Multidrug Resistance Phenotype Conferred by Overexpressing \(bfr2^+\)/\(pad1^+\)/\(sks1^+\) or \(pap1^+\) Genes and Mediated by \(bfr1^+\) Gene Product, a Structural and Functional Homologue of P-Glycoprotein in \(S. pombe\)

Manabu Arioka,‡ Mutsuo Kouhashi, Koji Yoda, Akira Takatsuki,* Makari Yamasaki,** and Katsuhiko Kitamoto

Department of Biotechnology, University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan
*Institute of Physical and Chemical Research (RIKEN), Hirosawa 2-1, Wako-shi, Saitama 351-0198, Japan
**Present address: College of Bioresource Sciences, Department of Food Science and Technology, Nihon University, Tokyo 154-0002, Japan

Received August 27, 1997

We investigated the mechanism of multidrug resistance conferred by overexpression of \(bfr2^+\)/\(pad1^+\)/\(sks1^+\) or \(pap1^+\) genes of \(S. pombe\). Overexpression of \(bfr2^+\) did not confer multidrug resistance on a \(pad1^+\)-disrupted strain. In a mutant with \(bfr1^+\) (a putative membrane transporter which belongs to the ATP-binding cassette superfamily) disrupted, overexpression of either \(bfr2^+\) or \(pap1^+\) did not confer multidrug resistance. These findings suggest that \(bfr1^+\) acts as the most downstream effector of the multidrug resistance conferred by \(bfr2^+\) and \(pap1^+\) genes.

Key words: ATP-binding cassette (ABC) superfamily; brefeldin A; multidrug resistance (MDR); \(bfr1^+\) gene; \(pad1^+\) gene

Living organisms can respond to various environmental conditions to protect themselves from damage and to maintain intracellular homeostasis. Energy-dependent export of chemicals is one of those cellular responses, in which efflux pumps in the plasma membrane transport substances out of the cells in an ATP hydrolysis-dependent way. Proteins that belong to the ATP-binding cassette (ABC) superfamily, such as mammalian P-glycoprotein, have a central role in that process. P-glycoprotein was first identified as a substrate overproduced in cancer cells with acquired resistance to various anticancer drugs, a phenomenon known as multidrug resistance. Later studies showed that unicellular organisms such as yeasts also have P-glycoprotein-like membrane transporters, and that their overproduction is involved in resistance to various antibiotics. We previously screened for genes that confer resistance to brefeldin A (BFA) when carried on a multicopy plasmid. A structural gene encoding a homologue of P-glycoprotein in \(S. pombe\), \(bfr1^+\), was isolated and was found to be associated with the multidrug resistance phenotype. We now report here the isolation and characterization of the \(bfr2^+\) gene obtained by further screening for BFA-resistance genes.

\(S. pombe\) cells were transformed by the method of Ito et al. with an \(S. pombe\) genomic library constructed on the multicopy vector pDB248. Among the more than \(1 \times 10^6\) prototrophic \(Leu^+\)-transformants examined, a plasmid we named pBFR2 was selected for further analysis, since it conferred more BFA resistance than the other plasmids. Cells harboring pBFR2 were resistant to up to \(12 \mu\)g/ml BFA, but those harboring vector pDB248 did not grow in the presence of \(4 \mu\)g/ml BFA. Subcloning analysis of a 9-kb insert carried on pBFR2 showed that the smallest region that could confer BFA resistance was 5 kb long. The nucleotides of the entire region were sequenced. A search for protein coding sequences predicted an open reading frame consisting of 308 amino acids; we named it \(bfr2^+\) (brefeldin A resistance). By search of the DDBJ nucleotide database for similar sequences, the \(bfr2^+\) gene was found to be identical to the \(pad1^+\)/\(sks1^+\) gene, isolated as a gene responsible for resistance to the protein kinase inhibitor, staurosporine and K-252a, respectively. These results suggest that the \(bfr2^+\)/\(pad1^+\)/\(sks1^+\) gene is involved in multidrug resistance. To test this idea, we examined the resistance of \(S. pombe\) cells carrying pBFR2 to drugs other than BFA. We monitored the growth of cells grown in a liquid culture by recording the optical density at 550 nm, rather than using solid medium, because this method could detect small differences in the growth rate, especially when a medium containing SDS was used to increase drug sensitivity. Wild-type \(S. pombe\) cells carrying pBFR2 were resistant to staurosporine, K-252a, actinomycin D, and cytochalasin B, indicating that \(bfr2^+\) was a multidrug resistance gene (Table I).

