The product of the SHR3 orthologue of Aspergillus nidulans has restricted range of amino acid transporter targets

Z. Erpazoglou, P. Kafasla, V. Sophianopoulou *  
Institute of Biology, National Center for Scientific Research, "Demokritos" (NCSR "D"), Agia Paraskevi, 153 10 Athens, Greece

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Abstract

The shrA gene of Aspergillus nidulans codes for a structural and functional homologue of Shr3p, a yeast ER membrane protein, which plays a crucial role in the secretory pathway of yeast amino acid permeases. shrA is a single-copy gene, whose expression is early activated during germination of A. nidulans conidiospores. ShrA is localized in the ER of the fungal cells and partially complements the shr3Δ phenotype. Differently from Saccharomyces cerevisiae, where SHr3p is necessary for membrane localization of the majority of amino acid permeases, deletion of the shrA locus in A. nidulans impairs a limited number of amino acid uptake activities, including those responsible for proline and aspartate transport. Strongly reduced membrane levels of a PrnB-sGFP fusion in a shrAΔ background clearly suggest a direct role of ShrA in the topogenesis of the proline specific transporter.

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1. Introduction

Polytopic membrane proteins reach their membrane-target via the general secretory pathway of the organism. However, accessory protein factors that mediate the interaction of the cargos with the general secretory components are also required and tend to be family-specific.

Shr3p of Saccharomyces cerevisiae is the most upstream family-specific, accessory protein identified so far (Ljungdahl et al., 1992). Its presence is necessary for the ER-exit (Ljungdahl et al., 1992) and incorporation into COPII coated vesicles (Kuehn et al., 1996) of S. cerevisiae amino acid permeases Gap1p and Hip1p. Deletion of the SHR3 locus results in a serious impairment of the majority of amino acid uptake systems of the organism (Ljungdahl et al., 1992).

Shr3p is an ER resident protein with four transmembrane segments and a carboxy-terminal cytoplasmic tail (Kota and Ljungdahl, 2005; Ljungdahl et al., 1992). The hydrophobic domain of Shr3p acts as a chaperone for the membrane-spanning segments of amino acid permeases, preventing their aggregation in the ER membrane (Ljungdahl et al., 1992). Its hydrophilic carboxy-terminal domain interacts with COPII components and probably recruits them in the vicinity of cargo-proteins for the formation of coated vesicles (Gilstring et al., 1999). These combined properties of Shr3p indicate a possible involvement in the ER quality control. Shr3p seems to be a well-conserved protein in fungi. Orthologues have been characterized in Schizosaccharomyces pombe (Psh3p; Martinez and Ljungdahl, 2000) and Candida albicans (Csh3p; Martinez and Ljungdahl, 2004) and appear to function similarly and interchangeably (Martinez and Ljungdahl, 2000; Martinez and Ljungdahl, 2004; Matijekova and Sychova, 1997).

Accessory proteins with similar function but no sequence homology to Shr3p have also been characterized in yeast. They are involved in the ER exit of a subset of hexose transporters (Gst2p), the phosphate transporter (Pho86p) or the chitin synthase III (Chs7p; Kota and Ljungdahl, 2005; Martinez and Ljungdahl, 2000). Though no data are available...
concerning the regulation of transcription of the SHR3 gene or its orthologues, expression of Chs7p (Trilla et al., 1999) and Pho86p (Lau et al., 2000) is regulated at the transcriptional level. The expression pattern of these proteins depends on the developmental stage or/and the growth conditions and is, often, in agreement to the expression pattern of their cognate substrates. More precisely, gene csh7p is expressed constitutively during vegetative growth and is induced during sporulation. Its expression is also enhanced under conditions where chitin synthesis increases (Trilla et al., 1999). On the other hand, expression of genes coding for both ER integral membrane protein Pho86 and its substrate, the phosphate transporter Pho84p, is highly induced in response to phosphate starvation (Lau et al., 2000).

In the present work, we have cloned and functionally characterized the SHR3 orthologue of Aspergillus nidulans, shrA. As in all ascomycetes, the amino acid transporters of this filamentous fungus belong to the APC (Amino acid Polyamine organoCation) superfamily (Saier, 2000; Sophianopoulou and Diallinas, 1995). Approximately 30 hypothetical amino acid transporters have been identified in silico in the A. nidulans genome sequence. However, so far only the major proline transporter PrnB has been thoroughly characterized in a genetic, molecular, biochemical and cellular level (Gómez et al., 2003; Sophianopoulou and Scazzocchio, 1989; Tavoularis et al., 2001, 2003; Tazebay et al., 1995, 1997). PrnB is a high-affinity, high-capacity proline transporter, transcriptionally and post-transcriptionally regulated according to developmental stage and nutritional conditions. Cellular expression of PrnB wild-type or mutant molecules has been studied via the Green Fluorescent Protein (GFP) technology. Additionally, gabaA of A. nidulans codes for a GABA permease (Arst et al., 1980; Bailey et al., 1979; Caddick et al., 1986; Hutchings et al., 1999), whereas uptake of aspartate/glutamate has also been detected (Kinghorn and Pateman, 1975; Pateman et al., 1974) and seems to be mediated by a single transport activity, encoded by the agtA gene (Apostolaki and Scazzocchio unpublished data).

