

Functional expression of human P-glycoprotein in *Schizosaccharomyces pombe*

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Human *MDR1* cDNA was introduced into the human cultured cells KB-3-1 and *Schizosaccharomyces pombe* *pmd1* null mutant KN3. The drug sensitivity of KB-G2 and KN3/pgp, expressing human P-glycoprotein, was examined. KB-G2 was resistant to the peptide antibiotics valinomycin and gramicidin D as well as having a typical multidrug resistance (MDR) phenotype. KN3/pgp was resistant to valinomycin and actinomycin D, but not to adriamycin. The ATP-hydrolysis-deficient mutant did not confer KN3 resistance to these antibiotics. Human P-glycoprotein expressed in *S. pombe* seemed to lack N-glycosylation. The N-glycosylation-deficient mutant, however, conferred a typical MDR phenotype on KB-3-1. These results suggest that human P-glycoprotein functions as an efflux pump of valinomycin and actinomycin D in the membrane of *S. pombe*.

P-glycoprotein; Multidrug resistance; Yeast, *Schizosaccharomyces pombe*; ATP hydrolysis; Glycosylation

1. INTRODUCTION

The development of multidrug resistance (MDR) is a major obstacle in cancer chemotherapy. Typical MDR acts against various anticancer drugs that have no structural similarity. P-glycoprotein is a 170-kDa membrane protein coded by the *MDR1* gene in humans, and is believed to be involved in MDR. P-glycoprotein binds various anticancer drugs including *Vinca* alkaloids, anthracyclines and actinomycin D, and pumps them out of the cell using the energy from ATP hydrolysis [1,2].

P-glycoprotein also interacts with peptides. Overproduction of human P-glycoprotein confers resistance against the peptide ionophore antibiotic gramicidin D [3]. Chinese hamster and human P-glycoproteins were reported to be capable of transporting a tripeptide, *N*-acetyl-leucyl-leucyl-norleucinal, which is an inhibitor of various intracellular proteases [4]. It was also reported that the mouse *mdr3* gene but not the human *MDR1* gene, carrying the G185V mutation, could complement yeast *STE6*, which is a homologue of mammalian *mdr* genes and mediates export of α -factor mating peptide in *Saccharomyces cerevisiae* [5,6]. By using a transcellular

transport system, it was shown that cyclosporin A, a cyclic undecapeptide with strong immunosuppressive effects, is transported by P-glycoprotein [7].

Expression of human proteins in a heterologous host sometimes gives important information on their mechanisms of action. *Schizosaccharomyces pombe* (*S. pombe*) has been reported to have the *MDR1* homologue gene *pmd1* [8]. Because the *pmd1* null mutant KN3, derived by gene disruption, was hypersensitive to the MDR-related drug actinomycin D, *S. pombe* KN3 was expected to be useful as a host for expressing human P-glycoprotein to analyse its function. Thus, human P-glycoprotein was expressed in *S. pombe* KN3 and a functional comparison was done with P-glycoprotein expressed in human cultured cells.

It is shown here that human P-glycoprotein confers on *S. pombe* resistance to valinomycin, a cyclic dodecapeptide antibiotic, and actinomycin D, but not to adriamycin. Because a mutant that was expected to be ATP-hydrolysis deficient did not confer resistance to valinomycin or actinomycin D, ATP hydrolysis is suggested to be necessary for extruding these agents out of cells.

2. EXPERIMENTAL

2.1 Cell culture and transfection

KB-3-1 cells were transfected with a human P-glycoprotein expression vector pSKGA [9], along with pSV2neo. Transfected cells were first selected in medium containing 0.8 mg/ml of G418. G418-resistant cells were pooled and selected for the expression of the *ADA* gene, which is contained in pSKGA as a selectable marker, in 10 nM

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Abbreviations: MDR, multidrug resistance; *S. pombe*, *Schizosaccharomyces pombe*; nt, nucleotide; PCR, polymerase chain reaction.

