Biochimica et Biophysica Acta 1864 (2017) 393–398

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Critical role of the proton-dependent oligopeptide transporter (POT) in the cellular uptake of the peptidyl nucleoside antibiotic, blasticidin S

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ARTICLE INFO

Article history:
Received 26 September 2016
Received in revised form 22 November 2016
Accepted 29 November 2016
Available online 1 December 2016

Keywords:
POT family
Oligopeptide transporter
Blasticidin S
Peptidyl nucleoside
Ptr2
Yeast

ABSTRACT

Blasticidin S (BlaS) interferes in the cell growth of both eukaryotes and prokaryotes. Its mode of action as a protein synthesis inhibitor has been investigated extensively. However, the mechanism of BlaS transport into the target cells is not understood well. Here, we show that Ptr2, a member of the proton-dependent oligopeptide transporter (POT) family, is responsible for the uptake of BlaS in yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. Notably, some mutants of Ptr2 that are dysfunctional in dipeptide uptake were still competent to transport BlaS. Mouse-derived oligopeptide transporter PepT1 conferred BlaS sensitivity in the *S. cerevisiae ptr2*Δ mutant. Furthermore, bacterial POT family proteins also potentiated the BlaS sensitivity of *E. coli*. The role of the POT family oligopeptide transporters in the uptake of BlaS is conserved across species from bacteria to mammals.

1. Introduction

Blasticidin S (BlaS) is an antibiotic produced by *Streptomyces griseochromogenes* [1]. BlaS was first used in agriculture as a natural non-mercurial fungicide to kill *Pyricularia oryzae*, the fungus that causes blast disease in rice. BlaS is a broad spectrum antibiotic and inhibits cell growth in prokaryotes, fungi, plants, and mammalian cells. BlaS occupies the P-site of the large ribosomal subunit and induces conformational changes in tRNA at the P site, thus effectively inhibiting protein synthesis [2]. Structurally, BlaS is a peptidyl nucleoside in which cytosine and modified arginine are linked to deoxyglucuronic acid [3]. Since this cytidine moiety binds to the corresponding guanine base at the P-site, cytosine is crucial for the inhibitory action of BlaS. Some microorganisms, which are naturally resistant to BlaS, produce an enzyme that converts BlaS to an inactive form by deaminating the pivotal cytosine moiety. Two non-homologous genes encoding the BlaS deaminase, named *bsd* and *bsr*, were isolated from *Aspergillus terreus* and *Bacillus cereus*, respectively. Heterologous expression of these BlaS deaminases confers resistance on BlaS-sensitive host cells. This trait enables the utilization of the *bsd* and *bsr* genes as dominant selectable markers. Combination of BlaS and the genes that confer BlaS resistance is now widely used for gene transfer [4–7].

Although the molecular mechanism of how BlaS inhibits cell growth has been investigated extensively, molecular mechanisms involved in the cellular uptake or transport of BlaS are still not fully understood. Recently, it has been reported that inactivation of a leucine-rich repeat-containing protein 8D (LRRC8D) confers resistance to BlaS in cultured mammalian cells [8]. The membrane protein LRRC8D is one of the subunits of a heteromeric complex called volume-regulated anion channel (VRAC) that regulates cell volume in response to changes in the concentration of external osmolytes [9]. However, LRRC8 protein is found only in chordates, and not evolutionarily conserved in bacteria, plants, and lower eukaryotes such as fungi, in spite of the fact that BlaS actively kills all these organisms. Therefore, non-chordates might be expressing a different BlaS transporter.

The proton-dependent oligopeptide transport (POT) family proteins, also termed the peptide transporter (PTR) family, are conserved in all organisms except the Archaea [10–13]. POT family proteins, including mammalian PepT1 and PepT2, are responsible for the uptake of extracellular di- and tripeptides, a variety of peptidomimetic molecules, and drugs of various chemical structures [14–17]. *S. pombe* and *S. cerevisiae*, two widely used model yeasts, have a single POT family protein named Ptr2 [18,19]. Recently, a novel fungal oligopeptide transporter (FOT) family, unrelated to the POT family, has been identified in some fungal species [20,21]. In *S. cerevisiae*, only the strains used for wine brewing contain this transporter, whereas laboratory strains lack the gene for the same [22].