Our results demonstrate that protein ShrA is important for the functional expression of at least three distinct amino acid transport activities in A. nidulans. Additionally, PrnB topogenesis to the plasma membrane is seriously impaired due to the shrAA mutation.

2. Materials and methods

2.1. Strains, media and microbial techniques

Minimal (MM) and complete (CM) media and growth conditions for A. nidulans have been previously described (Cove, 1966). Supplements were added when necessary. Nitrogen sources, urea, l-aspartate, l-leucine, l-phenylalanine and l-proline were used at a final concentration of 5 mM. Uric acid was used at a concentration of 595 μM. The carbon source, glucose, was used at a final concentration of 1% w/v. Toxic amino acid analogues d-serine and p-fluoro-d,l-phenylalanine were used in a final concentration of 0.3–1 mM and 10 μg/mL, respectively.

The A. nidulans strains used have the following genotypes: pabaA1 used as the wild-type strain for the cloning of the shrA gene and the corresponding cDNA clone and in northern blot analysis, yA2 pantoB100 argB2, yA2 pabaA1 prnB377 (prnBC397 shrAA), yA2 pantoB100 prnBC397 (prnBC397), pabaA1 riboB2 uapA3 uapA4 agtAA (agtA), wA3 biA1 pyroA4 agtAA (agtAA, Apostolaki and Scazzocchio, unpublished data), pabaA1 pyrG89 prnBgfpl prnBC397 is a deletion starting at the PrnB gene and extending up to the PrnC gene (Tavoularis et al., 2001; Tavoularis et al., 2003), prnB377 is a deletion in the open reading frame of the prnB gene that was described previously in Tazebay et al. (1995). pabaA1 pyrG89 prnBgfpl was isolated by crossing strain pabaA1 pantoB100 pyrG89 with strain yA2 pabaA1 argB2 prnBgfpl (Vagelatos and Sophianopoulou, unpublished data). Strains pabaA1 prnBgfpl shrA+ (prnBgfpl shrA+) and pabaA1 prnBgfpl shrAΔ (prnBgfpl shrAA) were isolated after transformation of strain pabaA1 pyrG89 prnBgfpl with plasmid pGEM:pyr4 (see below) and the 8 kb D-J PCR product (see below), respectively. Strains yA2 pantoB100 prnB+ shrA+ (shrA+) and yA2 pantoB100 prnB+ shrAA (shrAA) were isolated by crossing strain pabaA1 prnBgfpl shrAA with strain yA2 pantoB100 argB2. Strain yA2 prnB377 shrAA (prnB377 shrAA) was isolated by crossing strain yA2 pantoB100 prnB+ shrAA with strain yA2 pabaA1 prnB377, pantoB100, pabaA1, pyrG89, riboB2, biA1, pyroA4 and argB2 indicate auxotrophies for α-pantothenic acid, p-aminobenzoic acid, uracil, riboflavin, biotin, pyridoxine and l-arginine, respectively. yA2 and wA3 indicate yellow and white conidia, respectively. These markers do not affect the regulation of gene products involved in nitrogen sources uptake and catabolism. A. nidulans protoplast transformation was carried out as described in Tilburn et al. (1983).

Standard S. cerevisiae growth conditions and manipulations were performed. The yeast strain used was FGY145 (MATα ura3-52 his4A29 shr3A6 GAL+; Gilstring et al., 1999). Toxic amino acid analogues p-fluoro-d,l-phenylalanine and l-azetidine-2-carboxylate were used at a final concentration of 400 and 500 μg/mL, respectively. l-proline in SPD was added at a final concentration of 1 g/L. The concentration of Yeast Nitrogen Base in SPD supplemented with 30 mM histidine is four times higher than the concentration used in standard SD medium, since this amount was found to enhance the toxicity of histidine (Ljungdahl et al., 1992). Histidine to a final concentration of 1 mM was added as a supplement, when required. Yeast transformation was carried out according to Gietz et al. (1992).

