The ATP-binding cassette (ABC) transporter Bpt1p mediates vacuolar sequestration of glutathione conjugates in yeast

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Abstract Vacuolar sequestration or cellular extrusion of glutathione-conjugated xenobiotics and catabolites by ATP-binding cassette (ABC) transporters is an important detoxification mechanism operating in many species. In this study, we show that the yeast ABC transporter Bpt1p, a paralogue of Ycf1p, acts as an ATP-dependent vacuolar pump for glutathione conjugates. Bpt1p, which is inhibited by vanadate and glibenclamide, accounts for one third of the total vacuolar transport of glutathione conjugates. Furthermore, immunoblot analyses show that Bpt1p levels are strongly elevated in early stationary phase, consistent with a function of Bpt1p in vacuolar detoxification.

Key words: Yeast; Vacuole; Glutathione; ATP-binding cassette transporter; Detoxification; Stationary phase

1. Introduction

Conjugation of environmental toxins as well as cellular catabolites and their subsequent elimination from the cytosol represents an important pathway for cellular detoxification in eukaryotic cells. Thus, covalent attachment of xenobiotics to glutathione, glucuronide or sulfate, followed by vacuolar sequestration of conjugates, constitutes an important mechanism of detoxification, at least in plant and fungal species. The actual membrane translocation steps are mediated by MRP-like (multidrug resistance-related protein) transporters, a subfamily of the ubiquitous ATP-binding cassette (ABC) transporter family [1–3]. A total of four human MRPs have been identified as epithelial glutathione conjugate pumps involved in detoxification processes. For instance, MRP1 (ABCC1), the major transporter for the glutathione-conjugated leukotriene LTC_4 [4], is expressed ubiquitously in humans and protects cells and tissues by pumping conjugated xenobiotics into the body [5]. By contrast, MRP2 (ABCC2), localized mainly at the apical side of polarized epithelia in liver and kidney, mediates terminal excretion of conjugated compounds into the bile or urine [6–8]. Finally, another human ABC protein related to MRPs is the cystic fibrosis transmembrane conductance regulator (CFTR) [9,10]. Mutations in the CFTR chloride channel cause cystic fibrosis, the most frequent genetic disease in caucasians [11].

Vacuolar sequestration of conjugated compounds, heavy metals and catabolites is also a prominent way of cellular detoxification in plants [12]. For example, four *Arabidopsis thaliana* MRPs, AtMRP1, AtMRP2, AtMRP3 and AtMRP5, exhibit glutathione transport activity [13–16]. Furthermore, AtMRP2 and AtMRP5 pump glucuronides [16,17], suggesting that certain vacuolar MRPs display ATP-dependent transport activities for artificial and plant-specific glucuronides [18–20]. In the yeast *Saccharomyces cerevisiae*, six members of the MRP family exist [21], some of which reside in the vacuole. Ycf1p, the cadmium resistance factor [22], represents an important mediator of cellular detoxification through vacuolar sequestration of a wide variety of glutathione conjugates [22–25], as well as cadmium tolerance through ATP-dependent transport of bis(glutathionato)cadmium complexes [26]. Notably, functional expression in yeast revealed that human MRP1, as well as plant AtMRP1, AtMRP2, AtMRP3 and AtMRP5, can complement the organic anion transport activity of Ycf1p [13–17,24]. Because Ycf1p shares some homology with human CFTR, it was used as a model for the characterization of prevalent CFTR mutations found in cystic fibrosis patients [27,28]. For instance, mutations in the nucleotide-binding domains impair cadmium resistance or LTC_4 transport [27,28].

Two additional yeast ABC proteins have been implicated in vacuolar transport processes. The Ybt1p/Bat1p (*YLL048c*) transporter translocates taurocholate and other bile acids across vacuolar membranes [29]. Furthermore, Bpt1p (bile pigment transporter-1/*YLL015w*), the closest yeast homologue of Ycf1p, was originally discovered as a pump mediating vacuolar uptake of unconjugated bile pigments and magnetic resonance contrast agents [30,31]. However, little information about the biochemical properties of these ABC transporters and potential physiological substrates is available. In this study, we report that Bpt1p, like Ycf1p, works as a vacuolar pump for glutathione conjugates. Bpt1p function accounts for one third of the conjugate uptake activity, and we show that vacuolar Bpt1p levels increase when cells approach stationary

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Abbreviations: ABC transporter, ATP-binding cassette transporter; DNB-GS, *S*-dinitrobenzeneglutathione; CDNB, 1-chloro-2,4-dinitrobenzene; CFTR, cystic fibrosis transmembrane conductance regulator; MRP, multidrug resistance-related protein; AMP-PNP, adenosine 5′-(β,γ-imino)triphosphate

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phase. Our data demonstrate that Bpt1p and Ycf1p are the major players for vacuolar sequestration of glutathione conjugates in yeast.