In this study, we show that sensitivity to BlaS in yeasts depends on the presence of the POT family transporter Ptr2 [18,19,23]. Using a

Abbreviations: BlaS, blasticidin S; POT, proton-dependent oligopeptide transporter; FOT, fungal oligopeptide transporter.

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http://dx.doi.org/10.1016/j.bbamcr.2016.11.030
0167-4889/

yeast model system, we examined mouse-derived POT family protein PepT1, and a wine yeast-derived novel peptide transporter Fot1, for their role in the BlaS sensitivity. We have also examined the involvement of the bacterial POT family proteins in BlaS sensitivity in *E. coli*. Our findings suggest that di/tripeptide transporter Ptr2 imports BlaS into the yeast cells, and other POT family proteins in mouse and *E. coli* play a similar role.

2. Results

2.1. Ptr2 oligopeptide transporter is critical for BlaS sensitivity in yeasts

In the fission yeast *S. pombe*, the Ubr11 ubiquitin ligase stimulates the expression of the oligopeptide transporter Ptr2 [19] by degrading Upa1, a transcriptional repressor of the *ptr2* gene (Kitamura, unpublished). Since both *ubr11* and *ptr2* mutants are defective in the uptake of dipeptides, we tested whether the two mutants shared other phenotypes. We reported earlier that a mutant of the *ubr11* ubiquitin ligase in *S. pombe* showed weak resistance to some drugs such as inhibitors of ergosterol synthesis (terbinafine) or protein synthesis (hygromycin B and anisomycin) [24]. Unlike the *ubr11* mutant, the *ptr2* mutant is sensitive to hygromycin B, similar to the wild type strain (Fig. 1A). Interestingly, both *ptr2* and *ubr11* mutants were resistant to BlaS, another protein synthesis inhibitor (Fig. 1A and B). This resistant phenotype was suppressed by the ectopic expression of the Ptr2 from the heterologous *nmt* promoter in both mutants (Fig. 1A, compare second and third rows for *ubr11*Δ, and fourth and fifth rows for *ptr2*Δ), indicating that loss of Ptr2 expression was responsible for the BlaS resistance. When the BlaS sensitive wild type strain was cultured on BlaS containing agar medium, some BlaS resistant colonies appeared spontaneously. All the seven independent colonies, which were randomly chosen from ten such resistant colonies, failed to utilize dipeptide Ala-Gln as a sole nitrogen source, unlike the parental strain (data not shown). This observation further supported the close relationship between BlaS resistance and dipeptide uptake defect.

The simplest hypothesis that accounts for this finding is that BlaS is transported through Ptr2. Addition of soy peptides (di- and tripeptide-enriched mixture) to the medium abolished the sensitivity to BlaS (Fig. 1A), lending support to this hypothesis. In addition, the presence of an excess amount of naturally occurring dipeptides (Ala-Gln, Gly-Gly, Leu-Gly, Gly-Leu) mitigated the sensitivity to BlaS in the Ptr2 expressing strains (Fig. 1A and C), possibly by competing with BlaS in the uptake.

We examined whether Ptr2 was responsible for the uptake of BlaS in the evolutionarily distant yeast *S. cerevisiae*. Similar to *S. pombe*, *ptr2* mutants of *S. cerevisiae* could grow in the presence of BlaS (Fig. 2A). As expected, sensitivity in two different wild type strains was weakened in the presence of naturally occurring dipeptides (Fig. 2B), confirming that mitigation of BlaS toxicity by dipeptides was not a strain-specific effect. Unlike Gly-L-Leu, Gly-D-Leu was ineffective in suppressing the sensitivity. Contrasting effects of Gly-L-Leu and Gly-D-Leu were also observed in *S. pombe* (data not shown). Although D-amino acids are generally toxic to *S. cerevisiae*, Gly-D-Leu itself had no adverse effects for cell growth in both yeasts (data not shown), suggesting the unsuitability of Gly-D-Leu as a substrate for the Ptr2. Among the three *S. cerevisiae* strains tested, two laboratory strains (∑1278b and S288c) were sensitive to BlaS. However, EC1118, which is used for brewing wine, was resistant (Fig. 2C). We confirmed that both *PTR2* alleles of the intrinsically diploid EC1118 strain were inactive because of mutations (data not shown; [20]), further supporting the strong correlation between functionality of Ptr2 and BlaS sensitivity.

