}C for 18 h with continuous rotation. The resin was washed with a 10-bed volume of buffer A containing 0.1% DDM three times and then with five volumes of buffer A containing 0.1% DDM plus 40 mM imidazole. MDR1 was eluted with five volumes of buffer A containing 0.1% DDM plus 300 mM imidazole. Protein concentration was estimated by the Bradford method (Bio-Rad).

Photoaffinity labeling by [^{3}H] glibenclamide- 100 ng of Purified SUR1 and glibenclamide (1 μM [^{3}H]-glibenclamide plus cold glibenclamide) were mixed and incubated in dark at room

temperature for 5 min. To separate from the free glibenclamide, SUR1 was recovered with 5 μl of Ni-NTA agarose and UV-irradiated for 5 min (at 254 nm, 5.5 milliwatts/cm^2) on ice. After UV irradiation, samples were electrophoresed on a 7% SDS-polyacryl-amide gel. The binding of [^{3}H] glibenclamide was detected by scanning with a radioimaging analyzer (BAS2000, Fuji Photo Film Co.).

ATPase assay- For lipid stocks, L-α-lecithin was solubilized in 40 mM Tris-Cl, pH7.5, 0.1 mM EGTA at a concentration of 20 mg/ml by sonicating in a bath sonicator (Bioruptor CD-200 TM, COSMO BIO) until the suspension clarified. Lipid stocks were stored at 4^{o}C. Purified MDR1 and lipid stocks were mixed at protein/lipid ratio at 1/5 (w/w) in 40 mM Tris-Cl, pH 7.5, 0.1 mM EGTA, 2 mM DTT at 23 ^{o}C for 20 min, then sonicated at 4 ^{o}C for 30 s in a bath sonicator. Reconstituted protein was reacted in 16 μl of 40 mM Tris-Cl (pH7.5), 0.1 mM EGTA, 1 mM NaATP and 1 mM $MgCl_2$. The reaction was stopped by adding an equal volume of 12% SDS. ATPase activity was estimated by quantifying the released inorganic phosphate as described previously[11]. In brief, after the reaction was stopped by SDS, 10 μl of H_2O was added on sample. Color development started with the addition of 40 μl of equal mixture of 1% ammonium molybdate and 6% ascorbic acid in 1 N HCl. After 7 min incubation at room temperature, 60 μl of 2% sodium citrate, 2% sodium arsenite, and 2% acetic acid were added and incubated at 37^{o}C for 10 min. The color absorbance was measured at 850 nm by UV-VIS spectrophotometer UV mini 1240 (Shimadzu).

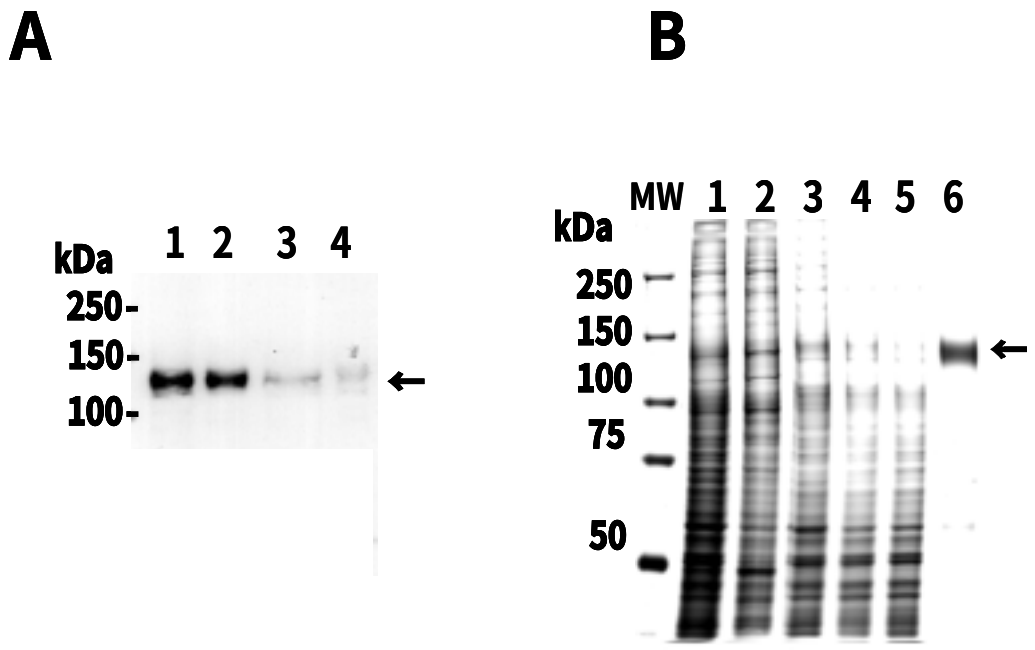

Fig. 1. **Solubilization and purification of human MDR1 expressed in insect cells.** Aliquots of each step from purification were subjected to SDS-PAGE on 8% polyacrylamide gel and visualized by penta-His antibody immunoblot(A) or silver staining(B). (A) Lane 1, microsome from humanMDR1 expressed Sf9 cells; lane 2, 0.8% DDM soluble microsomal protein; lane 3, 0.8% DDM insoluble protein; lane 4, Ni-NTA unbound fraction. (B)Lane 1, microsome from humanMDR1 expressed Sf9 cells; lane 2, peripheral proteins removed from microsomes by 0.5 M NaCl; lane 3, integral membrane proteins recovered by centrifugation after wash out the peripheral proteins; lane 4, 0.8% DDM soluble microsomal protein; lane 5, Ni-NTA unbound fraction; lane 6, Ni-NTA eluate (300 mM imidazole). 2 μg and 0.3 μg of proteins were loaded on lane 1-5 and lane 6, respectively. The allows show the position of MDR1

RESULTS

Expression and purification of human MDR1 in insect cells

Baculovirus encoding human MDR1 was generated as described in experimental procedures. Western blot analysis revealed that human MDR1 was successfully expressed in microsomes of insect cells (data not shown). To optimize the recombinant gene expression, Sf9 cells were infected with various multiplicity of infection (MOI) and recovered at the different periods after the infection. The expression levels reached maximum at 48h post

infection and further cultivation caused degradation of MDR1 (data not shown). The expression levels were similar when infected with MOI higher than five. From these observations, the expression procedures were fixed as MOI of 5 and recovery at 48h post infection. As shown in Fig. 1A, almost all MDR1 expressed in microsome was solubilized by 0.8% *n*-dodecyl-β-D-maltoside (DDM) and adsorbed by Ni-NTA agarose (Fig.1A). With the one-step purification protocol, human MDR1 was obtained with the purity of more than 95% judging from silver staining (Fig. 1B). Membrane proteins (350 mg) were obtained from 15 g of Sf9 cells grown in 1 L culture, and about 3.5 mg of the purified MDR1 protein was extracted from DDM-solubilized membrane. Because loss of MDR1 in each purification step appeared to be quite low, the expression level of MDR1 may be calculated as about 1% of total membrane protein.

ATPase activity of purified MDR1

To examine the quality of purified MDR1, ATPase activity of purified MDR1 was measured in the presence of well known substrate and inhibitors. Purified MDR1 was mixed with soybean crude lipid extract and subjected to ATP hydrolysis reaction as described in experimental procedures. The purified protein showed the typical ATPase activity of MDR1 (Fig. 2) as reported[12-16]: i) low basal activity, ii) a strong stimulation by verapamil, iii) dependence on Mg^{2+}, and iv) complete inhibition by phosphate-analogs ortho-vanadate (Vi) and beryllium fluoride (BeFx). These data indicate that purified protein retains its functional structure in transmembrane domains as well as nucleotide binding domains. Verapamil-induced ATPase activity was hardly detected without reconstitution to a liposome, suggesting the lipid environment is quite important for the function of MDR1 as reported before[12, 17]. The K_m value for ATP of verapamil-stimulated ATPase activity was 0.3 mM

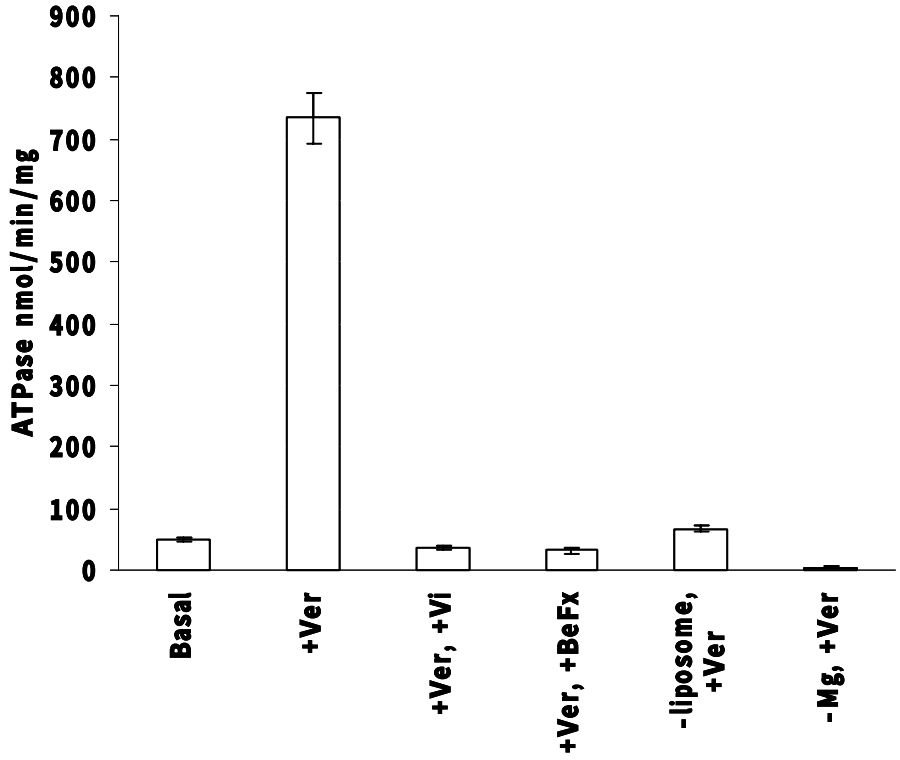

Fig. 2. **Characteristics of ATPase activity of purified human MDR1.** The purified MDR1 (100 ng) was reconstituted in liposome as described in Materials and Methods. Reactions were done in the absence or in the presence of 0.1 mM verapamil (Ver), 0.4 mM ortho-vanadate (Vi) and 0.1 mM beryllium fluoride (BeFx). ATPase activity was estimated by quantifying the released inorganic phosphate by colorimetric detection as described in Experimental procedures. Data are presented with the standard deviations (n =3).