2. Materials and methods

2.1. Yeast strains and plasmid constructions

Unless otherwise indicated, all yeast strains were cultivated at 30°C. The isogenic yeast strains DTY7 (*MAT*α *ura3-52 leu2-3,112 his6*), DTY168 (Δ*ycf1*::*hisG*), YMK1 (Δ*bpt1*::*LEU2*) and YMK2 (Δ*ycf1*::*hisG* Δ*bpt1*::*LEU2*) were used for uptake experiments. YMK1 and YMK2 were obtained by integrating a DNA fragment containing the regions −520 to +18 and +4032 to +4524 of *YLL015w* along with the *LEU2* marker into the strains DTY7 and DTY168, respectively. Heavy metal resistance assays were carried out in strain W303-1A (*MAT***a** *ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100*) and its isogenic derivatives YHW11 (Δ*ycf1*::*HIS3*), YYA2 (Δ*bpt1*::*loxP-kanMx-loxP*) and YYA3 (Δ*bpt1*::*loxP-kanMx-loxP* Δ*ycf1*::*HIS3*). The strains YYA2, YYA3 and YHW11 contained start-to-stop Δ*bpt1* and Δ*ycf1* deletions, respectively, generated by genomic integration of appropriate PCR products from the plasmids pFA6a-His3Mx6 [32] or pUG6 [33]. Strain YYA1 (W303-1A, *BPT1*::*3HA-KanMx6*) was obtained by genomic tagging of *BPT1* with the triple *Haemophilus influenzae* (HA) epitope using the plasmid pFA6a-3HA-KanMx6 [34] and appropriate oligonucleotides to amplify the *BPT1-HA* PCR fragment. Strain YYA9 (*BPT1*::*GFP-His3Mx6*) was obtained by genomic integration of a PCR product generated from plasmid pFA6a-GFPS65T-His3Mx6 [34] into YYA1, thereby replacing the HA tag by green fluorescent protein (GFP). All PCR products were transformed into yeast cells by routine laboratory methods [35], and correct genomic integration was verified by PCR analysis. For construction of the high-copy plasmid pMK1, the entire *BPT1* gene (*YLL015w*) was PCR-amplified from genomic DNA using appropriate oligonucleotides and the Expand high fidelity PCR system (Roche, Switzerland). The *BPT1* gene was then recloned as a *Not*I restriction fragment into the corresponding site of pNEV, placing *BPT1* expression under the control of the plasma membrane ATPase promoter [36].

2.2. Yeast cell extracts, fluorescence studies and cytotoxicity assays

About 5–10 OD_{600} cell equivalents were harvested, washed once and resuspended in ice-cold water. Cell extracts for SDS–PAGE analysis and immunoblotting were prepared exactly as described previously [37]. For fluorescence analysis, cells were cultured to the exponential growth phase in YPD containing additional adenine to prevent staining of the vacuolar lumen. Vacuolar membranes were stained by incubating cells with 8 μM FM4-64 fluorescent dye (Molecular Probes, USA) for 15 min at 30°C. Cells were inspected on a DMR confocal microscope (Leica Microsystems, Germany) using a Leica TCS 4D operating system equipped with a fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) filter set to detect GFP or FM4-64, respectively. Digital images were pseudocolored using Adobe Photoshop 6.0 (Mountain View, USA), with red attributed to FM4-64 and green to GFP.