2'-deoxycoformycin and 4 μ M 9- β -D-xylofuranosyl adenine for 2 weeks. Several transformants were isolated and insertion of the *MDR1* gene was confirmed by Southern blot analysis. One transformant (KB-G2) was propagated in 200 ng/ml of vincristine to maintain the high expression of P-glycoprotein

2.2 Construction of a *Schizosaccharomyces pombe* expression plasmid

For the construction of an *S. pombe* expression plasmid, *Sal*I and *Bam*HI sites were created at the 5' and 3' ends of human wild-type *MDR1* cDNA just before the first ATG codon and just after the termination codon by the polymerase chain reaction (PCR) and linker insertion, respectively. Then, *MDR1* cDNA was inserted into *Sal*I-*Bam*HI sites of pART1 vector, where the *MDR1* gene was under the control of the constitutive promoter of the *adh* gene.

2.3. Construction of ATP-hydrolysis-deficient and N-glycosylation-deficient mutants

For the construction of the ATP-hydrolysis deficient mutant, methionine was substituted for lysine-433 as follows. A PCR fragment generated with oligonucleotides GGGAAATTTGGAATTCAG (nt 1167-1184) and GCATCAGCTGGACTGTTGTGCTCATCCCACAGCC (nt 1321-1288) was digested with *Eco*RI and *Pvu*II, and was replaced with the corresponding fragment of the full-length *MDR1* cDNA.

To substitute aspartic acid for asparagine in three predicted N-glycosylation sites (asparagine-91, -94, and -99) of the full-length *MDR1* cDNA, site-directed mutagenesis was first done with the oligonucleotide AGTGATATCGATGATACAGG (nt 285-305) to create a *Cl*aI site at nt 292, which also resulted in the mutagenesis of the third glycosylation site (asparagine-99). Then, a synthetic linker shown below was

GATCTGATGTCAGACATCACTGATAGAAGTGATAT
ACTACAGTCTGTAGTGACTATCTTCACTATAGC

inserted into the *Bgl*II-*Cl*aI site of the full-length *MDR1* cDNA to mutagenize the other two glycosylation sites (asparagine-91 and -94).

2.4. Drug sensitivity of *Schizosaccharomyces pombe*

Drug sensitivity of cells was assayed by measuring the minimum inhibitory concentration. The cells were grown in Edinburgh minimal medium without leucine to an OD_{600} of 0.4. The same number of cells were streaked on YPD plates containing various concentrations of each drug, and incubated at 30°C for 5-7 days

3. RESULTS AND DISCUSSION

Although P-glycoprotein has been suggested to interact with some oligopeptides, interaction with peptide antibiotics is still obscure: MDR cells expressing P-glycoprotein have been reported to be resistant to gramicidin D, a linear pentadeca-peptide, but resistance to valinomycin, a cyclic dodecapeptide, was very weak or marginal [10]. Therefore, human MDR cell line KB-G2 was established by introducing human *MDR1* cDNA isolated from a normal adrenal gland [11] into drug-sensitive KB3-1 cells to examine the involvement of P-glycoprotein in the resistance to peptide antibiotics.

KB-G2 cells, expressing human P-glycoprotein (Fig. 1a), showed a typical MDR phenotype, resistance to various drugs that have no structural similarity, including adriamycin (Table I) and actinomycin D (data not shown). KB-G2 also showed resistance to the peptide antibiotics valinomycin, gramicidin D, and alamethicin. Resistance to valinomycin and gramicidin D was over-