(data not shown), also in good agreement with previously reported K_m values of MDR1 for ATP.

Expression and purification of SUR1 in insect cells

Next, the author tried to express SUR1 and SUR1 was successfully expressed in microsome in similar levels of MDR1. The ATP binding assay revealed that ATP binds to SUR1 tightly [18](data not shown). N-glycosidase treatment revealed that SUR1 was glycosylated with about 10 KDa sugar chain (data not shown). To find optimal detergent for purification, the author examined the solubility of SUR1 in various detergents (Fig. 3).

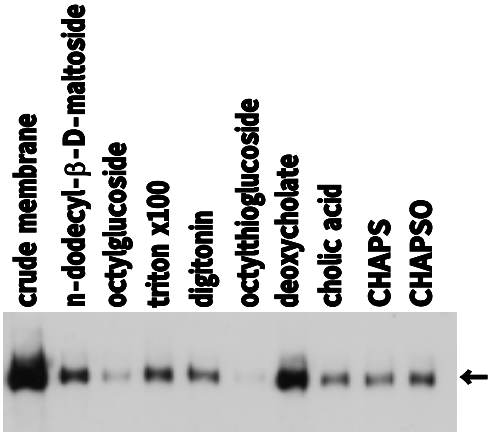

Fig.3. Solubilization of SUR1 expressed microsome. Microsome expressed SUR1 was incubated with indicated detergent (1%) and insoluble protein was removed by centrifugation. Soluble protein was subjected to SDS-PAGE on 7% polyacrylamide gel and visualized by penta-His antibody immunoblot. The allow shows the position of SUR1

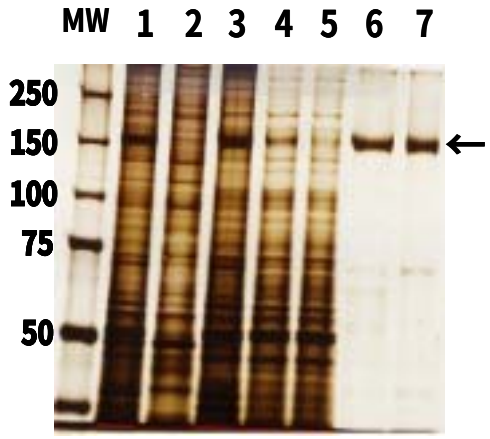

Fig. 4. Purification of wild type SUR1 and MM mutant. Aliquots of each step from purification were subjected to SDS-PAGE on 8% polyacrylamide gel and visualized by silver staining. Lane 1, microsome from SUR1 expressed Sf9 cells; lane 2, peripheral proteins removed from microsome by 0.5 M NaCl; lane 3, integral membrane proteins that recovered by centrifugation after wash out the peripheral proteins; lane 4, 1% DDM plus 0.4% sheep brain lipid soluble microsomal protein; lane 5, Ni-NTA unbound fraction; lane 6, Ni-NTA eluate (300 mM imidazole) of wild type SUR1; lane 7, Ni-NTA eluate of MM-mutant SUR1. 2 μg and 0.3 μg of proteins were loaded on lane 1-5 and lane 6,7, respectively. The allow shows the position of SUR1

Approximately 20% of SUR1 in microsome were solubilized by 1% DDM. The amounts of solubilized SUR1 were similar with triton X-100, digitonin, cholate, CHAPS, and CHAPSO. Deoxycholate was most effective for solubilizing SUR1 than any other detergents. However,

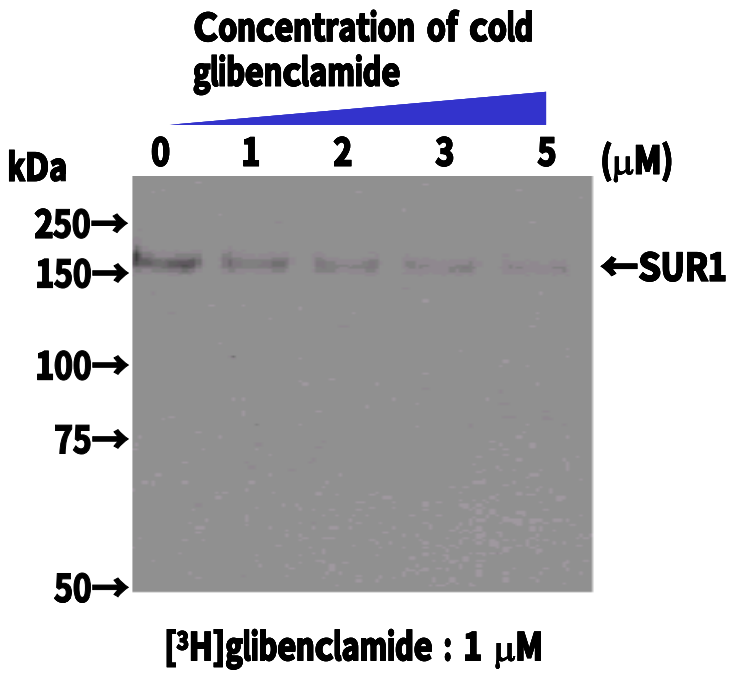

Fig. 5. Photoaffinity labeling of purified SUR1 by glibenclamide. Purified SUR1 and glibenclamide (1 μM [³H]-glibenclamide plus cold glibenclamide as above) was mixed and incubated in dark place at room temperature for 30 min. After UV irradiation, samples were electrophoresed with 7% SDS-polyacryl-amide gel. Label of [³H]-glibenclamide was detected with autoradiography.

the nonionic detergents are more suitable than ionic detergents for maintaining the protein activities, DDM was selected as a solubilizing agent for purification. Further analysis revealed that the best solubilizing method for SUR1 was the treatment with 1% DDM in the presence of 0.4% sheep brain lipid extract (data not shown). SUR1 was successfully purified by Ni-NTA agarose with 90% purity judging from silver stained SDS-PAGE gel slab (Fig. 4). The author also purified MM (K719M, K1385M) mutant of SUR1, in which the essential lysine residues for nucleotide binding in walker A sequences of both NBF1 and NBF2 were replaced by methionine. The purity of SUR1 mutant was slightly lower compared to the wild type because of its poor solubility with detergent.

Photoaffinity labeling of purified SUR1 by glibenclamide

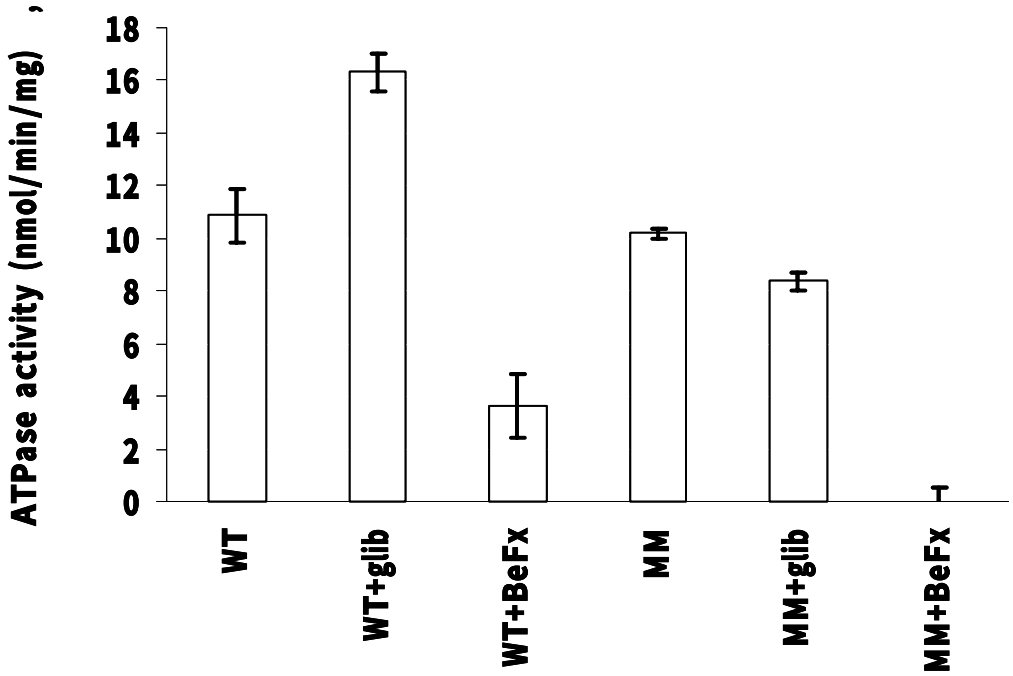

Fig. 6. Characterization of ATPase activity of purified SUR1. 50 ng of purified protein was reconstituted in 500 ng of soybean lipid and subjected to ATP hydrolysis reaction. ATPase activity of wild type (WT) and Walker A lysine mutant (MM) was measured in the presence of 10 μM of glibenclamide (Glib) or 125 μM of beryllium fluoride (BeFx). ATPase activity was estimated by quantifying released inorganic phosphate as described in experimental procedures. Data are presented with the standard deviations (n =3).

It has been demonstrated that SUR1 binds glibenclamide, a potent anti-diabetic sulfonylurea agent with a high affinity in pancreatic β-cells or in heterologously expressed mammalian cells [19, 20]. Thereby, the quality of purified SUR1 was examined in the ability of glibenclamide binding (Fig. 5). Purified SUR1 was photoaffinity labeled by 1 μM [^{3}H] glibenclamide and the labeling was inhibited by glibenclamide in a dose dependent manner.

ATPase activity of SUR1

Purified and reconstituted SUR1 showed ATPase activity of 11 nmol/min/mg (Fig. 6). ATPase activity was increased by the addition of 1 μM glibenclamide and partly inhibited by

125 μM of beryllium fluoride (BeFx). In contrast to ATPase activity of MDR1, ATPase activity of SUR1 did not affected by vanadate lower than 0.5 mM (data not shown). The purified SUR1 MM mutant showed similar ATPase activity to the wild type, although the key lysine residues for nucleotide binding in both NBFs were replaced to methionine. However, ATPase activity of MM mutant did not affected by glibenclamide and completely inhibited by 125 μM of BeFx. Tolbutamide also increased ATPase activity of the wild type but did not affect the activity of MM mutant (data not shown). By fitting the data into the Michaeris-Menten equation with least-square method, K_m value of the wild type SUR1 for tolbutamide was determined as 347 μM.