2.3. Preparation of yeast membrane vesicles and transport studies in vitro

Yeast membrane vesicles for in vitro transport studies were essentially isolated as described elsewhere [24,38] using the following modifications. Briefly, strains DTY7, DTY168, YMK1 and YMK2 were grown overnight in YPD to an OD_{600} of 2–5. YMK2 transformed with pMK1 or the control vector pNEV were grown overnight in selective medium to an OD_{600} of 1–2, washed once with water, resuspended in YPD and incubated for an additional 2 h. Cells were washed twice with water and resuspended to an OD_{600} of 1–2 in lyticase buffer (1.1 M sorbitol, 20 mM Tris–HCl pH 7.6, 1 mM dithiothreitol (DTT)) containing 57 units of lyticase per ml. After digestion was completed, spheroblasts were collected by a 10 min centrifugation at 1200×*g*. Cell lysis was achieved on ice in 25 ml of breaking buffer (1.1 M glycerol, 50 mM Tris–ascorbate pH 7.4, 5 mM EDTA, 1 mM DTT, 1.5% polyvinylpyrrolidone, 2 mg/ml bovine serum albumin (BSA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin), using a Dounce homogenizer and 40 strokes with a tight-fitting glass piston. Unbroken cells and debris were removed by washing and two centrifugations at 4000×*g* for 10 min. The supernatants were pooled and spun at 100 000×*g* for 45 min. The microsomal membrane pellet was suspended to an OD_{600} of about 4 in vesicle buffer (1.1 M glycerol, 50 mM Tris–ascorbate pH 7.4, 1 mM EDTA, 1 mM DTT, 2 mg/ml BSA, 1 mM PMSF, 1 μg/ml leupeptin). Small aliquots were immediately used for transport assays or stored at −80°C until use.

[^{14}C]*S*-2,4-Dinitrobenzeneglutathione ([^{14}C]DNB-GS; 10 mCi/mmol) was prepared exactly as described previously [39]. Uptake of [^{14}C]DNB-GS into membrane vesicles was measured by the rapid filtration technique using nitrocellulose filters (0.45 μm pore size; Schleicher and Schuell). Vesicles were mixed with six parts of transport buffer (0.4 M glycerol, 100 mM KCl, 20 mM Tris–MES pH 7.4, 1 mM DTT) and incubated with 40 μM [^{14}C]DNB-GS at 25°C in the absence or presence of 5 mM Mg-ATP, unless stated otherwise. Filters were washed, dried and then subjected to liquid scintillation counting. Counts were corrected for background and quenching. Quantification and calculations were done using Sigma Plot (Jandel Scientific, USA).

3. Results

3.1. Bpt1p localizes to the vacuolar membrane

A previous study characterized Bpt1p (*YLL015w*) as a transporter mediating uptake of unconjugated bilirubins into vacuolar vesicles [30], implying vacuolar localization of Bpt1p. To prove this assumption, we produced strain YYA9 expressing a functional Bpt1p-GFP fusion protein at chromosomal levels. Wild type W303-1A and YYA9 cells were cultivated to the exponential growth phase, and aliquots of cultures were treated for 15 min with FM4-64 to stain vacuolar membranes. After 90 min of growth in YPD lacking the dye, inspection of W303-1A and YYA9 cells by confocal microscopy revealed a perfect co-localization of Bpt1p-GFP and the FM4-64 fluorescence signals (Fig. 1A–C) in YYA9 cells, while W303-1A control cells lacked any detectable GFP fluorescence signals

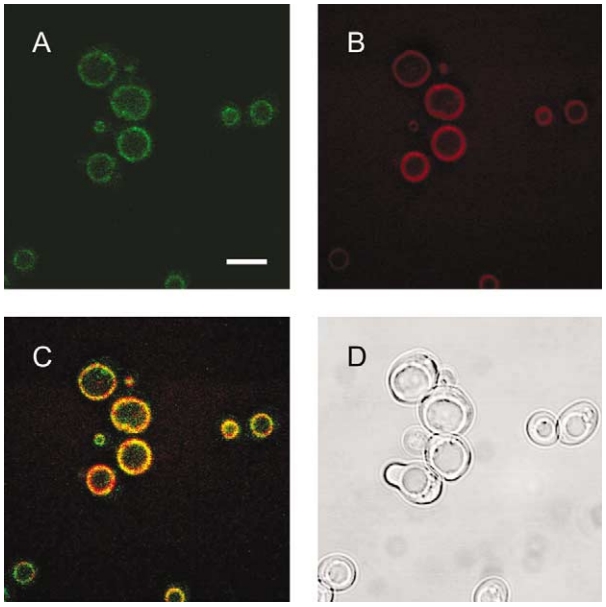

Fig. 1. Bpt1p is a vacuolar membrane protein. Strain YYA9 (Bpt1p-GFP) was cultivated in YPD to the exponential growth phase. Vacuolar membranes were stained in vivo by adding FM4-64 to the growth medium for 15 min as stated in Section 2. Fluorescence in living cells was visualized in a confocal microscope using FITC and TRITC filter sets. A: GFP fluorescence. B: FM4-64 staining of the vacuolar membrane. C: Pseudo-colored and merged images with GFP in green and FM4-64 in red. D: The corresponding transmitted light image. Bar = 5 μm.