2.2. DNA and RNA manipulations, plasmid constructions and isolation of the shrA disruption cassette

Plasmid isolation from Escherichia coli strains and standard DNA manipulations were performed as previously described (Sambrook et al., 1989). Genomic DNA from A. nidulans was isolated based on a protocol adapted from
Hoffman and Winston (1987), whereas total RNA extraction was carried out with the TRIzol® Reagent (GIBCOBRL). Polymerase chain reaction (PCR) was carried out using Taq DNA polymerase (New England Biolabs). DNA sequencing of plasmid constructions was carried out using the ABI 310 Genetic Analyser at the Institute of Biology, NCSR, Athens, Greece. Reverse transcription for the isolation of the shrA cDNA clone was carried out using the PowerScript™ reverse transcriptase (Clontech), oligo-dT as primer and total RNA extracted from the wild-type strain, grown on minimal medium supplemented with urea for 6h at 37°C.

Oligonucleotide primers used for the amplification, cloning and sequencing of the shrA gene and cDNA clone were:

**SHRA-NotI**: 5’ATAAGAATGCGGCCGCAACCGCTTC TCCTCTAACTCG3’

**SHRA-NotI**: 5’ATAAGAATGCGGCCGCAACCGCTTC TTCATAACCCACCATC3’

Plasmid pDB21 has derived by replacing the NotI-fragment of plasmid pPM1 (Martinez and Ljungdahl, 2000) with the shrA cDNA clone.

Plasmid pAN:ShrAGFP was constructed by cloning of the shrA-sgfp sequence (see below) to the BamHI and XbaI sites of plasmid pAN335:argB (Argyrou et al., 2001). The shrA-sgfp sequence was amplified by primers SHRA-ArgB (5’GAAGATCTCCGCTATGGGTCCTTC3’) and GXP-XbaI (5’GGCTCTAGACTCTCTGACGTCGACTCC3’). Plasmid pBSC:ShrAGFP was constructed by cloning of the XbaI restriction fragment of plasmid pAN225 (Hull et al., 1989; Tavoularis et al., 2001) into pBluescript sequentially cloning the shrA cDNA amplified by primers SHRA-F and SHRA-EcoRV (5’TTTTTTGATATTCGTGCTTTCTTTCTTGATCG3’).

The shrA pry4 disruption cassette was constructed by applying the D-J PCR technique, as described in Yu et al. (2004), with the exception that overlapping sequences were generated at the ends of both the shrA flanking regions and the pry4 gene, for the assembly of the three molecules. The pry4 sequence of Neurospora crassa was used for the complementation of the pry489 mutation (Balance and Turner, 1985; Oakley et al., 1987), in order to avoid homologous recombination at the marker genetic locus (pryG). The upstream and downstream to the shrA gene regions were amplified using primers UPRSHRA-F (5’AACTTCTTGTGCACCTTACCCCTTGAG3’), UPRSHRA-R (5’GGTGTTGAGTCTACTTCATTGAATGGCGACGTG3’) and DONSHRA-R (5’GGGAGCTACTTTCACTCTTGAGC3’) and DONSHRA-F (5’CCGGGAAAGCCGGACAGTGAAGATGCTCTTCTTACG3’) and DONSHRA-R (5’TTATCCTCTGTTGTTGCTCGATGG3’). Marker gene pry4 was amplified by primers PRY4SHRA-F (5’CAAGGCGAGACTTTTCCCTTACTGACTTAAAGCTTACACCTTATAGATAGTGGG) and PRY4SHRA-R (5’GTCGAGATCTCATGATAGAAATGGG).

The Expand High Fidelity PCR system (Roche Molecular Biochemicals, Mannheim, Germany) and nested primers NSHRA-F (5’AGTGCTTGGTGCTCGAGATG3’) and NSHRA-R (5’TGAATCCAGCTCCGACATCG3’) were used for the construction and amplification of the 8 kb disruption cassette.

Southern blot analysis was carried out according to Lockington et al. (1985). Northern blot analysis was carried out using the glyoxal method as described by Tazebay et al. (1995). The shrA-specific fragment, amplified from wild-type genomic DNA by primers SHR-A-PstI (5’AAGTGTCGCCTTGAGAATGGC3’) and SHR-A XbaI (5’GCTC TTAGAGGACCCGAGCAAGC3’) was used as a probe in northern blots. The ~1.8 kb PstI restriction fragment of plasmid pAN225 (Hull et al., 1989; Tavoularis et al., 2001) was the template for the prnB-specific probe, whereas the ~2.5 kb BamHI-KpnI restriction fragment of plasmid pSF5 (Fidel et al., 1988) was the template for the acnA-specific probe.