DISCUSSION

In this chapter the author described the establishment of the expression and purification techniques for ABC transporters. Baculovirus expression system has several advantages in expressing eukaryotic proteins compared to other expression systems. First, compared to the prokaryotic expression systems, the expressed protein is more close to its native one both in its structure and function. Second, compared to other eukaryotic expression systems, the higher expression levels can be achieved. Finally, because insect cells can grow well in suspension cultures, foreign proteins can be expressed in large scales.

Using the baculovirus expression system, MDR1 and SUR1 were successfully expressed in microsome and purified with more than 95% (MDR1) and 90% (SUR1) purity by a single purification step. The amount (mg protein from 1 L cultured cells) and the purity

are high enough not only for analyzing biochemical properties of ABC transporters but also for crystallization for the structural analysis.

To elucidate the qualities of purified proteins, ATPase activity of MDR1 and glibenclamide binding ability of SUR1 were examined. ATPase activity of purified and reconstituted MDR1 was significantly increased by the addition of verapamil and inhibited by well known MDR1 ATPase inhibitors, vanadate and BeFx. The verapamil-stimulated ATPase activity was comparable to that of MDR1 purified from mammalian cells[21]. The characteristics of ATPase activity clearly show that MDR1 retains its functional structure which recognizes the substrates in the drug-binding site and hydrolyzes ATP coupled to drug binding. It has been reported that the binding of glibenclamide is highly specific to SUR1 and requires functional structure. For example, the effect of glibenclamide on K_{ATP} channel formed by Kir6.2 and SUR2A was weak and reversible[22], although SUR1 and SUR2A are highly homologous in their amino acid sequences. Mutation of the single serine residue between TM15 and TM16 of SUR1 abolishes selective inhibition by sulfonylureas[23]. Thus, the specific binding of glibenclamide (Fig. 5) suggests that the structure of purified SUR1 is close to that of the native one.

In this study, the author provides the first direct evidence that SUR1 functions as ATPase. ATPase activity of purified and reconstituted SUR1 was calculated as 11 nmol/min/mg. The activity is similar to that of maltose-binding protein conjugated NBF2 of SUR2A (18 nmol/min/mg)[24] and CFTR (50-70 nmol/min/mg)[25, 26]. The turnover rate of ATP hydrolysis by SUR1 was as low as one-fiftieth of verapamil-stimulated ATPase activity of MDR1 purified by the same procedure (Fig.6 compared to Fig.2) and previously reported activity(1000-6500 nmol/min/mg)[13, 27-29]. The functional difference between MDR1 and SUR1 may well explain the different rate of ATP hydrolysis by these two proteins.

Because the transport cycle of MDR1 was fueled by ATP hydrolysis, the higher ATPase activity is required for efficient transport. On the other hand, like GTP-binding proteins, SUR1 acts as a molecular switch which controls the channel gating. It is proposed that SUR1 binding ATP keeps the channel close and SUR1 binding ADP opens the channel. If it is the case, single ATP hydrolysis cycle is sufficient for channel activation. It is conceivable that the ATPase activity of SUR1 is lower than that of MDR1.

Based on the results of this chapter, the author proposes the model for the mechanism of K_{ATP} channel gating and action of sulfonylureas (Fig.7). K_{ATP} channel is activated when SUR1 binds ATP at NBF1 and ADP at NBF2 (state II). Other states （I, III, IV, and V) are inactive. Under physiological conditions, the intracellular concentration of ATP is thought to be several mM, which is more than 10-fold higher than that of ADP. Therefore, SUR1 is supposed to be mainly in state I, in which ATP binds to both NBFs. However, because SUR1 has low ATPase activity, which the author speculates to be derived from NBF2, bound ATP is hydrolyzed to ADP at NBF2, state I constantly becomes state II (the active state). Because nucleotide binding affinity of NBF2 for ATP is high, ATP would not dissociate from NBF2 without ATP hydrolysis. When the MgADP dissociates from NBF2 followed by binding of MgATP, the three states (I, II, and III) of SUR form a cycle. Therefore, ATP hydrolysis at NBF2 is important to determine the open probability of K_{ATP} channel current.

Potassium channel blockers which specifically bind to SUR1, increased ATPase activity. It was reported that sulfonylurea destabilizes the ATP binding to NBF1[30]. Recently, crystal structures of NBF domain revealed that two NBFs form a stable dimer which holds two nucleotides within a dimer interface[31-33]. Because these nucleotides are essential for dimer formation, destabilization of ATP binding to NBF1 by sulfonylurea may destabilize the MgADP binding to NBF2 simultaneously. The release of nucleotide from NBF2 would

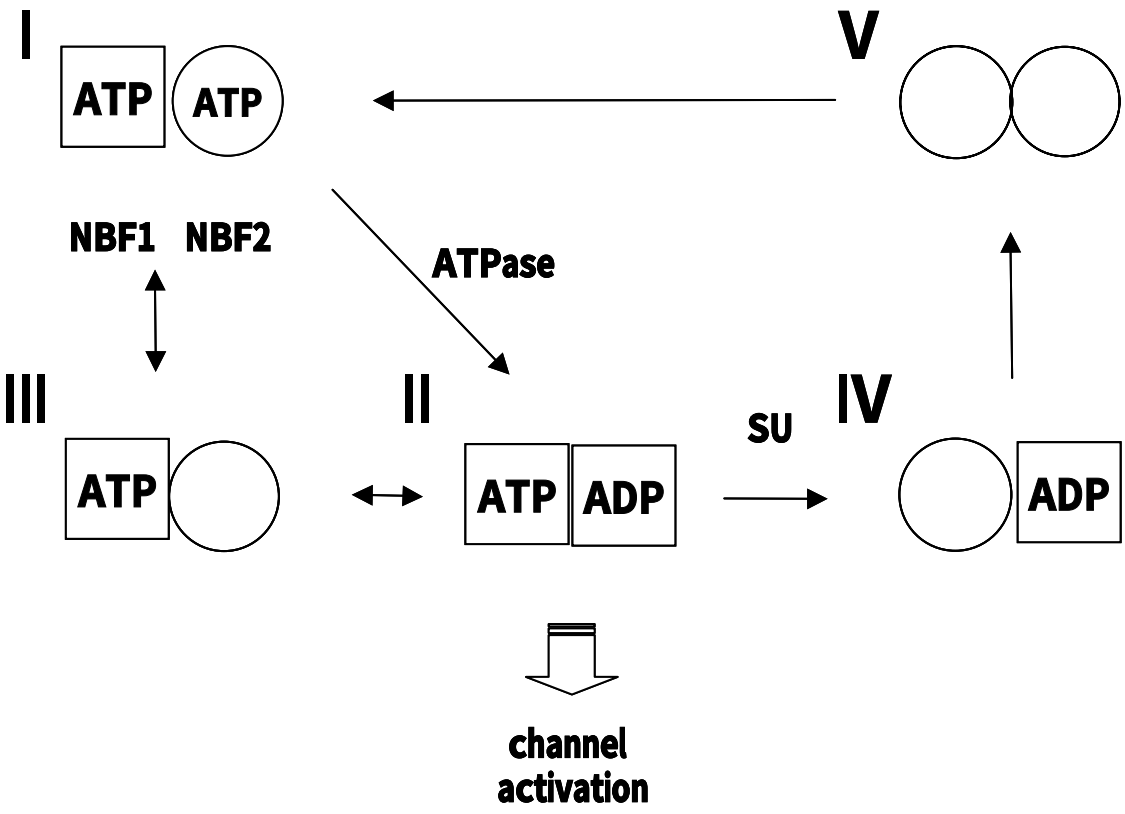

Fig. 7. Model for nucleotide activation of the K_{ATP} channel by SUR1 subunit. NBFs in the activate and inactive states are depicted as squares and circles, respectively. Under physiological conditions (in the presence of high levels of intracellular ATP), ATP binds to both NBFs (state I). ATP hydrolysis at NBF2 by intrinsic ATPase activity directly activates the channel (state I to II). Double headed arrows indicate equilibrium reactions. Sulfonylurea (SU) causes destabilization of ATP binding on NBF1 and promote a release of ADP from NBF2, concomitantly. The release of nucleotides from NBFs prompts new ATP hydrolysis cycle (state I, II, IV and V).

promote another ATP hydrolysis cycle (The cycle of states I, II, IV, and V), thus the apparent stimulation of ATP hydrolysis would be observed.

It has been shown that the dose-response curve for tolbutamide is two-phased with high and low affinity sites[34]. The K_m value (347 μM) of ATPase activity for tolbutamide is 70-fold higher than the K_i value of high-affinity block of channel current. K_{ATP} channel contains four sulfonylurea binding sites within a single channel. If binding of sulfonylurea to one site per channel complex is sufficient to close the channel as reported previously[35],

the K_i value of channel current is estimated as 10-fold lower than binding constant. Furthermore, SUR1 was purified without the channel subunit Kir6.2 in this study. It is suggested that inter-subunit interaction between SUR1 and Kir6.2 is important for ligand binding and channel gating of K_{ATP} channel [36, 37]. Thus it would be conceivable that the binding of tolbutamide to SUR1 alone is weaker than to SUR1-Kir6.2 complex.

It is unexpected that MM mutant of SUR1 showed ATPase activity as the wild type because the lysine residue in walker A motif is thought to be important for ATP binding[18, 38]. However, in contrast to the wild type SUR1, the ATPase activity of the mutant was not affected by glibenclamide and completely inhibited by BeFx. These results suggest that amino acid substitution of lysine residue alters the conformation of the ATP binding site, which does not affect the basal ATPase activity but affects the coupling between drug binding and ATP hydrolysis.

Recently, it became clear that many ABC transporters are involved in cellular lipid homeostasis[39]. It is difficult to characterize the transport activity of these transporters because of high hydrophobicity of their transport substrates. In addition, there are many ABC transporters whose functions are still obscure. These transporters also require ATP hydrolysis for substrate transport, measuring its specific ATPase activity would be efficient for elucidating these transporters. The baculovirus expression and purification system described here, may be a powerful tool to facilitate our understandings of various ABC transporters.

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Chapter 2

Development of a microanalysis method for ATPase of ABC transporters

MDR1 is a plasma membrane-located protein that confers multidrug resistance to mammalian cells by actively transporting various compounds out of cells. MDR1 is clinically important not only because it is involved in multidrug resistance of cancer cells [1, 2], but also because it affects the pharmacokinetics of various drugs [3]. MDR1 in the mucosal epithelium of intestinal cells is a major limiting factor for the rate of uptake of some drugs from the intestinal lumen [4-7]. Screening of drugs which are not transported by MDR1 is important for developing drugs of high oral availability. Because drug transport by MDR1 is dependent on ATP hydrolysis and because most substrates transported by MDR1 stimulate ATP hydrolysis, examining the effect on ATPase activity is imperative for understanding the interaction between drugs and MDR1. However, no reliable and convenient screening system based on ATPase activity of human MDR1 has been developed yet.

