Localization and Function of the Yeast Multidrug Transporter Tpo1p*

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In Saccharomyces cerevisiae four transporters, Tpo1p-Tpo4p, all members of the major facilitator superfamily, have been shown to confer resistance to polyamines. It was suggested that they act by pumping their respective substrate into the lumen of the vacuole depending on the proton gradient generated by the V-ATPase. Using sucrose gradient ultracentrifugation we found that an hemagglutinin (HA)-tagged Tpo1p as well as its HA-tagged Tpo2p–4p homologues co-localize with plasma membrane markers. Because the HA-tagged Tpo1p carrier protein proved to be functional in conferring resistance to polyamines in TPO1 knockouts, a function of Tpo1p in transport of polyamines across the plasma membrane seemed to be likely. The polyamine transport activity of wild type cells was compared with the respective activity of a TPO1 knockout strain. The results obtained strongly suggest that Tpo1p is a plasma membrane-bound exporter, involved in the detoxification of excess spermidine in yeast. When studying polyamine transport of wild type cells, we furthermore found that S. cerevisiae is excreting putrescine during the fermentative growth phase.

Polyamines are essential compounds occurring in virtually all prokaryotic and eukaryotic cells (1). Because these compounds are toxic in higher concentrations, their intracellular content is tightly regulated by controlling biosynthesis and degradation and also by transport into intracellular storage compartments or out of the cell. In the yeast Saccharomyces cerevisiae the polyamines putrescine, spermidine, and spermine are found. All three compounds are actively transported into the vacuole by a substrate/nH⁺ antiport mechanism (2). Four transport proteins, Tpo1p–Tpo4p, have been postulated to be responsible for the polyamine transport capability of the yeast vacuolar membrane (3, 4). According to their protein sequences, these four proteins are all members of a family of multidrug resistance transporters within the major facilitator superfamily. Tpo1p was identified by its sequence similarity to the Bacillus subtilis multidrug transporter Blt, which is involved in spermidine excretion (5). The other transporters of this group were found on the basis of sequence similarity to Tpo1p (4). The function of all Tpo proteins was deduced from their plasma membrane markers. Because the HA-tagged Tpo1p as well as its HA-tagged Tpo2p–4p homologues co-localize with plasma membrane markers, the HA-tagged Tpo1p carrier protein proved to be functional in conferring resistance to polyamines in TPO1 knockouts, a function of Tpo1p in transport of polyamines across the plasma membrane seemed to be likely. The polyamine transport activity of wild type cells was compared with the respective activity of a TPO1 knockout strain. The results obtained strongly suggest that Tpo1p is a plasma membrane-bound exporter, involved in the detoxification of excess spermidine in yeast. When studying polyamine transport of wild type cells, we furthermore found that S. cerevisiae is excreting putrescine during the fermentative growth phase.

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1 The abbreviations used are: HA, hemagglutinin; MES, 4-morpholineethanesulfonic acid; HPLC, high pressure liquid chromatography.
**Yeast Multidrug Transporter Tpo1p**

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### TABLE I

**S. cerevisiae strains used in this study**

| Strain | Genotype | Source |
|--------|----------|--------|
| 23344c | MATα ura3 | Bruno André (Brussels, Belgium) |
| 6044c  | MATα YCR023c-3xHA kanMX ura3 | Wolf Frommer (Tübingen, Germany) |
| RK 25  | MATα TPO1-3xHA kanMX ura3 | This work |
| RK 15  | MATα TPO2-3xHA kanMX ura3 | This work |
| RK 13  | MATα TPO3-3xHA kanMX ura3 | This work |
| RK 11  | MATα TPO4-3xHA kanMX ura3 | This work |
| RK 26  | MATα tpo1::lacZ kanMX ura3 | This work |

**Plasmid Constructions**—The yeast expression vector pDR199 was obtained from Wolf Frommer (Tübingen, Germany). It is a derivative of pDR195 (14), which in turn was generated from YEplac195 (15). The plasmid contains a copy of the PMA1 promoter and the terminus region of the ADH1 gene. The TPO1 gene was amplified via PCR using genomic DNA of strain 23344c as template and cloned between promoter and terminator using an XmaI and an XhoI site. The PCR primers for TPO1 were 5'-GGGTCCTCGAGTTAGGCGGCGTAGTCAGGAAC, with the underlined sequences indicate the XmaI and XhoI sites, respectively. The TPO1–3xHA fusion gene was amplified via PCR with genomic DNA from strain RK 25 as template using the same 3′ primer as for the amplification of the unmodified TPO1. The sequence of the 3′ primer was GCAGTGTTAGCCGCGTAGTCAGGAAC, with the XhoI site underlined. The PCR fragment was inserted into pDR199 by using the XmaI and the XhoI site between PMA1 promoter and ADH1 terminator. Accuracy of the constructs was verified by sequencing. The plasmids were transformed into yeast according to Gietz (17). Phenylalanine transport measurements were performed according to Sato et al. (20), and spermine import was determined after Kakinuma et al. (21).