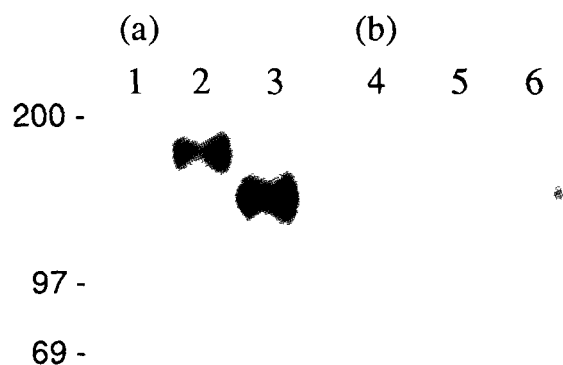


Fig. 1. Western blot of membrane fractions using monoclonal antibody C219 as a probe for P-glycoprotein. 10 mg of crude membrane protein was put on each lane. (a) Lane 1, KB-3-1; lane 2, KB-G2; lane 3, KB-NC1 (b) lane 4, KN3/pgp; lane 5, KN3; lane 6, KN3/pgpMK. Molecular size standards are indicated in kDa at the left

come in the presence of excess verapamil, cepharanthin, or cyclosporine A, which are known as MDR modulators, as in the case of resistance to vinblastine. These results suggest that P-glycoprotein functions as an efflux pump of valinomycin and gramicidin D.

KB-G2 did not show resistance to melitin. Melitin is a 26-amino acid peptide with a molecular weight of 2,847. Because most substrates for P-glycoprotein are hydrophobic with molecular weights of around 1,000, melitin could be too large to be a substrate for P-glycoprotein. Alternatively, the amphipathic nature of melitin could be a reason, although KB-G2 did not show resistance to gramicidin S, a hydrophobic cyclic decapeptide (data not shown). It is not clear why resistance to alamethicin was not overcome in the presence of MDR modulators (Table I).

Next we attempted to examine the function of human P-glycoprotein expressed in *S. pombe* KN3 as a host. *S. pombe* KN3/pgp expressing human P-glycoprotein showed resistance to actinomycin D and valinomycin but interestingly not to adriamycin (Table II). Because KN3 was not sensitive to other drugs examined, the difference of sensitivity could not be measured although KN3/pgp showed marginal resistance to VP16. We examined the effect of the MDR modulators verapamil and cyclosporin A, but unexpectedly cyclosporin A (100 mg/ml) did not overcome resistance of *S. pombe* KN3/pgp to valinomycin or actinomycin D (data not shown). Verapamil (100 mg/ml) showed a marginal effect on actinomycin D resistance (Table II), but did not show an obvious effect on valinomycin resistance (data not shown). This could be due to the poor penetration of the MDR modulators into the plasma membrane of *S. pombe* to interact with human P-glycoprotein. Because

Table I
Relative resistance of KB-G2 cells expressing P-glycoprotein and effects of MDR modulators

Antibiotics	–		+Cyclosporin A		+Cepharanthin		+Verapamil	
	3-1	G2	3-1	G2	3-1	G2	3-1	G2
Adriamycin	1 ^a	12	nd ^b	nd	nd	nd	nd	nd
Vinblastine	1	93	0.9	2.9	0.9	3.8	0.8	2.9
Valinomycin	1	6.9	0.9	1.3	1.0	0.9	1.1	1.4
Gramicidin D	1	104	0.2	0.5	0.8	1.8	0.9	1.8
Alamethicin	1	4.1	0.4	3.6	0.5	4.1	0.8	3.6
Melittin	1	0.7	nd	nd	nd	nd	1.1	0.9

The IC₅₀ values of KB-G2 cells against each drugs were divided by those of the parent KB-3-1 cells in the absence or presence of 1 μ M MDR modulators.

^a IC₅₀ values of KB-3-1 cells: adriamycin, 18 ng/ml; vinblastine, 0.84 ng/ml; valinomycin, 0.52 ng/ml; gramicidin D, 39 ng/ml; alamethicin, 1.7 μ g/ml; melittin, 7.4 μ g/ml.

^b not determined.

S. pombe is tolerant to valinomycin and actinomycin D more than 1000-fold when compared to KB-3-1 cells (Tables I and II), it is plausible to assume that *S. pombe* is also tolerant to the MDR modulators. Alternatively, the membrane environment might affect the function of human P-glycoprotein, as discussed later.