The commonly used method to measure ATPase activity is the spectrometric measurement of phosphomolybdate complexes formed by the reaction between ammonium molybdate and inorganic phosphate released from ATP (Pi-Mo method) [8]. However, the Pi-Mo method is not sensitive enough to measure low ATPase activity, especially if a large

amount of purified enzyme is not available. Luciferase assay is highly sensitive to quantify ATP, but it can not be used to measure low ATPase activity at physiological concentrations (2-3 mM) of ATP. Reversed-phase high-performance liquid chromatography (HPLC) has been reported as a highly sensitive method to measure amounts of ATP and ADP with linearity over a wide range from 0.05 to 10 nmol [9]. However, because these methods took 10 to 15 min for one assay, it may not be applicable for high through-put screening[9-11]. Determination of radioactive phosphate from [^{32}P]ATP is sensitive, but this method needs extra radioisotope facilities.

TiO_2 selectively adsorbs organic phosphates as reported by Matsuda *et al*. [12], and can be used to separate adenine nucleotides. The author applied HPLC equipped with a titanium dioxide column for measuring ATPase activity of human MDR1. This column greatly improved the sensitivity and reduced the time required for each assay.

EXPERIMENTAL PROCEDURES

Materials- L-α-lecithin, ATP, ADP, AMP and adenosine were from Sigma-Aldrich. Titanium dioxide column (Titansphere^{TM} TiO) was purchased from GL Sciences.

Purification of MDR1- Microsomes were thawed on ice and diluted to 4 mg protein/ml with ice cold buffer A plus protease inhibitors. An equal volume of 1.2% w/v DDM in buffer A plus protease inhibitor was added to the solution, and the suspension was mixed by inversion and kept on ice for 10 min. Insoluble proteins were removed by centrifugation (60,000 x *g*, 30 min). Then, Ni-NTA agarose resin (Qiagen) was added to the supernatant at a ratio of 0.5 ml of resin per 100 mg of solubilized microsomal protein. The slurry was incubated at

4°C for 18 h with continuous rotation. The resin was washed with a 10-bed volume of buffer A containing 0.1% DDM three times and then with five volumes of buffer A containing 0.1% DDM plus 40 mM imidazole. MDR1 was eluted with five volumes of buffer A containing 0.1% DDM plus 300 mM imidazole. Protein concentration was estimated by the Bradford method (Bio-Rad).

ATP hydrolysis reaction- MDR1 was purified and reconstituted in liposomes as described in Experimental Procedures of chapter 1. Reconstituted protein was reacted in 15 µl of 40 mM Tris-Cl (pH7.5), 0.1 mM EGTA, 1 mM NaATP, 1 mM $MgCl_2$ and 0.1 mM verapamil in the presence or absence of 0.4 mM ortho-vanadate and 0.1 mM beryllium fluoride at 37 °C for 30 min. The reaction was stopped by heating the reaction mixture at 100 °C for 5sec by thermal cycler PTC-200 (MJ-Reserch) in the case of HPLC detection, and by adding an equal volume of 12% SDS in the case of colorimetric detection. MDR1 completely lost its ATPase activity with these treatments. For HPLC detection, 5 µl of heat inactivated sample was analyzed.

HPLC apparatus and chromatographic conditions- The liquid chromatographic equipment consisted of LC-10ADVP pump and SPD-M10AVP diode array detector (Shimadzu). Data were recorded and analyzed with CLASS-VP software system Version 5.03 (Shimadzu). The detection wavelength was set at 260 nm. ATP and ADP were separated on a Titansphere TiO column (4.6 mmID x 100 mm, GL Sciences). Chromatographic determination was performed at 40°C and at a 2 ml/min flow rate. The mobile phase was composed of NaH_2PO_4 buffer (50 mM, pH 7) and 50% v/v acetonitrile (Wako).

Colorimetric detection-The colorimetric detection was done as described previously [8]. After the reaction was stopped by SDS, 10 µl of H_2O was added on sample. Color

development started with the addition of 40 μl of equal mixture of 1% ammonium molybdate and 6% ascorbic acid in 1 N HCl. After 7 min incubation at room temperature, 60 μl of 2% sodium citrate, 2% sodium arsenite, and 2% acetic acid were added and incubated at 37^{o}C for 10 min. The color absorbance was measured at 850 nm by UV-VIS spectrophotometer UV mini 1240 (Shimadzu).

RESULTS

Nucleotide separation with a titanium dioxide column

It has been reported [9] that ATP and ADP can be separated with a reversed-phase HPLC column with linearity over a wide range from 0.05 to 10 nmol. However because the method with a reversed-phase HPLC column takes about 10-15 min for one assay [9-11], it may not be applicable for high through-put screening. Therefore, the author tried to use a titanium dioxide column. A chromatogram of nucleotides is shown in Fig. 1A. Adenosine, AMP, ADP, and ATP were separated within 3 min, and the retention time of each compound was 0.62 min, 0.92 min, 1.39 min, and 2.37 min, respectively. The relationship between ADP amount and chromatogram peak area was linear from 5 pmol to 10 nmol with a coefficient of determination (r^2) of 1.00 as shown in Fig. 1B.

Sensitivity of titanium dioxide column method to drug-stimulated ATPase activity

Because ADP, as low as 5 pmol, can be detected with titanium dioxide column method (Fig. 1B), this new method is expected to be much more sensitive in measuring ATPase activity than conventional ones. We examined the sensitivity of this method to measure drug-stimulated ATPase activity of purified MDR1 and compared it to the Pi-Mo

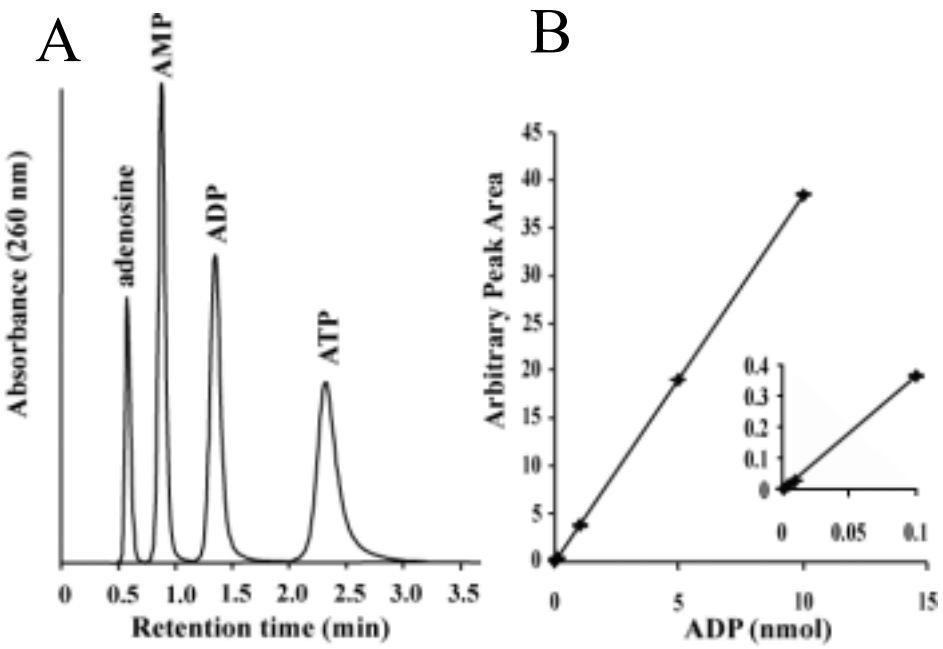

Fig.1. Nucleotide separation by a titanium dioxide column and relationship between chromatogram peak area and amount of ADP. (A) Separation of nucleotide. The mixture of nucleotide (adenosine: 5 nmol, AMP, ADP, ATP: 10 nmol) was injected to titanium dioxide column (4.6 mmID x 100 mm) at 0 min. Separation was carried by 50 mM Na-Pi buffer (pH7.0), 50% v/v acetonitrile at 40ºC. Absorbance at 260 nm was recorded. Adenosine, AMP, ADP, and ATP were eluted at 0.62 min, 0.92 min, 1.39 min and 2.37 min, respectively. (B) Relationship between peak area and amount of ADP. The inset shows a relationship at low concentrations of ADP. The straight line was drawn by linear regression. Experiments were done in triplicate.

method. The relationships between amount of the purified MDR1 and released ADP measured by these methods were both linear with a coefficient of determination (r^2) of 0.977 (Fig. 2A) and 0.971, respectively (Fig. 2B). The drug-stimulated ATPase activity of MDR1 was clearly detected with 0.5 ng of the purified protein with titanium dioxide column method; however, at least 10 ng was required with Pi-Mo method.

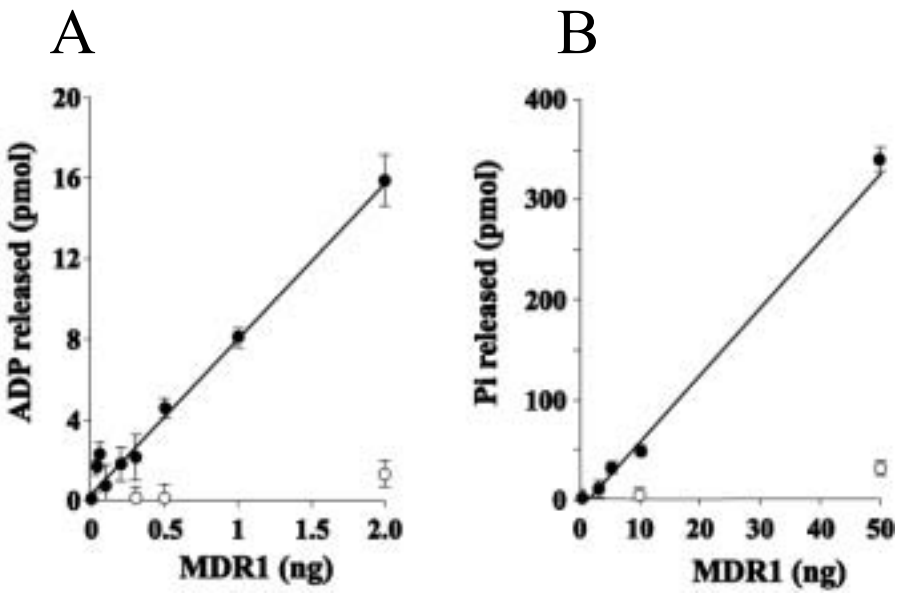

Fig.2. **Relation between released ADP and amount of the purified MDR1.** (A) Amount of released ADP was measured by using HPLC. (B) Amount of inorganic phosphate was measured by Pi-Mo method. Reaction was done in the presence (●) or in the absence (○) of 100 μM verapamil. The coefficients of determination (r^2) were 0.977 (A) and 0.971 (B), respectively. The straight line was drawn by linear regression. Standard deviations (N=3) are shown by bars.