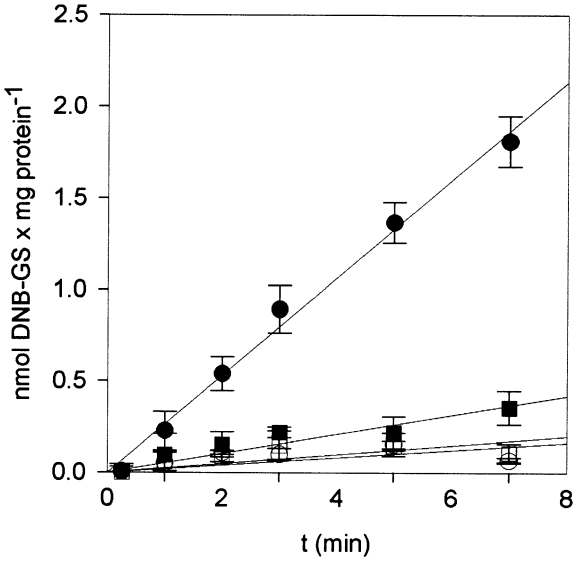

Fig. 2. Bpt1p mediates vacuolar sequestration of DNB-GS. Time-dependent uptake of [^{14}C]DNB-GS mediated by Bpt1p expressed from the multicopy plasmid pMK1. Total membrane vesicles isolated from YMK2 transformed with plasmid pMK1 (●) or the empty vector (■) were incubated with 40 μM [^{14}C]DNB-GS in the presence (filled symbols) or absence (open symbols) of 5 mM Mg-ATP. All experiments were carried out in triplicate. Error bars indicate standard deviations.

(data not shown). Bpt1p-GFP signals in YYA9 cells were only detected in vacuolar membranes, but not in any other cellular membrane compartment, showing that Bpt1p is exclusively localized in the yeast vacuole.

3.2. *Bpt1p mediates ATP-dependent vacuolar uptake of glutathione conjugates*

Bpt1p is the closest homologue to Ycf1p, sharing more than 42% sequence identity with Ycf1p, a vacuolar transporter for glutathione conjugates [23,24]. Hence, we performed in vitro transport experiments using radiolabeled [^{14}C]DNB-GS and membrane vesicles prepared from wild type strain (DTY7) and strains carrying Δ*ycf1* (DTY168), Δ*bpt1* (YMK1) or a Δ*ycf1* Δ*bpt1* double deletion (YMK2). Specific ATP-dependent [^{14}C]DNB-GS uptake into vesicles from wild type DTY7 cells was set as 100% and compared to the transport in vesicles isolated from isogenic deletion strains (Table 1). As

Table 1
Bpt1p and Ycf1p mediate ATP-dependent vacuolar transport of DNB-GS

Strain	pmol DNB-GS × (min × mg protein)$^{-1}$	% of wild type activity
DTY7 (wild type)	339 ± 93	100.0
DTY168 (Δ*ycf1*)	85 ± 63	25.0
YMK1 (Δ*bpt1*)	236 ± 9	69.7
YMK2 (Δ*ycf1* Δ*bpt1*)	2 ± 2	5.2

Uptake of 40 μM [^{14}C]DNB-GS into membrane vesicles isolated from strains DTY7, DTY168, YMK1 and YMK2 was measured in the presence of 5 mM ATP. Incubations were terminated after 7 min and aliquots removed for liquid scintillation counting. Values were corrected for the radioactivity bound to the filters after 30 s of uptake. Corresponding control assays were performed in the absence of ATP. The data correspond to five independent experiments, each of which was performed in triplicate.

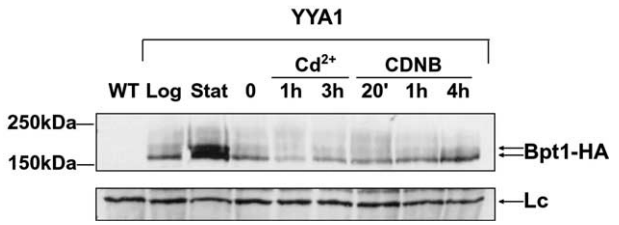

Fig. 3. Bpt1p levels increase in cells approaching early stationary phase. Immunoblotting of cellular extracts from wild type (WT) and strain YYA1 expressing genomic levels of Bpt1p-HA in exponentially growing cells (Log) and in early stationary phase cultures (Stat). Cells were exposed to 100 μM cadmium or 5 μM CDNB for various time periods. Extracts were subjected to immunoblotting using the monoclonal HA antibody 16B12. Lc, loading control.