A POT family transporter is a proton-driven symporter. Since it uses the proton electrochemical gradient across the membrane to drive import, uptake of peptides is strongly affected by extracellular pH. The pH of unadjusted SD medium was 5.4 before starting the cell culture. High pH has a negative effect on yeast growth. However, the sensitivity to BlaS was significantly alleviated in the high pH (6.5 and 7.0) medium

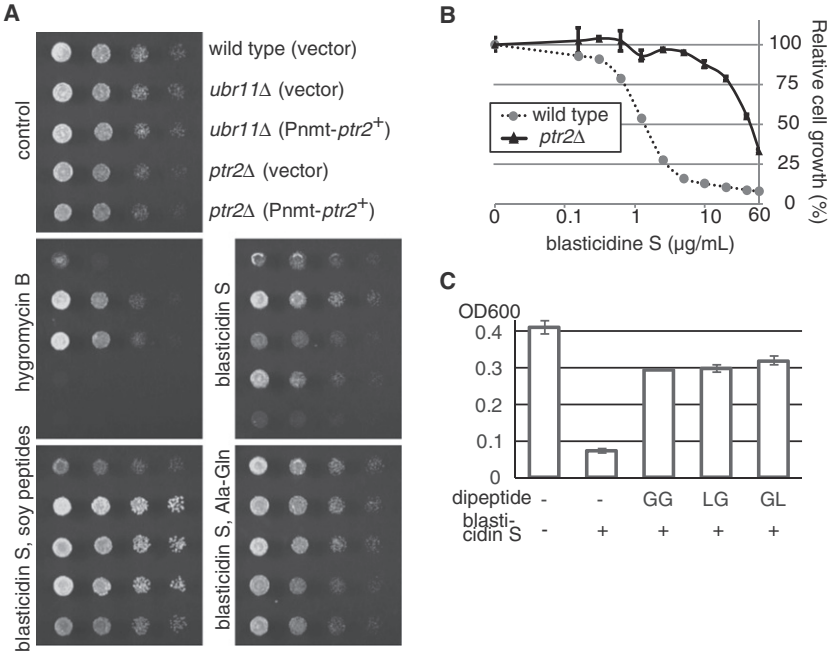

Fig. 1. Ptr2 is critical for BlaS sensitivity in fission yeast *S. pombe*. (A) Serially diluted *S. pombe* cells were spotted on the EMM medium containing hygromycin B (40 μg/mL), BlaS (6 μg/mL), soy peptides (0.1% *w*/*v*), and Ala-Gln (0.1% *w*/*v*). Strains: wild type (vector), KSP3168; *ubr11*Δ (vector), KSP3165; *ubr11*Δ (Pnmt-*ptr2*$^+$), KSP3166; *ptr2*Δ (vector), KSP3195; *ptr2*Δ (Pnmt-*ptr2*$^+$), KSP3196. (B) Wild type (L972) and *ptr2*Δ (KSP2422) strains were inoculated in liquid EMM containing BlaS at the indicated concentration. After 18 h, growth was monitored by measuring absorbance and expressed as percentage relative to BlaS-free culture. (C) Wild type (L972) cells were cultured in EMM containing BlaS (5 μg/mL) with or without indicated dipeptide (0.1% *w*/*v*). Amino acids in the dipeptides are L-forms and shown in single letter convention (e.g., LG means L-Leu-Gly). Absorbance (OD_{600}) was measured after 20 h.