**Export Measurements of Accumulated Spermidine**—The cells were grown overnight in succinate-buffered CBS medium in the presence of 10 mM spermidine and harvested at A600 = 1.3–1.5 by centrifugation. The cell pellets were washed three times in medium without polyamines but containing only 1 g/l of NH4SO4 and finally resuspended in the same medium. The cell suspension was incubated at 30 °C with agitation, and aliquots were taken at defined time points. To analyze spermidine efflux, the cells were immediately separated from the medium by centrifugation (11,000 × g for 3 min), and released spermidine was quantified using high performance liquid chromatography according to Price et al. (21).

**Determination of Total Polyamine Content**—Extraction of total polyamines was performed according to Tomitori et al. (4). Yeast cells were incubated in 10% trichloroacetic acid at 65 °C for 1 h. Derivatization and quantification by high performance liquid chromatography were done as described above.

**RESULTS**

**Tpo1p Is Localized in the Cytoplasmic Membrane**—In the course of an approach to identify new vacuolar transporters, we determined the subcellular localization of various putative transport proteins that have been fused C-terminally to a triple HA epitope tag. A version of Tpo1p with the same tag was generated as bona fide control, because we considered it to be an established vacuolar transporter (3, 4). The intracellular localization was determined by sucrose density gradient centrifugation of membrane preparations isolated from yeast cells carrying the respective fusion construct as the only gene copy. Defined fractions were separated by SDS-PAGE and subsequently analyzed by immunoblotting (Fig. 1). Although the 3xHA-tagged version of Ycr023p co-localized with the 100-kDa subunit of the vacuolar ATPase (Vph1p), the Tpo1p–3xHA fusion co-localized with the plasma membrane ATPase (Pma1p), thus raising the question of whether tagging of Tpo1p leads to mislocalization, although the HA tag is not known to direct proteins to the plasma membrane.

To get further evidence we tested the functionality of the HA

5.5, and supplemented with 10 mM putrescine and 1 mM of each spermine and spermidine to an A600 of 2–3. The cells were harvested and resuspended in 100 mM Tris-sulfate, pH 9.4, 10 mM dithiothreitol to yield a concentration of 0.5 g of cell fresh weight/ml. The cells were shaken for 10 min at 30 °C, centrifuged, and resuspended to 0.15 g of cell fresh weight/ml in SOB (1.2 mM sorbitol, 5 mM MES-Tris, pH 6.9). The spheroplasts were generated by incubation with lysing enzymes (Sigma) (1 mg/5 × 106 cells) for 30 min at 30 °C. The spheroplasts were washed twice in SOB and resuspended in buffer A (12% Ficoll, 10 mM MES-Tris, pH 6.9, 100 μM MgCl2), protease inhibitors (Complete; Roche Molecular Biochemicals) were added, and the suspension was homogenized by seven strokes of a Wheaton A Dounce homogenizer. The resulting suspension was transferred to a centrifuge tube, overlaid with half the volume of buffer A, and centrifuged at 60,000 × g for 1 h and 15 min. The white floating layer that appeared after centrifugation was transferred to an ultracentrifuge tube, adjusted with buffer A (see above) to 6 ml, and overlaid with 6 ml of buffer B (8% Ficoll, 10 mM MES-Tris, pH 6.9, 100 μM MgCl2). After further centrifugation (60,000 × g for 1 h and 15 min), vacuolar vesicles formed a white layer on top of the solution. They were aspirated, suspended in 100 mM MES-Tris, pH 6.9, 100 mM KCl, 20 μM MgCl2, frozen under liquid nitrogen, and stored for further use at −80 °C. The enzyme assays were done according to Roberts et al. (19). Phenylalanine transport measurements were performed according to Sato et al. (20), and spermine import was determined after Kakinuma et al. (21).
fused to Tpo1p. Disruption of TPO1 leads to increased polyamine sensitivity of the mutant cells, when grown under Mg²⁺-limiting conditions (3). This phenotype should be rescued by expression of the HA-modified version of the transporter if this fusion is functional. We thus transformed a plasmid encoded version of TPO1–3xHA into a strain in which TPO1 had been replaced by a lacZ-kanMX deletion cassette. The fused gene on the plasmid was expressed under the control of the PMA1 promoter. The resulting yeast cells were tested for spermidine sensitivity in comparison with the wild type and the tpo1::lacZ strain, each transformed with the empty vector (pDR199). We found that synthesis of the HA-tagged Tpo1p leads to markedly increased spermidine tolerance. Because the functionality of the HA fusion of Tpo1p could be demonstrated, we tried to get further evidence for the Tpo1 protein being located in the plasma membrane, where it should contribute to polyamine transport.