expected, Δ*ycf1* vesicles displayed a DNB-GS uptake that was reduced by about 75% when compared to wild type cells. Vesicles prepared from the Δ*bpt1* deletion strain showed a consistent reduction of DNB-GS transport by about 30% when compared to control cells (Table 1). However, DNB-GS uptake was essentially abolished in vesicles from the Δ*ycf1* Δ*bpt1* deletion strain. These data show that Bpt1p and Ycf1p are both transporters of glutathione conjugates and mediate the bulk of total DNB-GS uptake into vacuoles (Table 1).

To confirm that Bpt1p mediates DNB-GS transport, we overexpressed Bpt1p in the Δ*ycf1* Δ*bpt1* strain YMK2 using the high-copy plasmid pMK1. Membrane vesicles were prepared from transformants and used to determine DNB-GS uptake activities (Fig. 2). Vesicles from cells carrying pMK1 accumulated significant levels of [^{14}C]DNB-GS only in the presence of ATP, whereas vesicles from cells harboring the empty vector control displayed only insignificant DNB-GS uptake (Fig. 2). Therefore, Bpt1p expressed from a multicopy plasmid can restore ATP-dependent glutathione conjugate transport in Δ*ycf1* Δ*bpt1* cells.

To analyze the properties of Bpt1p-mediated conjugate transport, we also investigated the effects of trinucleotides, a non-hydrolyzable ATP analogue and different inhibitors (Table 2) on Bpt1p transport into vesicles from the strain YMK2 containing pMK1. As for Ycf1p [40], GTP can partially replace ATP as the energy source, since it could restore 70% of the ATP-dependent uptake. As expected, the non-hydrolyzable ATP analogue adenosine 5′-(β,γ-imino)triphosphate (AMP-PNP) failed to significantly drive DNB-GS transport

Table 2
Effect of nucleotides and inhibitors on Bpt1p-mediated [^{14}C]DNB-GS transport

Nucleotide	Inhibitor	% of activity
ATP	–	100
GTP	–	70.7 ± 3.2
AMP-PNP	–	12.9 ± 2.2
ATP	1 mM vanadate	33.7 ± 1.4
ATP	0.1 μM bafilomycin A1	100.2 ± 0.9
ATP	5 mM NH_4Cl	87.9 ± 12.7
ATP	150 μM glibenclamide	19.5 ± 6.2

Membrane vesicles isolated from strain YMK2 harboring pMK1 were incubated for 7 min with 40 μM [^{14}C]DNB-GS in the presence or absence of 5 mM Mg-ATP, Mg-GTP, Mg-AMP-PNP and the compounds indicated. Values were corrected for unspecific binding and controls were performed with membrane vesicles isolated from YMK2 cells carrying the empty control vector in the presence and absence of Mg-ATP. Each experiment was performed in triplicate.

(Table 2). The presence of 1 mM vanadate, a known inhibitor of the ATPase activity of ABC transporters, reduced transport to about 33% of the control value, while bafilomycin A1 did not affect [^{14}C]DNB-GS uptake. Finally, ammonium chloride slightly decreased [^{14}C]DNB-GS transport to about 88% of the control value. By contrast, addition of 150 μM glibenclamide to the reaction efficiently blocked 80% of the ATP-dependent uptake.

3.3. Bpt1p levels increase when cells approach stationary phase

We have demonstrated that Bpt1p and Ycf1p share overlapping functions in vacuolar sequestration. Expression of *YCF1* is regulated by the stress mediator Yap1p and induced by substrates such as cadmium. Thus, we tested whether or not expression of *BPT1* mRNA or Bpt1p protein levels are regulated by exposure to cadmium and 1-chloro-2,4-dinitrobenzene (CDNB) or during various growth stages. Cells expressing epitope-tagged *BPT1-HA* were thus grown to the mid-exponential (OD_{600} of 1.0) and early stationary growth phase (OD_{600} of about 8.0). Exponentially growing cells were exposed to 100 μM cadmium and 5 μM CDNB for various times as indicated. Extracts were subjected to immunoblotting using the monoclonal HA antibody 16B12. Bpt1p-HA migrated as an apparent double band, particularly in stationary phase cells, which could reflect either processing intermediates or post-translational modifications. However, Bpt1p-HA levels were not influenced by either cadmium or CDNB (Fig. 3, lanes 4–9), as were mRNA levels (data not shown). Nevertheless, Bpt1p levels increased more than five-fold when cells approached early stationary phase when compared to logarithmically growing cells (Fig. 3, lanes 2 and 3), whereas the internal loading control, a cross-reaction to the HA antibody, remained unchanged. Hence, Bpt1p levels increase sharply when cells enter early stationary phase, which is consistent with an important role for Bpt1p in vacuolar detoxification.