DISCUSSION

In this chapter, the author describes a novel method for measuring the ATPase activity of human MDR1 using purified protein and HPLC equipped with a titanium dioxide column. This method is highly sensitive to detect any subtle changes in ATPase activity of MDR1 induced by drugs, and is suitable for automated high through-put screening.

Application of HPLC equipped with a titanium dioxide column in measuring ADP made it possible to reduce the amount of the purified MDR1 required for the reaction to one-twentieth of the amount used in conventional colorimetric inorganic phosphate detection assays[8]. From a 1L-culture of Sf9 cells, 3.5 mg of the purified human MDR1 can be obtained, allowing more than 3 million reactions to occur. Furthermore, the titanium

dioxide column showed good separation of nucleotides in much less elution time compared to the reversed phase HPLC analysis previously reported.

Measuring ATPase activity by using the purified MDR1 has several advantages. This assay system shows very low basal ATPase activity, and is suitable for detecting stimulation of MDR1 ATPase. This assay system does not include any inhibitors to repress other endogenous membrane ATPase unlike other assay systems, such as using microsomes from Sf9 insect cells expressing human MDR1[13, 14]. The direct effects of drugs on MDR1 ATPase can be measured by this method, making the interpretation of results much simpler.

MDR1 is an important target for developing drugs, not only because it is involved in multidrug resistance of tumor cells but also because it may be involved in tumorigenesis as reported recently[15, 16]. The effective screening system, reported here, for drugs interacting with MDR1 may facilitate the development of new chemotherapeutic drugs for preventing multidrug resistance and tumorigenesis and also for developing drugs of high oral availability.

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Chapter 3

Modulation of drug-stimulated ATPase activity of human MDR1/P-glycoprotein by cholesterol

The most fascinating feature of MDR1 is its broad specificity for transport substrates. Unlike other specific transporters, MDR1 recognizes structurally unrelated compounds and expels them from cells[1, 2]. To date, high resolution structural information about MDR1 is not available, it is not known how MDR1 recognizes such structurally diverse compounds. However, several biochemical studies revealed that MDR1 possesses multiple drug binding sites [3-5] and these sites are located at the middle of lipid bilayer [6]. Shapiro *et al.* demonstrated that MDR1 possesses at least three positively cooperating drug-binding sites [4]. The H site is selective for Hoechst 33342 and colchicine, and the R site is selective for rhodamine 123 and anthracyclines, and progesterone binds to another site. Drug binding to one site stimulates transport by the other. Moreover, rhodamine 123 and progesterone in combination stimulate the transport of Hoechst 33342 in an additive manner. Martin *et al.* [3] also assigned four drug-binding sites, three of which were classified as sites for transport and another for regulation of the transport.

Recently, many ABC proteins are reported to function in lipid homeostasis. For example, ABCG5 and ABCG8 mediate the efflux of cholesterol and sitosterol from the intestine and hepatocytes into the intestinal lumen and bile duct [7]. ABCA1 mediates efflux of

cholesterol and phospholipids to form high density lipoprotein (HDL) [8, 9]. ABCB4 (MDR2), being highly homologous with MDR1, functions in the secretion of phosphatidylcholine into bile duct from hepatocytes [10]. Therefore, it is conceivable that MDR1 also interacts with membrane lipids. Indeed, it has been reported that cholesterol stimulates basal (i.e. without any drugs) ATPase activity [11, 12], and that cholesterol is recognized and transported as an endogenous substrate of MDR1 [13]. It was also shown that depletion of cholesterol reduced the transport activity of MDR1 resulting in the intracellular accumulation of drugs in cells [12, 14, 15].

In this chapter, the author analyzed the ATPase activity of MDR1 using purified human MDR1 reconstituted in liposomes containing 0-20% (w/w) cholesterol. Cholesterol increases the basal ATPase activity, and affects the drug-stimulated ATPase activity of MDR1. The effects differ from one drug to another and can be classified into five types, suggesting that cholesterol directly regulates substrate recognition and transport by MDR1.

EXPERIMENTAL PROCEDURES

Materials-Sf9 cells were obtained from Pharmingen. Lipids and ATP were purchased from Sigma-Aldrich. Media, pluronic F-68, and gentamycin were obtained from Invitrogen. *n*-Dodecyl-β-D-maltoside (DDM) was purchased from Anatrace. Ni-NTA agarose was from Qiagen. Other compounds were from Sigma-Aldrich or WAKO.

Expression and purification of human MDR1-A sequence encoding a thrombin- cleavage site, ten histidine codons, and a termination codon was inserted at the 3' end of human MDR1 cDNA [16]. The modified MDR1 was expressed in insect cells with the use of a recombinant baculovirus and purified by Ni-NTA chromatography as described in chapter 1.

Reconstitution into proteoliposomes- Phosphatydilcholine (PC), phosphatydil- ethanolamine (PE), phosphatydilserine (PS) and cholesterol were dissolved in chloroform mixed in a proper ratio, and dried under high vacuum for over 2.5 h to remove the chloroform. The lipid film was resuspended at 10 mg/ml in 40 mM Tris-Cl (pH7.4), 0.1 mM EGTA by sonication in a bath sonicator (Bioruptor CD-200 TM, COSMO BIO) until the suspension clarified. After sonication, lipids were kept on ice for 24 h and subjected to two cycles of freeze-thawing. Finally, lipids were sonicated again (5 cycles, 30 sec each. 2 min rests on ice between cycles). Lipid stocks were used within a week. For reconstitution, purified protein and lipid stocks were mixed at a protein: lipid ratio of 1:10 and incubated at 23ºC for 20 min, and sonicated for 15 sec in a bath sonicator as described in chapter 1.

MDR1 ATPase activity-The ATPase reaction was performed as described in chapter 2 with minor modifications. Reconstituted protein was reacted in 20 μl of 40 mM Tris-Cl (pH7.5), 0.1 mM EGTA, 1 mM NaATP, 1 mM $MgCl_2$ and various concentrations of drugs at 37ºC for 30 min. The reaction was stopped by adding 10 μl of 3% SDS and 10 mM vanadate instead of heating the reaction mixture at 100ºC.

Enzymatic parameters-The experimental data were computer-fitted to the Michaelis-Menten equation, $v = V_{max}[S]/(K_m + [S])$, where v is drug-stimulated ATPase activity and [S] is the drug concentration. Fitting was carried out using the least−squares method (KaleidaGraph) and values for the V_{max} and K_m were extracted.

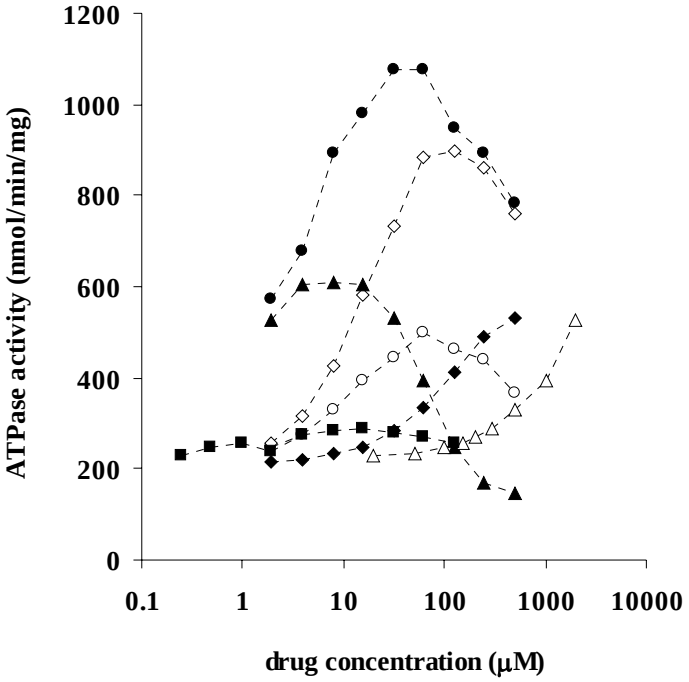

Fig.1. Drug-dependent ATPase activity of purified MDR1. Purified MDR1 was reconstituted in PC: PE: PS = 4: 4: 2 liposomes and ATPase activity was measured as described in experimental procedures. ●, verapamil; ○, rhodamine B; ◆, digoxin; ◇, rhodamine 123; ▲, vinblastine; △, colchicine; ■, paclitaxel.

RESULTS

ATPase activity of purified human MDR1

Human MDR1, fused with a histidine tag at the C-terminus, was expressed at high levels in insect cells with a MDR1 recombinant baculovirus, and purified as described in chapter 1 with minor modifications. MDR1 was extracted with 0.8% DDM, and extracted MDR1 was purified by Ni^{2+} affinity chromatography. MDR1 was recovered from the resin with 300 mM imidazole with a purity of more than 95% judging from silver staining (data not shown).

Purified MDR1 was reconstituted in liposomes (PC:PE:PS = 4:4:2), and ATPase activity was measured by HPLC with a titanium dioxide column as described in chapter 2. Various compounds increased ATPase activity (Fig. 1). A typical concentration-dependence with a

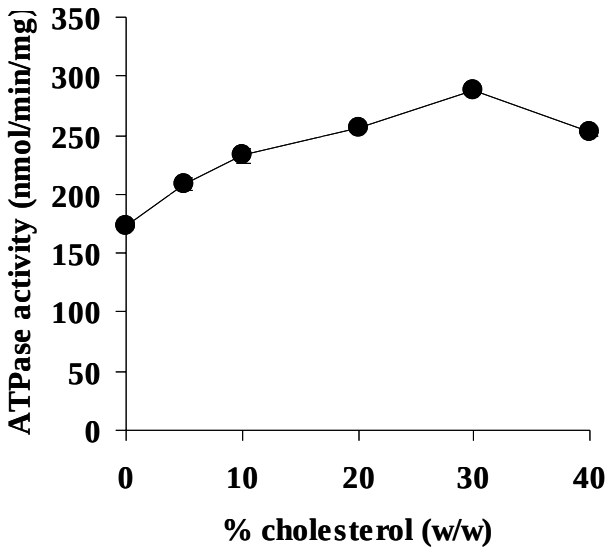

Fig. 2. Effects of cholesterol on basal ATPase activity. Purified MDR1 was reconstituted in PC: PE: PS = 4: 4: 2 liposomes containing 0, 5, 10, 20, 30 or 40% (w/w) cholesterol. Data are presented with the standard deviation (n = 3).

bell-shaped curve [17, 18] was obtained with verapamil and rhodamine 123: the ATPase activity increased as the concentration rose and peaked at about 30 μM and 125 μM, respectively, whereas it was rather suppressed at higher concentrations. Vinblastine stimulated MDR1 ATPase activity at 10 μM or less but showed strong inhibitory effects at higher concentrations and suppressed ATPase activity below the basal level (about 200 nmol/min/mg) at 200 μM or more. Colchicine increased the ATPase activity as its concentration rose, but maximal activity was not obtained even at 2 mM. Without the reconstitution to liposomes, ATPase activity was not stimulated by the addition of substrate drugs (data not shown), suggesting the lipid environment is quite important for the function of MDR1 as reported before [11].