The Polyamine Uptake Rate of Intact Cells Is Not Influenced by TPO1 Deletion—Because the localization of Tpo1p in the plasma membrane was contradictory to the proposed function in vacuolar polyamine transport, we tested first whether polyamine import into cells is influenced by TPO1 deletion. An earlier report had indicated that a TPO1 deletion strain shows spermine import rates similar to wild type cells (3), whereas a decreased import rate in TPO1 deletions was reported later (4). We found, however, no difference in spermine uptake activity between the tpo1::lacZ strain and wild type using 100 µM (Fig. 3) or 20 µM spermine (not shown), suggesting that Tpo1p is not involved in polyamine import of yeast cells and/or that Tp2p–Tpo4 may complement the knockout of Tpo1p.

Vascular Vesicles from a TPO1 Disruption Strain Do Not Show Any Impairment in Polyamine Uptake—In view of several reports describing Tpo1p as vacuolar polyamine importer, we tried to account for an impairment in vacuolar polyamine uptake after TPO1 deletion. Consequently, we prepared vascular vesicles from wild type cells and tpo1::lacZ mutants, respectively, grown in the presence of 10 mM putrescine, 1 mM spermidine, and 1 mM spermine. Polyamines were added to the growth medium because a stimulatory impact of externally added polyamines on TPO1 expression had been reported recently (4). The polyamine concentrations employed led to a markedly increased intracellular polyamine level (about 4-fold in the case of spermine and spermidine, and about 2-fold for spermine), which should be high enough to enhance the expression of TPO1.

We optimized the protocol for the isolation of vacuolar vesicles, which led to preparations of high purity (Table II). The ATPase activity of the vesicle preparation was completely inhibited by the addition of the V-ATPase-specific inhibitor concanamycin A (not shown), proving that significant contaminations of plasma membrane and mitochondria were virtually absent. The vesicles were used to determine the uptake of amino acids (20) and spermine (2). Because phenylalanine uptake measurements in particular were highly reproducible in isolated vascular vesicles, we used this carrier activity to normalize the spermine uptake rate and thus to eliminate possible variations caused by the vesicle preparation, a strategy that was not employed in the original report of Tomitori et al. (4), who reported a slightly decreased import activity for vacuolar vesicles prepared from a TPO1 deletion strain compared with wild type. But this correction might be crucial, because we found that although the absolute values varied slightly for each preparation, the relative uptake rates measured were essen-
the termined. It could be shown that the spermidine efflux rate of cultures of wild type and mutant cells, respectively, were decreased as compared with the wild type (Fig. 6A). The observed difference in efflux rates was not due to a significant difference between wild type and mutant cells in fact excrete putrescine but not spermidine until they reach the diauxic shift (Fig. 5) after ~24 h. The putrescine release of the two strains was indistinguishable, suggesting that Tpo1p is not involved in this process.

Polyamine Export of Growing Yeast Cells—Although spermidine is the most abundant polyamine in yeast cells (Ref. 9; see also Fig. 6B), we did not observe release of this solute under normal growth conditions. To challenge yeast cells for spermidine export, we increased the intracellular spermidine content by growing cells in the presence of this compound. The cells were cultured in the presence of 10 mM spermidine in succinate-buffered CBS medium under non-limiting Mg2+-conditions, where both wild type and mutants are more tolerant to polyamine stress, and the spermidine concentrations applied are thus not toxic for both strains. During the incubation the total intracellular spermidine content of yeast cells in the logarithmic growth phase increased significantly from about 8 to 25 nmol/mg cell dry mass (Fig. 6B). After washing and resuspending in polyamine-free medium, the accumulated spermidine was released. We found a significant difference between wild type and tpo1 deletion mutant under these conditions. Spermidine efflux of the tpo1::lacZ cells was significantly decreased as compared with the wild type (Fig. 6A). The observed difference in efflux rates was not due to a different preloading of cells with spermidine, as can be seen in Fig. 6B. To provide a solid basis for the observed difference in polyamine excretion, the initial efflux rates of 15 independent cultures of wild type and mutant cells, respectively, were determined. It could be shown that the spermidine efflux rate of the TPO1 deletion strain drops to 49 ± 28% of wild type rates (Fig. 6C). These experiments indicate a function of Tpo1p in spermidine detoxification. Preloading yeast cells with putrescine unfortunately led to widely varying results; however, in a series of experiments we did not observe a statistically significant difference between the wild type and the TPO1 deletion strain in terms of putrescine efflux activity (not shown).