Staurosporine resistance conferred by \(pad1^+\) is mediated by the \(pap1^+\) gene product, an \(S. pombe\) homologue of mammalian transcriptional activator AP-1; in the \(pap1^+\)-null mutant, overexpression of \(pad1^+\) does not confer staurosporine resistance. Overexpression of the \(pap1^+\) gene gave the wild-type cells resistance to the several drugs examined, suggesting that the multidrug resistance phenotype conferred by overexpression of the \(bfr2^+\) gene also depends on the \(pap1^+\) gene (Table I).

---

* Corresponding author. Department of Biotechnology, University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan. Tel. 03-3812-2111 ext.5163; Fax 03-5689-7294; e-mail: arioka@hongo.ecc.u-tokyo.ac.jp

Abbreviations: ABC, ATP-binding cassette; BFA, brefeldin A; kb, kilobase; \(\Delta\), deletion; SDS, sodium dodecyl sulfate
Table 1. Comparison of Drug Resistance of S. pombe Cells Carrying Various Genes on a Multicopy Plasmid.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Minimum inhibitory concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type&lt;sup&gt;a&lt;/sup&gt; harboring</td>
</tr>
<tr>
<td></td>
<td>pDB248&lt;sup&gt;+&lt;/sup&gt; pBF2 p(pap1&lt;sup&gt;+&lt;/sup&gt;) pBF9-1</td>
</tr>
<tr>
<td>BFA (μg/ml)</td>
<td>4 8 8 8 8 2 2 8 8 8 2 2 2 8 8</td>
</tr>
<tr>
<td>Staurorosporine (μM)</td>
<td>2 6 8 8 2 2 6 8 2 6 6 6</td>
</tr>
<tr>
<td>K-252a (μM)</td>
<td>2 5 5 5 1 1 5 5 1 1 1 5</td>
</tr>
<tr>
<td>Actinomycin D (μg/ml)</td>
<td>6 10 10 10 4 4 10 10 4 4 10</td>
</tr>
<tr>
<td>Cytochalasin B (μg/ml)</td>
<td>6 10 10 10 4 4 8 8 4 4 6 10</td>
</tr>
</tbody>
</table>

Cells harboring various plasmids were grown in a liquid SD medium (0.67% yeast nitrogen base without amino acids, 2% glucose) containing 0.006% sodium dodecyl sulfate (SDS) at 30°C for 2 to 3 days. The minimum inhibitory concentrations at which the growth of cells (optical density at 550 nm) was less than 20% of that in the absence of drugs were determined. Plasmids used were: pDB248+, the vector; pBF2, bfr2<sup>+</sup> carried on pDB248; p(pap1<sup>+</sup>), pap1<sup>+</sup> carried on pDB248+; pBF9-1, bfr1<sup>+</sup> carried on pDB248+.<sup>b</sup>

<sup>a</sup> Wild type strain JS333 (his<sup>+</sup> ade6-M216 leu<sup>+</sup>).

<sup>b</sup> Δpap1 strain TP108-3C (h<sup>+</sup> leu<sup>+</sup> ade6-M216 pap1<sup>+</sup> ura4<sup>+</sup>).