To confirm that the resistance to actinomycin D and valinomycin of KN3/pgp was dependent on the active efflux pump activity of human P-glycoprotein, we introduced a single amino acid substitution into human P-glycoprotein that is thought to abolish the ATPase activity and hence the drug transport activity [12]. *S. pombe* KN3/pgpMK expressing the mutant form of P-glycoprotein as much as the wild type in KN3/pgp (Fig. 1b) did not show any resistance to these drugs (Table II). These results suggest that human P-glycoprotein functions as an efflux pump of actinomycin D and valinomycin in the membrane of *S. pombe*.

Human P-glycoprotein expressed in *S. pombe* mi-

grated faster than that expressed in human cultured cells (Fig. 1) and did not confer resistance to adriamycin. This apparent small molecular weight of human P-glycoprotein expressed in *S. pombe* was considered to be due to the lack of glycosylation, because it has been reported that human P-glycoprotein expressed in *S. cerevisiae* is not glycosylated properly [5,6,14]. Thus we examined the effects of glycosylation on the function of human P-glycoprotein by expressing a mutant in which asparagine in the consensus sequences of three predicted N-glycosylation sites was replaced with aspartic acid. Although the human P-glycoprotein contains 10 consensus sequences for N-linked glycosylation, only three are predicted to be exposed to the out of the cell in the putative first extracellular loop [13].

The N-glycosylation-deficient mutant was transfected into KB-3-1, and the stable transformant KB-N1 expressing P-glycoprotein without N-glycosylation was isolated (Fig. 1a). KB-N1 showed resistance to colchicine, vinblastine, vincristine, and adriamycin (data not shown). The absence of N-glycosylation did not alter the pattern of cross-resistance.

These results suggest that other modifications than N-glycosylation or membrane circumstance affect the ability of human P-glycoprotein to confer resistance to adriamycin. We have reported previously that human P-glycoprotein expressed in *S. cerevisiae* did not confer adriamycin resistance or was not photoaffinity labeled by [³H]azidopine [14]. We have also reported that P-glycoprotein activity is dependent on the membrane environment in a reconstituted system [15]. The accumulation of drugs in a multidrug resistant cell line was recently reported to be sensitive to alteration of membrane properties [16]. These results suggest that the plasma membrane of *S. pombe* can support the interaction of P-glycoprotein with valinomycin and actinomycin D, but not with adriamycin. Valinomycin and actinomycin D contain a peptide moiety. P-glycoprotein also mediates export of α -factor mating peptide [6]. The

Table II

Drug sensitivity of *Schizosaccharomyces pombe* expressing human P-glycoprotein

Antibiotics	KN3/vector	KN3/pgp	KN3/pgpMK
Actinomycin D ^a	5 (1) ^c	10 (2)	4 (0.8)
+Verapamil (100 μ g/ml)	5 (1)	7.5 (1.5)	
Valinomycin ^a	3 (1)	20 (6.7)	0.5 (0.2)
Leptomycin B ^b	6 (1)	6 (1)	
Adriamycin ^a	75 (1)	75 (1)	
Gramicidin D ^a	>150	>150	
VP16 ^a	200	>200	
Puromycin ^a	>200	>200	

The minimum inhibitory concentration of KN3/vector, KN3/pgp expressing human P-glycoprotein and KN3/pgpMK expressing ATP-hydrolysis-deficient mutant were compared.

^a μ g/ml.

^b ng/ml.

^c Values in parentheses are the resistance relative to that of KN3/vector.

interaction of substrates which contain a peptide moiety with P-glycoprotein might be different from that of other substrates. The success of transfer of valinomycin resistance to *S. pombe* by human wild-type P-glycoprotein, which seems ATP-hydrolysis-dependent, will facilitate the analysis of the mechanism of P-glycoprotein function.

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