Complementation of the Impaired Export Activity—If the observed impairment of spermidine efflux was indeed a consequence of the TPO1 deletion, the introduction of plasmid-borne Tpo1p should lead to an increase of the spermidine export rate. Therefore, we constructed a plasmid expressing Tpo1p under control of the PMA1 promoter and transformed this construct into the tpo1::lacZ strain. Expression of the plasmid encoded gene was controlled by analyzing polyamine sensitivity of the resulting yeast, which was in fact highly decreased (not shown).

The initial spermidine export rates of this strain were determined and gave values in the same range as observed for the

| Marker enzyme | Enrichment | Yield |
|---------------|------------|-------|
| Vacuolar markers | | |
| Dipeptidyl aminopeptidase B | 47-fold | 10 |
| α-Mannosidase | 45-fold | 10 |
| Marker enzymes of contaminating fractions | | |
| Dipeptidyl aminopeptidase A (Golgi) | 11-fold | 2.3 |
| Cytochrome c oxidase (mitochondria) | 3-fold | 0.7 |

FIG. 4. Relative spermine uptake of isolated vacuolar vesicles. Vascular vesicles from wild type (filled bars) and tpo1::lacZ cells (open bars) have been prepared. The cells have been grown in phthalate-buffered CBS medium supplemented with 10 mM putrescine and 1 mM of both spermidine and spermine. Total uptake and the uptake rate of the vesicles were determined for spermine and phenylalanine. The ratio of spermine and phenylalanine uptake and uptake rate, respectively (relative uptake), is shown at the ordinate.

FIG. 5. Putrescine export of growing yeast cells. Wild type (○) and tpo1::lacZ cells (○) have been grown in succinate-buffered CBS medium from A600 = 0.5 to stationary phase. A, growth of wild type and TPO1 disruption cells. B, putrescine content of the medium.
We therefore generated three strains whether Tpo2p, Tpo3p, and Tpo4p are located in the plasma membrane raises the question on the location of the other described polyamine transporters in S. cerevisiae, i.e. whether Tpo2p, Tpo3p, and Tpo4p are located in the plasma membrane as well (4). We therefore generated three strains bearing 3xHA-tagged versions of TPO2, TPO3, and TPO4, respectively, as the only gene copy. Using sucrose density gradient centrifugation, we fractionated membrane preparations of these strains and found that all of the TPO gene products localize in the same fraction, which was identified as plasma membrane fraction before (Fig. 7; compare Fig. 1). An involvement of the three yeast polyamine transporters in transport of these solutes across the plasma membrane seems to be likely and will be analyzed in the future.

**FIG. 7.** Localization of the Tpo transporters. Cell lysates of yeast strains expressing triple HA-tagged versions of Tpo1p–Tpo4p, respectively, were separated by sucrose gradient centrifugation. The gradients were fractionated from top (fraction 1) to bottom (fraction 12) and analyzed by SDS-PAGE followed by immunoblotting using anti-HA antibody.

**DISCUSSION**

**Subcellular Localization of Tpo1p-4p Revised**—The expression and subsequent detection of epitope-tagged proteins has turned out to be a powerful method in unraveling the subcellular localization of proteins. By applying this approach we found that a triple HA-tagged version of Ycr023p co-localized with the vacuolar ATPase, using sucrose gradient centrifugation as the analytical method. Ycr023p is a member of the multidrug resistance family and most probably functions in the detoxification of hazardous compounds into the vacuolar lumen. It has been shown that its deletion confers resistance to allylglycine (25). Unexpectedly, triple HA-tagged versions of all four polyamine transporters, Tpo1p-4p, that have been described in *S. cerevisiae* so far co-localized with the plasma membrane ATPase. Despite the fact that tags in general might cause mislocalization of proteins, we decided to carefully re-evaluate whether these transporters were in fact plasma membrane bound and thus not involved in vacuolar transport of polyamines as had been described before (4). Direct localization studies of these proteins had not been performed so far; their intracellular localization had been deduced indirectly from physiological experiments with Tpo1p being the best studied protein of this group (3, 4, 24). We provide experimental evidence for a role of Tpo1p in transport across the plasma membrane, which is based on the observations that the HA-tagged protein is functional and that a TPO1 knockout is not impaired in uptake of polyamines into vacuolar vesicles. The observation of Tomitori *et al.* (3) that vesicles prepared from TPO1-overexpressing strains show an increased spermine import activity is most probably due to mislocalization of the overproduced protein. In this study we could show that a strain deleted in TPO1 shows a significant defect in spermidine secretion under conditions in which the cells are challenged by incubation in medium containing high levels of polyamines. We thus conclude that Tpo1p is a plasma membrane-embedded carrier protein. The arguments that Tpo2p, Tpo3p, and Tpo4p have the same subcellular location are so far exclusively based on results of wild type (Fig. 6C). When the *tpo1::lacZ* strain was transformed with the empty vector, the export rates were in the same range as observed for the *TPO1* deletion without any plasmid (not shown). The initially accumulated spermidine concentration was unchanged in both experiments. Because *TPO1* was found to complement the described phenotype, we consider Tpo1p to be a plasma membrane-bound exporter involved in spermidine export.