<sup>c</sup> Δbf1 strain KEN1 (h<sup>+</sup> ade6-M216 leu<sup>+</sup> ura4<sup>+</sup> bfr1<sup>+</sup> ura4<sup>+</sup>).

I). This suggestion was confirmed in an examination of the drug resistance of Δpap1 mutants carrying pBF2 or other plasmids. As expected, a pap1-null mutant (TP108-3C<sup>90</sup>) carrying pBF2 did not have a pleiotropic drug resistance phenotype, but such cells carrying pap1<sup>+</sup> or bfr1<sup>+</sup> on a multicopy plasmid (either p(pap1<sup>+</sup>) or pBF9-1, respectively) had resistance to these drugs (Table I). This pattern of resistance clearly indicated that the multidrug resistance conferred by overexpression of the bfr2<sup>+</sup> gene also depended on the presence of pap1<sup>+</sup>.

We next examined whether the bfr1<sup>+</sup> gene was involved in the drug resistance conferred by bfr2<sup>+</sup> or pap1<sup>+</sup>. The bfr1<sup>+</sup> gene is a structural and functional homologue of mammalian multidrug resistance P-glycoprotein and can confer multidrug resistance on S. pombe cells. Overexpression of bfr2<sup>+</sup> or pap1<sup>+</sup> genes did not confer Δbfr1 mutant resistance to BFA, K-252a, and actinomycin D, so bfr2<sup>+</sup>- or pap1<sup>+</sup>-mediated resistance to these drugs was dependent on the bfr1<sup>+</sup> gene. In contrast, resistance to staurorosporine and cytochalasin B was partly independent and dependent, respectively, of the presence of the bfr1<sup>+</sup> gene. Resistance to these drugs might involve pmd1<sup>+</sup>, another ABC-type putative membrane transporter of S. pombe, as overexpression of the gene leads to multidrug resistance.<sup>21</sup>

In the upstream region of the bfr1<sup>+</sup> gene, we found several repeats of AP-1-like consensus sequences, although none of them matched exactly the consensus T(T/G)AGTCA sequences (DBJ/EMBL/GenBank accession No. AB003671). In addition, sequences similar to the palindromic sequence reported to be essential for pap1<sup>+</sup>-dependent expression of the p52 gene in S. pombe<sup>10</sup> were found in the 5' region of bfr1<sup>+</sup> as well. These observations suggest that overexpression of the bfr2<sup>+</sup> and pap1<sup>+</sup> genes may lead to transcriptional activation of the bfr1<sup>+</sup> gene, conferring multidrug resistance. To test this possibility, we examined the expression of the bfr1<sup>+</sup> gene in cells carrying multicopy bfr2<sup>+</sup>, pap1<sup>+</sup>, or bfr1<sup>+</sup> genes by northern analysis. Total RNA (10 μg) from wild-type S. pombe cells harboring either vector alone or bfr1<sup>+</sup>, pap1<sup>+</sup>, or bfr2<sup>+</sup> on a multicopy vector, and from the Δbfr1 mutant carrying the vector were prepared, blotted onto nitrocellulose filters, and probed with a bfr1<sup>+</sup> probe. In cells overexpressing bfr1<sup>+</sup>, an intense band at 7 kb and its putative degradation product at 5 kb, both of which corresponded to bfr1<sup>+</sup> mRNA, were detected (Fig. 1A, lane 2). In cells carrying p(pap1<sup>+</sup>) or pBF2, however, little if any change in the expression of bfr1<sup>+</sup> was observed compared with cells carrying the vector alone (lanes 1, 3, and 4). These bands were not detected in the Δbfr1 mutant (lane 5), indicating that they were a bona fide transcript of bfr1<sup>+</sup>. We have shown that the bfr1<sup>+</sup> gene is needed for multidrug resistance of cells that overexpress bfr2<sup>+</sup> or pap1<sup>+</sup>. Because pap1<sup>+</sup> is needed for multidrug resistance conferred by overexpression of bfr2<sup>+</sup>, the bfr2<sup>+</sup> and pap1<sup>+</sup> genes are probably involved in the positive regulatory pathway for the expression of bfr1<sup>+</sup>. It is unlikely, however, that transcription of bfr1<sup>+</sup> is directly regulated by the pap1<sup>+</sup> gene product, as transcription of bfr1<sup>+</sup> did not increase in cells overexpressing bfr2<sup>+</sup> or pap1<sup>+</sup>. In addition, BFA or K-252a did not affect the mRNA levels of bfr1<sup>+</sup>, indicating that the transcription of bfr1<sup>+</sup> was not induced by these drugs (data not shown). This finding is in contrast to the observation with the PDR5 and SNO2 genes of Saccharomyces cerevisiae, the transcription of which increased after drug treatment.<sup>12</sup> Thus the drug resistance conferred by the bfr2<sup>+</sup> and pap1<sup>+</sup> genes seems to have only a post-transcriptional mechanism. The CI<sup>+</sup> channel activity of the product of the cystic fibrosis gene, CFTR, is regulated by phosphorylation,<sup>13</sup> so drug transport activity of the bfr1<sup>+</sup> gene product may be potentiated by some unknown modification of the protein.