4. Discussion

In yeast only one ABC transporter, Ycf1p, was hitherto known to act as a glutathione conjugate pump. However, Δ*ycf1* cells still display residual vacuolar uptake activities for conjugates. In this study, we demonstrate that the Ycf1p homologue Bpt1p functions as the second MRP-like ABC pump in the yeast vacuolar membrane. Bpt1p shares 42% sequence identity with Ycf1p, and displays a functional overlap with Ycf1p with respect to glutathione conjugate sequestration. This conclusion is based on several observations. First, we show here that the Bpt1p transport specificity includes glutathione conjugates, many of which may arise from metabolism or stress as physiological substrates. Second, microscopic imaging of live cells demonstrates that chromosomal levels of Bpt1-GFP, like Ycf1p, localize exclusively to the vacuolar membrane (Fig. 1). Third, Bpt1p comprises almost one third of the total vacuolar uptake of glutathione conjugates into membrane vesicles in vitro (Fig. 2, Table 1). Fourth, conjugate transport by Bpt1p is ATP-dependent and can be inhibited by vanadate and glibenclamide (Table 2). Finally, cells lacking Bpt1p exhibit reduced growth on medium containing Cd^{2+} ions (data not shown).

Ycf1p was first identified by its ability to confer cadmium resistance [22]. Moreover, Ycf1p-mediated heavy metal detoxification is based on vacuolar import as glutathione complexes [23]. We consistently observed a cadmium hypersensitivity phenotype of Δ*bpt1* cells, although it is much less strongly pronounced than in the Δ*ycf1* deletion strain (data not shown). Thus, Cd^{2+} transport might not represent the main activity of Bpt1p in yeast cells in vivo. The minor role in cadmium transport might be explained by the fact that Bpt1p actually represents a residue-specific 'natural mutant variant' of Ycf1p. Residue S1212 in Ycf1p, which is essential for cadmium resistance, is replaced by leucine in Bpt1p. Strikingly, the S1212L-Ycf1p variant is very inefficient in conferring cadmium resistance [28]. Hence, it is not surprising that Bpt1p has escaped discovery through genetic screens using heavy metal resistance phenotypes.

Although Bpt1p transport activities were weaker than those of Ycf1p with respect to heavy metal resistance and conjugate transport, our data show that deletion of Ycf1p reduced transport by about 75%, while Bpt1p accounted for more than 25% of the total uptake. The reduction of DNB-GS transport in YYA2 is a specific Δ*bpt1* phenotype, since Bpt1p expressed from the high-copy plasmid pMK1 could restore DNB-GS transport in a Δ*ycf1* Δ*bpt1* background. As expected for an ABC pump, Bpt1p-mediated transport is fueled by ATP hydrolysis, although GTP can also serve as the energy source, albeit at lower efficiency. The sulfonylurea compound glibenclamide, which has been shown to inhibit human MRP1 [41] and AtMRP5 from *A. thaliana* [16], also drastically blocks Bpt1p function in DNB-GS transport (Table 2).

Despite an overlapping function and substrate specificity of Ycf1p and Bpt1p, we present evidence for interesting differences concerning the regulation of these genes. *YCF1* expression is tightly regulated by Yap1p in response to heavy metal stress [26,42]. By contrast, Bpt1p expression appears not regulated either by its substrates cadmium and CDNB or under different stress conditions such as high osmolarity (data not shown). Nevertheless, we show that Bpt1p protein levels increase when cells approach early stationary phase. Hence, it is tempting to speculate that the main Bpt1p function is to combat metabolic challenges such as those yeast encounters during high-density growth. The overlapping substrate specificity of Bpt1p and Ycf1p, their identical cellular localization yet complementary functions and distinct regulation indicate that yeast cells are well-equipped to oppose both endogenous and exogenous challenges such as those imposed by environmental or metabolic stress.

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