**Triple HA Fusions of Tpo2p–4p Are Also Located in the Plasma Membrane**—The fact that Tpo1p was localized in the plasma membrane raises the question on the location of the other described polyamine transporters in *S. cerevisiae*, i.e. whether Tpo2p, Tpo3p, and Tpo4p are located in the plasma membrane as well (4). We therefore generated three strains...
sucrose gradient fractionation and need further experimental proof.

**Polyamine Excretion in** *S. cerevisiae*—Export of polyamines, especially of putrescine as the key metabolite in polyamine biosynthesis, had been described for some prokaryotic (22) and eukaryotic organisms (23), as well as for cultured mammalian cells (26). The biological significance of this process is most probably related to the fact that although polyamines are pivotal for many physiological processes, they are toxic when present in high levels, so that cells had to develop mechanisms for controlling the intracellular pools (27). Although the regulation of biosynthesis is certainly a possibility to control non-toxic levels (28), excretion provides an additional rationale. Taking into account that re-uptake is guaranteed by the presence of importers systems, a possible loss of precursors can be avoided. Putrescine export had not been studied in detail in *S. cerevisiae* before. When trying to establish a function of Tpo1p in polyamine export, we therefore asked whether yeast had developed mechanisms for putrescine homoestasis similar to other microorganisms and whether Tpo1p might be involved in that process. Although we were able to detect significant putrescine export which, interestingly, was restricted to the fermentative growth phase, Tpo1p is obviously not involved in this process to a significant extent, because wild type and the TPO1 disruption strain had equal export activity. We cannot rule out, however, that redundant transporters may adjust their putrescine export activities in the deletion strain to compensate for the missing TPO1 function.

**Polyamine Detoxification**—When cells are stressed under conditions of growth in the presence of high levels of polyamines, a detoxification mechanism has to exist to overcome toxic intracellular levels that may arise because of the presence of polyamine importers. This could in principle be achieved by regulating the uptake activity and/or the activity of exporters for these solutes. We therefore preloaded yeast cells with polyamines and followed the efflux after transfer of cells into polyamine-free medium. Tpo1p was shown not to contribute significantly to putrescine tolerance (3); thus we concentrated on the analysis of spermidine export, which was in fact affected by TPO1 deletion. The decrease in excretion activity was significant and could be reverted by introducing a plasmid-encoded copy of TPO1 proving that the phenotype observed was a direct consequence of the missing TPO1 gene. The rescued strain showed spermidine export rates comparable with the wild type (Fig. 6C), which was also the reason for the polyamine tolerance that we found when testing the functionality of the TPO1–3xHA construct (Fig. 2). In the latter experiment we even observed an increased tolerance of the *tpo1::lacZ* strain containing TPO1–3xHA on a plasmid, compared with wild type, which was most probably due to the experimental conditions applied. Spermidine tolerance was assayed under magnesium limitation, a condition that leads to enhanced polyamine uptake (9), thus generating higher intracellular levels than the wild type. This transporter has been reported to confer resistance to a variety of structurally nonrelated toxic compounds like quinidine and cycloheximide (24), mycophenolic acid (29), or 2-methyl-4-chlorophenoxyacetic acid and 2,4-dichlorophenoxyacetic acid (30), suggesting an broad substrate specificity, which is characteristic for multidrug resistance proteins (5). Furthermore, TPO1 was recently shown to be a target for the regulators Pdr1p (24) and Pdr3p (30), both of which mediate multidrug resistance. This further emphasizes the concept that the primary function of Tpo1p in *S. cerevisiae* is the detoxification of hazardous compounds, which includes excess spermidine.

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