Effects of cholesterol on MDR1 ATPase activity

It has been suggested that the basal ATPase activity of human MDR1 in native membrane

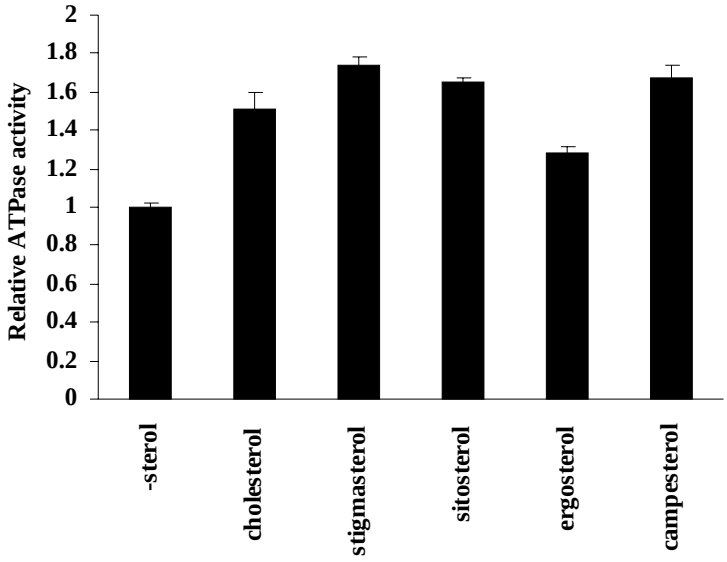

Fig. 3. Effects of sterols on basal ATPase activity. Purified MDR1 was reconstituted in liposomes containing 20% (w/w) cholesterol, stigmasterol, β-sitosterol, ergosterol or campesterol. Relative ATPase activity was presented with respect to that in the absence of sterol with the standard deviation (n = 3).

vesicles is highly dependent on the presence of cholesterol [12, 13], and also that the basal ATPase activity of partially purified hamster MDR1, reconstituted in PC:PE (9:1) liposomes, is dependent on the presence of cholesterol [11]. The author examined whether cholesterol affects the ATPase activity of purified human MDR1 by reconstituting the protein in liposomes (PC: PE:PS=4:4:2) containing various concentrations of cholesterol (Fig. 2). The ATPase activity increased as concentration of cholesterol rose and peaked at 30% (w/w) cholesterol. In the presence of 30% cholesterol, the ATPase activity of MDR1 was 1.7-fold that in the absence of cholesterol. This suggests that cholesterol directly interacted with MDR1 at drug-binding sites. Alternatively, cholesterol may have affected the lipid environment, fluidity for example, and indirectly increased the turnover of basal ATP hydrolysis.

Effects of various sterols on basal activity

To consider the possibility of an indirect effect of cholesterol on MDR1, the specificity of sterol species was examined (Fig. 3). Stigmasterol, sitosterol, and campesterol stimulated the MDR1 ATPase activity as efficiently as cholesterol. Ergosterol was less effective than other sterols. The specific effect of sterols on MDR1 ATPase activity may support the direct interaction of sterols with MDR1.

Effects of cholesterol on drug-stimulated ATPase activity of MDR1

MDR1 has been suggested to possess multiple drug-binding sites and a drug binding to one site allosterically modulates drugs binding to other sites [3-5]. Thus, if cholesterol directly interacts with MDR1 at substrate-binding sites, the effects of cholesterol on the drug-stimulated ATPase activity of MDR1 would be differ from one drug to another. Therefore, the author examined the drug-stimulated ATPase activity of MDR1 reconstituted either in liposomes (PC:PE:PS = 4:4:2) or in liposomes containing 20% (w/w) cholesterol. In the cases of rhodamine 123 and digoxin, the presence of cholesterol little affected the V_{max} of ATPase activity but significantly lowered the K_m value from 21 to 10 μM (Fig. 4A, Table I) and from 181 to 76 μM (Fig. 4B, Table I), respectively.

In contrast, the presence of cholesterol little affected the K_m for paclitaxel, but increased the V_{max} from 132 to 258 nmol/min/mg (Fig. 4C, Table I). These results suggested that effects of cholesterol on the drug-stimulated ATPase activity of MDR1 differ from one drug to another, and the presence of cholesterol increases the affinity of MDR1 for rhodamine 123 and digoxin, whereas it increases the paclitaxel-induced hydrolysis of ATP by MDR1. K_m values for ATP were not affected by cholesterol in the absence or presence of paclitaxel (data not shown).

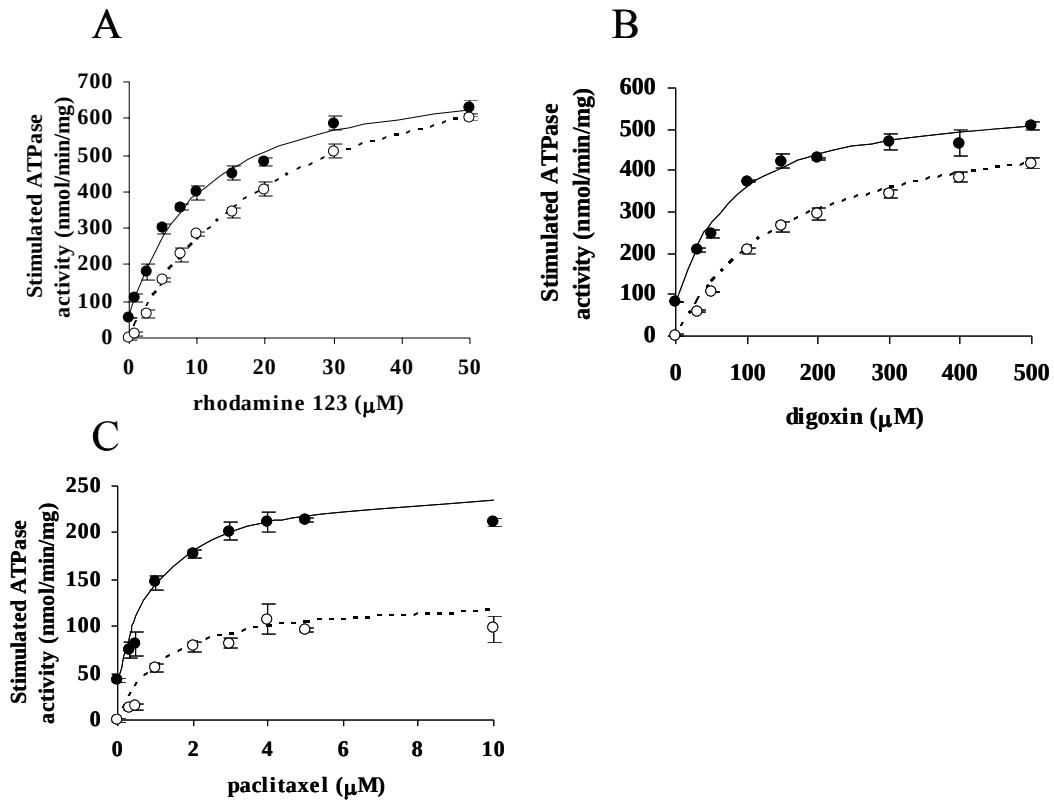

Fig. 4. The effect of cholesterol on drug-stimulated ATPase activity. Purified MDR1 reconstituted in liposomes containing 0% (open symbols) or 20% (filled symbols) cholesterol was reacted in the presence of various concentration of drugs. (A) rhodamine 123, (B) digoxin, (C) paclitaxel. Experiments were done in triplicate and averages and standard deviations are shown. After subtracted the basal (without drug and cholesterol) activity, data were fitted to the Michaelis–Menten equation and K_m and V_{max} values were extracted. Lines represent calculated best-fit curves.

To further analyze effects of cholesterol on the drug-stimulated hydrolysis of ATP by MDR1, MDR1 was reconstituted in liposomes containing 0, 5, 10, or 20% (w/w) cholesterol and the enzymatic parameters of ATPase activity for ten drugs, rhodamine 123, verapamil, dexamethasone, digoxin, nicardipine, rhodamine B, paclitaxel, vinblastine, vincristine, and valinomycin, were examined (Table I). Effects of cholesterol on K_m and V_{max} values of MDR1 ATPase activity differ from one drug to another, and may be classified into five types. The first type involves rhodamine 123, dexamethasone, verapamil, and digoxin: the K_m decreases in the presence of 20% cholesterol, but the V_{max} is little affected. The second type

Table I: K_m and V_{max} values of drug-stimulated ATPase activity of MDR1 reconstituted in liposomes containing various concentrations of cholesterol

