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Aureobasidin A, an antifungal cyclic depsipeptide antibiotic, is a substrate for both human MDR1 and MDR2/P-glycoproteins

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Abstract The human *MDR1* gene encodes the multidrug transporter P-glycoprotein (Pgp). Although the MDR2/Pgp shares about 80% identity at the amino acid level with the MDR1/Pgp, the MDR2/Pgp cannot act as a multidrug transporter. We examined the drug sensitivity of *Saccharomyces cerevisiae* expressing either the human MDR1/Pgp or MDR2/Pgp. The human MDR1/Pgp conferred about 4-fold resistance to aureobasidin A, a cyclic depsipeptide antifungal antibiotic, on the drug-sensitive yeast strains. Interestingly the human MDR2/Pgp also conferred about 2.5-fold resistance to aureobasidin A. The resistance to aureobasidin A conferred by the MDR2/Pgp as well as by the MDR1/Pgp was overcome by vinblastine, verapamil, and cyclosporin A, depending on their concentrations, but not by colchicine. Aureobasidin A probably interacts directly with Pgps, because it overcame multidrug resistance of human cells and inhibited azidopine photoaffinity labeling of MDR1/Pgp in human cell membranes. These results suggest the possibility that the human MDR1 and MDR2/Pgps have conserved domain(s) for drug recognition.

Key words: ABC transporter; P-glycoprotein; MDR1; MDR2; Aureobasidin A; Drug resistance; *Saccharomyces cerevisiae*

1. Introduction

Multidrug resistance (MDR) in cultured cells is caused by the overexpression of P-glycoprotein (Pgp) and hence Pgp is supposed to be involved in MDR in tumor cells. Pgps are encoded by a small gene family, which includes two members in humans, *MDR1* and *MDR2*, and three members in mice, *mdr1a, mdr1b, and mdr2*. Overexpression of human *MDR1*, or mouse *mdr*1a and *mdr*1b, in drug-sensitive cultured cells can confer multidrug resistance [1–3], while human *MDR2* and mouse *mdr2* cannot [4,5].

It has been proposed that the human MDR1/Pgp participates in the protection of organs against toxic xenobiotics and in transport of some natural steroid hormones in adrenal and in placental trophoblasts [6,7]. The mouse mdr2/Pgp has been shown to be indispensable for the secretion of phospholipids into bile [8] and to function as a phosphatidylcholine-specific translocase [9]. The mouse mdr1a or mdr1b/Pgp was unable to compensate for the function of the mdr2/Pgp [8]. These results suggest that the substrates and the physiological functions of the two types of Pgp are different.

Substrates for the human MDR1/Pgp include peptides such as valinomycin [10], gramicidin D [11], cyclosporin A [12], a tripeptide *N*-acetyl-leucyl-leucyl-norleucinal [13], and yeast a-factor mating peptide [14]. During our search for peptide antibiotics which interact with human Pgp, we found that *Saccharomyces cerevisiae* expressing human MDR1/Pgp showed resistance to aureobasidin A, a new antifungal cyclic depsipeptide antibiotic produced by *Aureobasidium pullulans* R106 [15,16]. Surprisingly the overexpression of MDR2/Pgp also conferred resistance to aureobasidin A. The resistance to aureobasidin A was overcome by vinblastine, verapamil, and cyclosporin A, depending on the concentrations, suggesting that two types of human Pgps have conserved domain(s) for drug recognition.

2. Materials and methods

2.1. Materials

Monoclonal antibody C219 was purchased from Centocor. [^{3}H]Azidopine (1850 GBq/mmol) was purchased from Amersham Corp. Aureobasidin A was kindly provided by Takara Shuzo Co., Ltd.

2.2. Yeast strains

S. cerevisiae W303-1A (*MATa ura3-52 leu2-3,112 his3-11,115 trp1-1 ade2-1 can1-100*) was kindly provided by Dr. Karl Kuchler. To isolate a drug-sensitive variant, W303-1A was treated with 5 mM ethyl methanesulfonate and was screened for resistance to 3 μg/ml nystatin. Several strains were tested for sensitivity to various drugs, and strain W303-1AY18 was isolated. Nystatin-resistant strains were reported to change the content of ergosterol in the membrane and hence to increase the sensitivity to various drugs [17]. However, the UV absorption spectra of the nonsaponifiable lipid extracts of the nystatin-resistant strain W303-1AY18 showed the presence of ergosterol in the membrane similarly to the wild-type W303-1A (data not shown). The reason that W303-1AY18 is resistant to nystatin and moderately sensitive to various drugs is unknown.