Turi and Rose<sup>14</sup> reported that in a bar1<sup>−</sup>/crr1<sup>−</sup> mutant, transcription of hba2<sup>+</sup> (identical to bfr1<sup>+</sup>) is elevated. It was previously shown that crr1<sup>+</sup> is a negative regulator of pap1<sup>+</sup> function,<sup>10</sup> but it is not known whether pap1<sup>+</sup> is involved in transcriptional activation of bfr1<sup>+</sup> in the bar1<sup>−</sup>/crr1<sup>−</sup> mutant.
Acknowledgments

We thank Prof. Mitsuhiro Yanagida (Kyoto University, Japan) and Dr. Takashi Toda (Imperial Cancer Research Fund, UK) for providing the pap1+ gene and pap1 disruptant. We also thank Prof. Masayuki Yamamoto (University of Tokyo, Japan) for the gift of the cam1+ gene. This work was supported by a grant from the New Energy and Industrial Technology Development Organization (NEDO), Japan, and by a grant-in-aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan.

References


Fig. 1. Expression of bfr1+ Gene in Cells Carrying Various Plasmids. Cells grown in a liquid SD medium (10 ml) at 30°C to the exponential phase of growth (with 1 to 2 × 10^6 cells/ml) were harvested by centrifugation, vortexed with 0.6 g of glass beads in 500 µl of lysis buffer (0.2 M Tris-HCl, pH 7.5, 0.5 M NaCl, 10 mM EDTA, and 1% SDS) plus an equal volume of a 24:24:1 mixture of phenol, chloroform, and isooamyl alcohol, and centrifuged. Extraction with phenol was done twice more and the resultant aqueous phase was precipitated with ethanol. Total RNA (10 µg) from wild-type S. pombe cells carrying either the multicopy vector pDB248' (lane 1) or the bfr1+ (lane 2), pap1+ (lane 3), or bfr2+ (lane 4) genes on pDB248', and from the A bfr1 mutant carrying pDB248' were prepared, separated on a 1% agarose gel, transferred to nitrocellulose membranes, and analyzed by northern blotting. A 2.6 kb Hind III fragment covering the central region of the bfr1+ protein coding sequence was used as the bfr1+ probe (A). This central region is completely deleted in the A bfr1 mutant. For the control probe, a 0.5 kb fragment containing the calmodulin gene (cam1+) of S. pombe was used (B). The signal intensities were measured, and the ratio of bfr1+ to cam1+ was calculated. The ratio was standardized in terms of the bfr1+ to cam1+ ratio of cells carrying the vector (C). Probes were labeled with a random primed DNA labeling kit (Boehringer) and [α-32P]dCTP. The positions of 25S and 18S rRNA are indicated in the figure.