Drug (MW)	cholesterol (%, w/w)			
	0	5	10	20
Rhodamine 123 (345)				
K_m (μM)	21 ± 2	16 ± 2 (76)	13 ± 1 (62)*	10 ± 1 (48)*
V_{max} (nmol/min/mg)	874 ± 28	886 ± 45 (101)	983 ± 24 (112)	757 ± 15 (87)
Dexamethasone (392)				
K_m (μM)	826 ± 139	527 ± 88 (64)*	423 ± 63 (51)*	394 ± 3 (48)*
V_{max} (nmol/min/mg)	690 ± 67	586 ± 68 (85)	620 ± 39 (90)	723 ± 23 (105)
Verapamil (455)				
K_m (μM)	4.1 ± 0.4	3.8 ± 0.3 (93)	2.7 ± 0.0 (66)*	2.2 ± 0.2 (54)*
V_{max} (nmol/min/mg)	690 ± 23	637 ± 9 (92)*	719 ± 4 (104)	646 ± 12 (94)
Digoxin (781)				
K_m (μM)	181 ± 11	120 ± 8 (66)**	111 ± 20 (61)**	76 ± 7 (42)**
V_{max} (nmol/min/mg)	578 ± 20	598 ± 2 (103)	633 ± 26 (110)*	576 ± 26 (100)
Rhodamine B (444)				
K_m (μM)	14 ± 3	9 ± 3 (64)*	11 ± 1 (79)	7 ± 1 (50)**
V_{max} (nmol/min/mg)	233 ± 21	291 ± 23 (125)*	305 ± 23 (131)*	314 ± 3 (135)*
Nicardipine (480)				
K_m (μM)	4.9 ± 0.3	3.0 ± 0.3 (61)**	1.9 ± 0.1 (39)**	1.2 ± 0.0 (24)**
V_{max} (nmol/min/mg)	437 ± 4	636 ± 18 (146)**	622 ± 8 (142)**	510 ± 17 (117)*
Vinblastine (811)				
K_m (μM)	1.7 ± 0.3	1.3 ± 0.1 (76)	1.2 ± 0.1 (71)	1.6 ± 0.3 (94)
V_{max} (nmol/min/mg)	347 ± 5	368 ± 7 (106)*	304 ± 2 (88)**	297 ± 2 (86)**
Vincristine (825)				
K_m (μM)	3.7 ± 0.6	2.7 ± 0.4 (73)	2.8 ± 0.4 (76)	3.9 ± 0.2 (105)
V_{max} (nmol/min/mg)	199 ± 20	307 ± 11 (154)	215 ± 6 (108)	196 ± 6 (98)
Paclitaxel (854)				
K_m (μM)	1.3 ± 0.2	1.4 ± 0.1 (108)	1.5 ± 0.1 (115)	1.1 ± 0.1 (85)
V_{max} (nmol/min/mg)	132 ± 15	136 ± 1 (103)	180 ± 3 (136)*	258 ± 2 (195)**
Valinomycin (1111)				
K_m (μM)	2.5 ± 0.2	2.2 ± 0.1 (88)	2.6 ± 0.2 (104)	3.5 ± 0.1 (140)**
V_{max} (nmol/min/mg)	400 ± 2	408 ± 13 (102)	508 ± 7 (127)**	418 ± 9 (105)

Values are average ± standard deviation (n=3). Relative values with respect to control (0% cholesterol) are shown in parentheses.
*P<0.05 with respect to control (0% cholesterol)
* * P<0.01 with respect to control (0% cholesterol)

involves nicardipine and rhodamine B: the K_m decreases and the V_{max} increases in the presence of cholesterol. The third type involves vinblastine and vincristine: neither the K_m nor the V_{max} is affected greatly. The forth type involves paclitaxel: the K_m is not much affected and the V_{max} increases in the presence of cholesterol. The last type involves valinomycin: the K_m increases in the presence of cholesterol, and the V_{max} is little affected.

Effect of cholesterol on steroid-stimulated ATPase activity of MDR1

The author initially presumed that cholesterol would compete with dexamethasone at the shared binding site and increase the K_m value for dexamethasone. However as shown in

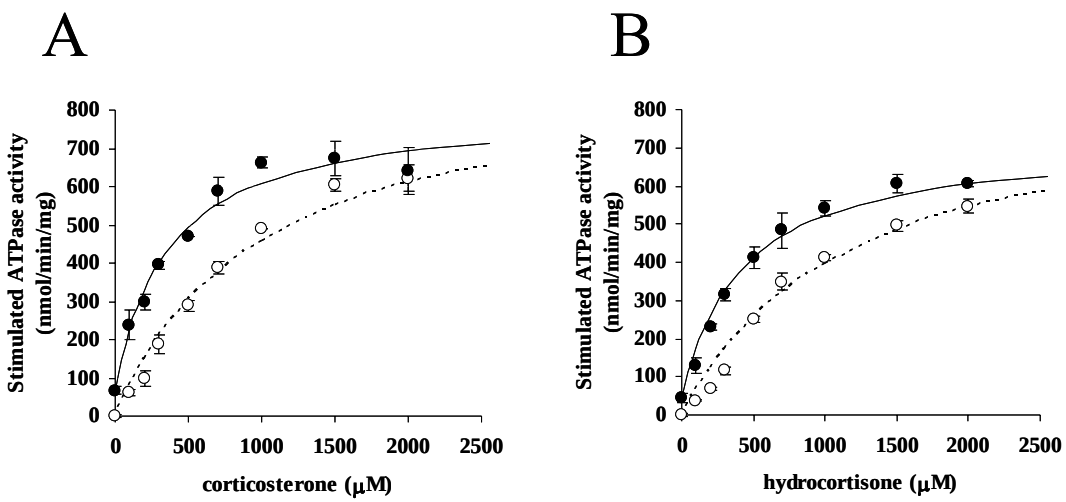

Fig. 5. Effects of cholesterol on corticosterone and hydrocortisone-stimulated ATPase activity. Purified MDR1 reconstituted in liposomes containing 0% (open symbols) or 20% (filled symbols) cholesterol was reacted in the presence of corticosterone (A) or hydrocortisone (B). Experiments were done in triplicate and average and standard deviations are shown. Lines represent calculated best-fit curves.

table I, cholesterol decreased the K_m value for dexamethasone, indicating that the binding site for cholesterol is different from that for dexamethasone. To further examine the effect of cholesterol, the author analyzed effects on the ATPase activity of MDR1 stimulated by corticosterone and hydrocortisone (Fig. 5). K_m values for both corticosterone and hydrocortisone decreased from 1007 μM to 365 μM and from 1175 μM to 422 μM, respectively when reconstituted in liposomes containing 20% cholesterol and these steroids were classified into type I drugs.

Effects of sterols on paclitaxel-stimulated ATPase activity of MDR1

Finally, the author examined effects of various sterols on the paclitaxel-stimulated ATPase activity of MDR1 (Table II). Sitosterol and campesterol increased V_{max} values to a similar extent as cholesterol. Interestingly, stigmasterol stimulated the activity more efficiently than

cholesterol. But ergosterol did not stimulate it. On the other hand, all of these sterols decreased the K_m value and increased the V_{max} value of the rhodamine B-stimulated ATPase activity of MDR1 as efficiently as cholesterol (data not shown). These results also suggest that the action of sterols is drug specific.

Table II: K_m and V_{max} values of paclitaxel-stimultated ATPase activity of MDR1reconstituted in liposome containing various sterols (20%, w/w)

	K_m (μM)	V_{max} (nmol/min/mg)
-sterol	1.0 $\pm$ 0.4	103 $\pm$ 19
cholesterol	1.2 $\pm$ 0.4	300 $\pm$ 36*
stigmasterol	1.1 $\pm$ 0.1	492 $\pm$ 25*
sitosterol	1.0 $\pm$ 0.2	291 $\pm$ 12*
ergosterol	0.6 $\pm$ 0.0	111 $\pm$ 3
campesterol	0.9 $\pm$ 0.0	290 $\pm$ 8*

*P<0.01 with respect to control (0% cholesterol)

DISCUSSION

The results presented in this chapter, show that cholesterol, in the lipid bilayer, modulates the ATPase activity of MDR1. Cholesterol increases the basal ATPase activity of purified human MDR1 reconstituted in liposomes (PC : PE : PS = 4 : 4 : 2), and the levels of activity was highest in liposomes containing 30% (w/w) cholesterol. Furthermore, cholesterol affects the drug-stimulated ATPase activity of MDR1, the effects differing from one drug to another.

In this study, purified human MDR1 was reconstituted in liposomes, and measured the

amounts of ADP released after the hydrolysis by HPLC with a titanium dioxide column as described in chapter 1 and 2. Inhibitors for other membrane-bound ATPases such as sodium azide and ouabain, which are necessary when membrane-bound or partially purified MDR1 is used in experiments [19, 20], were not needed in this study. A typical concentration-dependence with a bell-shaped curve [17, 18] was obtained, and drug-stimulated ATPase activity was as high as previously reported [21, 22]. These experimental conditions made it possible to examine the effect of cholesterol in the lipid bilayer on the MDR1 ATPase activity in detail.

The plasma membrane of animal cells usually contains 30-40 mol% (18-25% (w/w)) of cholesterol [23]. To inquire into the effect of cholesterol on the function of MDR1, the drug-stimulated ATPase activity of MDR1 was examined after reconstituted in liposomes containing 0, 5, 10, or 20% (w/w) cholesterol. The effects were classified into five types (Table I): Type I, K_m decreases in the presence of cholesterol, and V_{max} is little affected; Type II, K_m decreases and V_{max} increases; Type III, K_m or V_{max} is little affected; Type IV, K_m is little affected and V_{max} increases; and Type V, K_m increases, and V_{max} is little affected.

One characteristic function of MDR1 is to transport drugs with various structures and molecular weights, from 300 to well over 1000. When the ratio of the K_m of each drug in the absence and presence of 20% cholesterol was plotted versus the molecular weight of that drug, the author found a strong correlation between them except nicardipine (Fig. 6). At first glance, digoxin did not fit the correlation. But when the aglycon form of digoxin (MW: 390) was plotted on the graph, it fitted well. The binding affinity of drugs with small molecular weights, between 350 and 500, increased in the presence of 20% cholesterol, and these drugs (rhodamine 123, dexamethasone, verapamil, digoxin, corticosterone, hydrocortisone, nicardipine, and rhodamine B) are categorized into type I and type II (Table I, Fig. 5). The

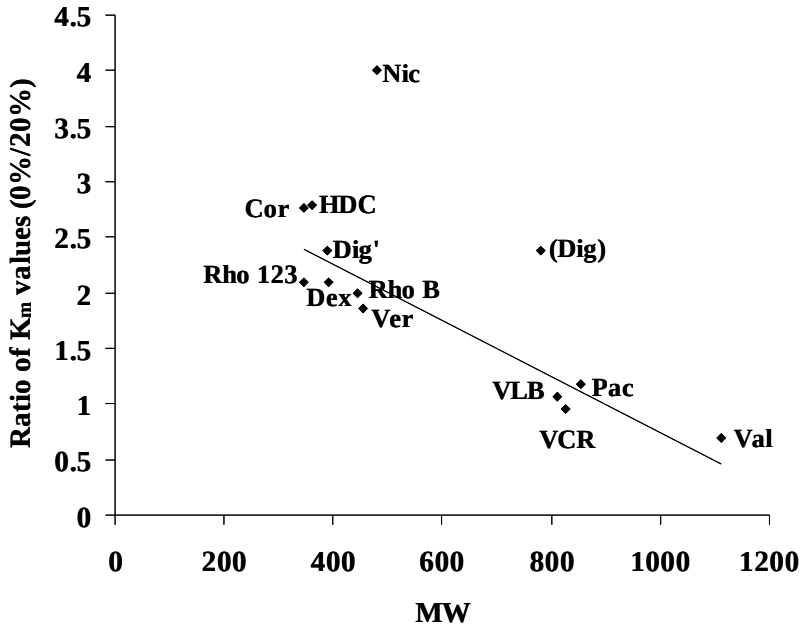

Fig. 6. Relationship between the ratio of K_m values in the absence and presence of 20% (w/w) cholesterol and molecular weights of drugs. The line represents the best fit linear regression (r^2=0.8732) except the points for nicardipine and digoxin. Rho123, rhodamine 123; Cor, corticosterone; HDC, hydrocortisone; Dig', aglycon form of digoxin; RhoB, rhodamine B; Ver, verapamil; Nic, nicardipine; Dig; digoxin; VLB, vinblastine; VCR, vincristine; Pac, paclitaxel; Val, valinomycin.

binding affinity of drugs with molecular weight between 800 and 900 are not affected by the presence of cholesterol, and these drugs (vinblastine, vincristine, and paclitaxel) are categorized into type III and type IV. The binding affinity of valinomycin (type V), whose molecular weight is over 1000, decreased in the presence of 20% cholesterol.