2.3. Plasmids and yeast transformation

The multi-copy plasmid YEp is used for expression in yeast cells of the human wild-type *MDR1* gene [18] (pYM/MDR1) or the *MDR2* gene [19] (pYM/MDR2) from the glyceraldehyde 3-phosphate dehydrogenase promoter. Yeast cells were transformed with pYM/MDR1 or pYM/MDR2 by the lithium acetate method. The human *MDR2* gene [19] was obtained from the American Type Culture Collection.

2.4. Drug resistance assays of yeast cells

Drug resistance was at first tested with an agar plate assay by streaking the overnight yeast cultures (diluted to $A_{595} = 1.0$) on SD plates containing appropriate concentrations of drugs. Growth was monitored after incubation at 30°C for 3 days. Growth inhibition assays were done in flat-bottom 96-well microplates, with wells containing equal volumes (50 μl) of YPD medium with or without aureobasidin A. Yeast cells grown to stationary phase were diluted in YPD ($A_{595} = 0.2$). Equal volumes of these cultures (50 μl) were added to each well and incubated at 30°C for 24 h. Growth was measured optically at 595 nm on a microplate reader after incubation at 30°C for 24 h.

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Abbreviations: MDR, multidrug resistance; Pgp, P-glycoprotein; PCR, polymerase chain reactions; aa, amino acid(s)

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3. Results and discussion

We introduced human *MDR1* and *MDR2* expression vectors into a drug-sensitive yeast strain W303-1AY18. Each transformant was examined to possess appropriate plasmids by PCR with MDR1- or MDR2-specific primers (data not shown). The membrane fractions were prepared from the transformants and analyzed by immunoblotting for the presence of the human MDR1 and MDR2/Pgps using monoclonal antibody C219 which recognizes both Pgps (Fig. 1). The membrane fraction from transformants showed a unique immunoreactive band with an apparent molecular mass of 140 kDa, which was absent from the membrane fraction from the control cells. Human MDR2/Pgp (1279 aa) migrated at approximately the same position as MDR1/gp (1280 aa). The apparent low molecular mass compared to those expressed in human cells was probably caused by the lack of glycosylation [14,20,21].

The colony formation assay was first used to assess functionality of human MDR1 and MDR2/Pgps expressed in drug-sensitive yeast strains, and the resistance to aureobasidin A, a new cyclic depsipeptide antifungal antibiotic, of those expressing the human MDR1/Pgp was noticeable (Fig. 2A). The human *MDR1* transformants formed colonies on plates containing 0.15 μg/ml aureobasidin A, while yeasts harboring the control vector did not. Surprisingly the human *MDR2* transformants also showed resistance to 0.1 μg/ml aureobasidin A (Fig. 2C).

The effects of aureobasidin A on cell growth were then examined in liquid cultures (Fig. 3). When growth rates at given aureobasidin A concentrations relative to growth in its absence were plotted as a function of aureobasidin A concentration, the IC_{50}s (concentrations required for inhibiting the growth rate by 50%) for W303-1AY18/MDR1 and W303-1AY18/MDR2 were 3.7 and 2.5 times higher than that for cells harboring the control vector, respectively.

We examined the possibility of direct interaction of aureobasidin A with the human MDR1/Pgp using the human MDR cultured cells KB-G2 [10], which were obtained by introducing the *MDR1* expression vector into drug-sensitive KB3-1 cells and had a typical MDR phenotype. In the presence of 2 μM aureobasidin A, the concentrations of vinblastine (Fig. 4A) and colchicine (data not shown) inhibiting the growth rate of KB-G2 by 50% were shifted as low as that for the drug-sensitive KB-3-1 cells. Furthermore aureobasidin A inhibited photoaffinity labeling with [^{3}H]azidopine of MDR1/Pgp in the membrane fraction prepared from KB-G2 (Fig. 4B). These results suggest that aureobasidin A interacts directly with the human MDR1/Pgp and competes with vinblastine, colchicine, and azidopine at common drug binding site(s).

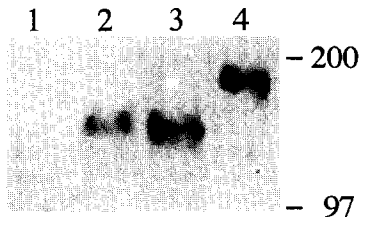

Fig. 1. Immunoblotting of membrane fraction from *S. cerevisiae* W303-1AY18/vector (lane 1), W303-1AY18/MDR1 (lane 2), W303-1AY18/MDR2 (lane 3) and human MDR cultured cells KB-G2 [10] (lane 4). 20 μg of membrane protein was put on each lane, and was reacted with monoclonal antibody C219 as a probe for P-glycoprotein. Molecular size standards are indicated in kDa on the right.

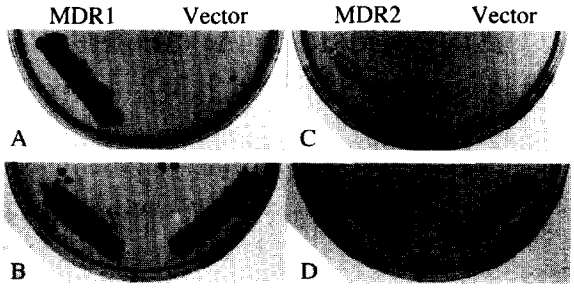

Fig. 2. Resistance of *S. cerevisiae* W303-1AY18 harboring human MDR1 or MDR2 expression vector. Overnight cultures of W303-1AY18 harboring the control vector, pYM/MDR1 or pYM/MDR2 were diluted to $A_{595} = 1.0$ by media and streaked on SD plates containing 0 (B and D), 0.1 (C), or 0.15 μg/ml aureobasidin A (A). The plates were incubated at 30°C for 3 days.

Because aureobasidin A has low toxicity for cultured cells and because we could not obtain an isotope-labeled aureobasidin A with a high specific activity, we could not examine if the human MDR1 and MDR2/Pgps would transport it. But if this is the case, substrates for the human MDR1/Pgp such as verapamil, cyclosporin A and vinblastine which have high affinity to the human MDR1/Pgp would be expected to depress the aureobasidin A resistance of yeast cells conferred by the human MDR1/Pgp.

The resistance to aureobasidin A conferred by MDR1/Pgp was overcome by vinblastine, verapamil, and cyclosporin A depending on the concentrations (Fig. 5A). Especially in the presence of cyclosporin A at a concentration of 5 μM, which is expected to be an efficient MDR modulator because of its high modulating activity [22], W303-1AY18/MDR1 became as sensitive as the control yeast cells. Colchicine, which is also a substrate for the human MDR1/Pgp but has low affinity to the human MDR1/Pgp [23], did not have any effect. These results suggest that in the plasma membrane of yeast strain W303-1AY18 the MDR1/Pgp retains functional drug binding site(s) with a substrate specificity similar to that formed in the

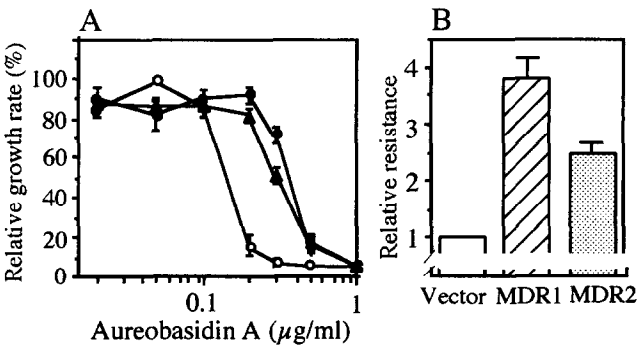

Fig. 3. A: Growth inhibition assay of W303-1AY18 harboring the control vector (○), pYM/MDR1 (●) or pYM/MDR2 (▲). The assay was done in triplicate as described in Section 2. The values are expressed relative to each transformant strain grown in the absence of the drug. B: Relative resistance of W303-1AY18 harboring the control vector, pYM/MDR1 or pYM/MDR2. Relative resistance was calculated by comparing the IC_{50}s. The results are presented relative to W303-1AY18 harboring the control vector. Each bar represents the average of three experiments.

plasma membrane of human cells and that aureobasidin A binds to the common drug binding site(s).

Next we examined these MDR modulators for resistance to aureobasidin A conferred by MDR2/Pgp, and found that the inhibitory effects of these MDR modulators were almost identical to the effect on the resistance conferred by MDR1/Pgp (Fig. 5B). These results strongly suggest that human MDR2/Pgp has the conserved domain(s) for drug recognition in common with the MDR1/Pgp.