Recently, the crystal structures of several bacterial multidrug transporters [24, 25] and multidrug-binding transcription factors [26, 27] were revealed. A common feature of their drug-binding pocket is the presence of several 'mini-pockets' within the larger drug-binding site, and there are no obstructions or structural barriers between the continuous mini-pockets. MDR1 has been suggested to possess several allosterically coupled drug-binding sites[3, 4, 28,

29] and to bind more than one molecule of drug at the same time [30, 31]. The strong correlation between the effect of cholesterol on the K_m for drugs and their molecular weights suggests that the drug-binding site of MDR1 has plasticity but may best fit drugs with a molecular weight of 800 to 900. When smaller drugs, with a molecular weight of 350 to 500, bind to MDR1, cholesterol (MW: 386.7) binds together and may support the binding of drugs to the drug-binding site by filling the empty space with the hydrophobic tetracyclic ring structure. In the case of nicardipine, cholesterol increases the binding affinity very strongly. This combination may make the best fit with the binding site. In previous studies, it has been demonstrated that the bulkiness of side chains at the position of His61 and its neighboring amino acid residues in the first transmembrane helix is important for substrate specificity [32, 33]. For example, the replacement of His61 by amino acids with bulkier side chains increased resistance to small drugs such as colchicine and VP16, while it lowered resistance to a large drug, vinblastine. These observations also suggest that filling the empty space of the drug-binding pocket is important for recognizing small drugs. It is conceivable that cholesterol, a major (about 20% (w/w) of lipids) and important constituent of the plasma membrane [23], is utilized by MDR1 to adjust the space of the drug-binding site for recognizing various compounds with a wide range of molecular sizes.

Cholesterol affected not only drug-binding but also the turnover of ATP hydrolysis. The V_{max} values for rhodamine B, nicardipine, and paclitaxel were significantly increased in the presence of 20% cholesterol. The ATP-dependent transport by MDR1 is supposed to involve five major steps as follows. First, a drug binds to a drug-binding site facing the lipid phase; second, the drug-binding triggers ATP-binding or hydrolysis; third, ATP-binding or hydrolysis causes a conformational change of MDR1, by which the drug-binding site faces the extracellular aqueous phase; forth, the drug dissociates from MDR1; and fifth, the

conformation is reset to the initial state after the release of ADP or hydrolysis of another ATP. If cholesterol affects the lipid environment, such as fluidity, and indirectly increases the turnover of ATP hydrolysis, an increase in the V_{max} should be observed for all the drugs. The specific effect of cholesterol on the V_{max} as well as K_m suggests that cholesterol accelerates the conformational change by cooperatively binding with the drug to the drug-binding site. The sterol-specific nature of the effect on paclitaxel-stimulated ATPase activity (Table II) also supports a cooperative effect of cholesterol and drugs. Stigmasterol had a greater effect on paclitaxel-stimulated ATPase activity. Ergosterol did not affect paclitaxel-stimulated ATPase activity whereas it affected rhodamine B-stimulated ATPase activity as effective as other sterols. The plasticity of the drug-binding site was demonstrated in QacC [34]: binding of the first drug causes the side chain of a key aromatic residue to rotate, which allows the simultaneous binding of the second drug to the otherwise partially overlapped binding pocket of the first drug. Sterols may cause conformational changes to the drug-binding site which affect the drug-binding in a drug-specific manner.

Because cholesterol, dexamethasone, corticosterone, hydrocortisone, and digoxin share a similar steroidal tetracyclic ring structure, it is a surprise that cholesterol does not compete but rather stimulates the binding of these compounds. Cholesterol, stigmasterol, sitosterol, and campesterol, which increase the paclitaxel-stimulated ATPase activity of MDR1, contain a divalent bond in the B-ring, while ergosterol, containing two divalent bonds in the B-ring, does not increase paclitaxel-stimulated ATPase activity. Dexamethasone, corticosterone, hydrocortisone, or digoxin does not contain the C-3 hydroxyl group or a divalent bond in the B-ring. These results suggest that the binding site of sterols, which contain the C-3 hydroxyl group and a divalent bond in the B-ring, is different from the binding sites of other steroids, and that these structures may be important for cholesterol to support the function of MDR1.

The most puzzling feature of MDR1 is its recognition and transport of drugs with such a wide variety of structures and molecular sizes. To function as an efflux pump for various lipophilic and toxic xenobiotics, it is necessary for MDR1 to recognize them as they pass through the lipid bilayer. To do this, MDR1 has probably acquired two important features in the course of evolution: one is a plasticity of the drug-binding site, and the other is the ability to utilize cholesterol to fill the empty space when binding small drugs. Because it is now realized that one of the major physiological roles of ABC proteins is cholesterol homeostasis[35], binding cholesterol at the drug-binding site could be an inherent ability of ABC proteins. These findings will facilitate our understanding not only of the mechanisms of substrate recognition by MDR1 but also of the evolution of ABC proteins.

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CONCLUSIONS

The purposes of this research presented in this thesis are the understanding of the molecular basis of ABC proteins to exhibit different functions and the characterization of the transport mechanism of MDR1. For this purpose, the author developed the large scale expression and purification systems. The author also developed a microanalysis method for ATPase activity of ABC proteins. The results described in each chapter can be summarized as follows.

In chapter 1, the author established the large scale expression and purification system for ABC proteins. Large scale expression and purification system is a powerful tool for characterizing the protein function in detail. To date, expression and purification of ABC proteins have not been feasible. Using baculovirus expression system, human MDR1 was successfully overexpressed in microsomes of insect cells and purified with a purity of 95% by a Ni-affinity chromatography. ATPase activity of purified and reconstituted MDR1 was significantly enhanced by the addition of verapamil and completely inhibited by ortho-vanadate or beryllium fluoride. This result suggests that MDR1 has the functional structure in a purified form.

To reveal the difference between a transporter and a channel regulator, the author expressed and purified SUR1, channel regulator, with the same method. Specific binding of glibenclamide, a potent K_{ATP} channel blocker, suggests functional expression and purification of SUR1 in baculovirus expression system. ATPase activity of purified and reconstituted SUR1 was as low as one-fiftieth of verapamil-stimulated ATPase activity of MDR1. This result suggests that the turnover rate of ATP hydrolysis is the important factor to determine the

function of ABC proteins. Both glibenclamide and tolbutamide stimulated the ATPase activity of SUR1. From these results, the author proposes a new model on the mechanisms of the action of sulfonylureas on K_{ATP} channel. Baculovirus expression and purification system, the author established in this study, may significantly improve our understandings of the functions of ABC proteins.

In chapter 2, the author described the development of a microanalysis method for ATPase activity of MDR1 using high-performance liquid chromatography equipped with a titanium dioxide column. The amount of ADP produced by the ATPase reaction was determined within 2 min with this method and the relationship between ADP amount and chromatogram peak area was linear from 5 pmol to 10 nmol. This method made it possible to reduce the amount of purified MDR1 required for a reaction to 0.5 ng, about one-twentieth required for the conventional colorimetric inorganic phosphate detection assay. This method is sensitive enough to detect any subtle changes in ATPase activity of MDR1 induced by drugs, and can be applied to measure ATPase activity of any protein.

In chapter 3, the author demonstrated that the cholesterol, an important constituent of lipid bilayer of eukaryotic cells, modulates the drug-stimulated ATPase activity of MDR1. Purified MDR1 was reconstituted in proteoliposomes containing various concentrations of cholesterol and enzymatic parameters of drug-stimulated ATPase were examined. The effects of cholesterol on drug-stimulated ATPase activity differ from one drug to another. Cholesterol increases the binding affinity of small drugs (MW<500), but does not affect that of drugs with a molecular weight of between 800 and 900, and suppresses that of valinomycin (MW>1000). V_{max} values for rhodamine B, nicardipine and paclitaxel are also increased by

cholesterol suggesting that cholesterol affects turnover as well as drug binding. Paclitaxel-stimulated ATPase activity of MDR1 is enhanced in the presence of stigmasterol, sitosterol and campesterol, as well as cholesterol, but not ergosterol. The presence of cholesterol lowered the K_m for corticosterone, hydrocortisone, and digoxin, transport substrates of MDR1. These results suggest that MDR1 possesses a sterol-specific binding site within the drug-binding pocket and utilized cholesterol to adjust the space of the pocket for recognizing various compounds with a wide range of molecular sizes.

LIST OF PUBLICATIONS

A. Kimura, Y., Shibasaki, S., Morisato, K., Ishizuka, N., Minakuchi, H., Nakanishi, K., Matsuo, M., Amachi, T., Ueda, M. & Ueda, K. (2004) Microanalysis for MDR1 ATPase by high-performance liquid chromatography with a titanium dioxide column, *Anal Biochem. 326*, 262-6

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D. 小林　綾、木村泰久、松尾道憲、植田和光　創薬ターゲットとしての ABC タンパク質 (2005) *日本薬理学雑誌 (Folia Parmacol. Japan) 125,* 185-193

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F. Matsuo, M., Kimura, Y. & Ueda, K. K_{ATP} channel interaction with adenine nucleotides, *J. Mol. Cell. Cardiol*.
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