It has been suggested that the physiological roles and the substrate specificity of MDR1/Pgp and MDR2/Pgp are different. But MDR2/Pgp shares about 80% identity at the amino acid level with MDR1/Pgp. Several groups have identified the mutations to amino acids that modulate the substrate specificity of human and mouse Pgps, which are in the 4th, 6th, 8th, 10th, 11th, and 12th transmembrane domains and the 1st, 2nd, and 4th cytoplasmic loops [24–26]. However, all the reported amino acids were conserved between MDR1 and MDR2/Pgps. Recently it was reported that the mouse mdr2/Pgp-mediated phosphatidylcholine translocation was inhibited by verapamil [9], suggesting that binding sites for verapamil, which is known as a Pgp inhibitor but is also a substrate for MDR1/Pgp to transport ([27], our unpublished results), are conserved between MDR1/Pgp and MDR2/Pgp. These results have suggested that a part of the mechanism of drug binding by MDR2/Pgp overlaps with that of MDR1/Pgp. In this paper, we reported that MDR2/Pgp expressed in yeast cells retains an ability to interact with a variety of typical MDR1 substrates. Nevertheless, overexpression of human MDR2/Pgp or mouse mdr2/Pgp cannot confer multidrug resistance on drug-sensitive mammalian cells, which has been confirmed by several groups [4,5]. We have reported [7] that human MDR1/Pgp transports cortisol, aldosterone, and dexamethasone, but not progesterone, although progesterone directly binds MDR1/Pgp, inhibits azidopine photoaffinity labeling of MDR1/Pgp, and increases the sensitivity of multidrug-resistant cells to vinblastine. These results suggest that substances that efficiently bind to Pgp are not necessarily transported by Pgp.

In this paper we showed for the first time that human

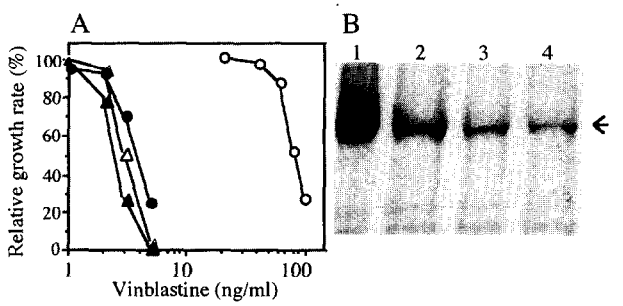

Fig. 4. A: Effects of aureobasidin A on the growth of human MDR cultured cells KB-G2 (○, ●) and drug-sensitive KB-3-1 cells (△, ▲). Growth inhibition was analyzed in the absence (○, △) or presence (●, ▲) of 2 μg/ml aureobasidin A by MTT assay as previously described [7]. B: Azidopine photolabeling of P-glycoprotein. 10 μg of membrane protein from human MDR cultured cells KB-G2 was photolabeled with 0.2 μM [^{3}H]azidopine in the absence (lane 1) or presence of 5 μM (a 25-fold excess) (lane 2), 10 μM (a 50-fold excess) (lane 3), 20 μM (a 100-fold excess) (lane 4) of aureobasidin A as previously described [7]. P-glycoprotein is indicated by an arrow on the right.

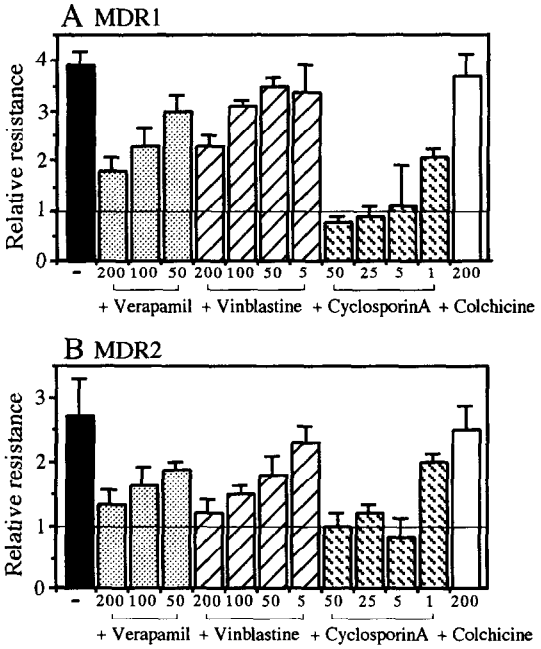

Fig. 5. Effects of MDR modulators on aureobasidin A resistance of W303-1AY18 harboring pYM/MDR1 (A) or pYM/MDR2 (B). Growth inhibition assay was done in the absence (−) or in the presence of verapamil (50, 100, and 200 μM), vinblastine (5, 50, 100, and 200 μM), cyclosporin A (1, 5, 25, and 50 μM), or colchicine (200 μM) as described in Section 2. The results are presented relative to W303-1AY18 harboring the control vector. MDR modulators of these concentrations have no effect on the growth of W303-1AY18. Each bar represents the average of at least three experiments.

MDR1 and MDR2/Pgps would recognize a common substrate. Because MDR2/Pgp is considered to interact with phosphatidylcholine in the inner leaflet of the lipid bilayer and to translocate it to the outer leaflet, it is conceivable that MDR1/Pgp also removes some of its substrates from the inner leaflet of the plasma membrane. These results will promote the understanding of the mechanisms of drug recognition and transport and the physiological functions of the human MDR1 and MDR2/Pgps.

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