2.3. Fluorescence microscopy–confocal laser microscopy

Conidia (5 x 10^4) per milliliter inoculated on sterile cover slips embedded in appropriate liquid culture media were incubated for 16 h at 25°C (Tavoularis et al., 2001, 2003). Expression of the chimeric sequences shrA-sgfp or prnB-sgfp was induced by the addition of uric acid (to a final concentration of 0.1 mg/mL) or L-proline (to a final concentration of 20 mM), respectively, for 2h. Cover slips were washed with phosphate-buffered saline (PBS) and observed under the epifluorescence microscope or the confocal laser microscope.

Labelling of A. nidulans nuclei was carried out with DAPI. Cells were grown as described above. Cover slips were placed upside down on a 200 µL drop of appropriate medium containing DAPI at a concentration of 20 µg/mL, incubated for 15 min at RT and washed with PBS.

Epifluorescence microscopy was carried out with appropriate filters in a Bio-Rad Radiance 2000 microscope. Confocal laser microscopy was carried out on a BIO-RAD MRC 1024 CONFOCAL SYSTEM (Laser Sharp Version 3.2) Bio-Rad software, zoom x2, excitation: 488 nm/Blue, samples at Laser Power 10%; Kalman filter N=5-6, 0.3 µm cut, iris: 7-8, crypton/argon laser, Nikon DIAPHOT 300 Microscope, x60 (oil immersion) lens, emission filter 522/DF35, lens reference: Plan Apo 60/1.40 oil DM, Nikon Japan 160175, 60 DM/Ph4, 160/0.17).

2.4. Radioactive substrate transport assays

[2,3,4,5-3H] L-proline, [2,3-3H] L-aspartate and [8-3H]-xanthine uptake were assayed in germinating conidia at 37°C as previously described (Meintanis et al., 2000; Tavoularis et al., 2001, 2003; Tazebay et al., 1995). Standard uptake assays for the determination of initial uptake rates were performed in A. nidulans MM (pH 6.5) by using 12 nM [2,3,4,5-3H] L-proline (specific activity 80 Ci/mmol−1;
Moravek, Biochemicals, Brea CA, USA), 26 nM [2,3-3H] L-aspartate (specific activity 15–50 Ci mmol−1; Amersham Biosciences, UK) and 10 nM [8-3H]-xanthine (specific activity 2 Ci mmol−1; Moravek, Biochemicals, Brea CA, USA). Initial uptake rates were expressed in pmol of substrate aspartate (specific activity 15–50 Ci mmol−1; Amersham Biosciences, UK) and 10 nM [8-3H]-xanthine (specific activity 2 Ci mmol−1; Moravek, Biochemicals, Brea CA, USA). Radioactivity was determined in sediment and supernatant by liquid scintillation counting (Beckman Instruments). Transport measurements were repeated independently, and the reported results represent the mean values of at least three to five different experiments. The apparent Michaelis constant (Km) and maximal velocity (Vmax) values were determined from double reciprocal plots of the initial uptake rates against substrate concentration. The kinetic parameters of the secondary proline transport activities were determined in a prnB377 genetic background (strains prnB377 shrA+ and prnB377 shrA−).

3. Results

3.1. Cloning of a SHR3 homologue in A. nidulans

In *S. cerevisiae* a genetic approach has lead to the isolation of the SHR3 gene (Ljungdahl et al., 1992). Functional homologues in *S. pombe* (Psh3p; Martinez and Ljungdahl, 2000) and *C. albicans* (Csh3p; Martinez and Ljungdahl, 2004) have also been characterized. Using Sh3p as an in silico probe (http://www.broad.mit.edu/annotation/fungi/aspergillus/faq.html) we found a 600 bp putative ORF in contig 1.29 (scaold 2), coding for a polypeptide chain with 30% amino acid identity and 49% similarity to the yeast Shr3p. We named this protein sequence ShrA, based on its nomenclature rules (Martinelli and Kinghorn, 1994). ShrA is composed of 200 amino acid residues and has a predicted molecular mass of 21.5 kDa (http://au.expasy.org/tools/protparam.html; Gasteiger et al., 2005, date 05/05). Based on its hydropathy profile (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html), date 05/05 ShrA is a membrane protein with four transmembrane helices and a hydrophilic cytoplasmically oriented carboxy-terminal tail.

Southern blot analysis of total genomic DNA extracted from the wild-type strain (see Section 2) has shown that ShrA is encoded by a single copy gene, shrA (data not shown). This is in agreement with the in silico analysis result, where only one putative SHR3 homologue was identified. shrA gene maps on chromosome VII and contains an intron of 109 bp near its 5′ end (between nucleotides +46 and +155, with respect to the adenine base (+1) of the ATG initiation codon). We obtained a complete shrA cDNA clone (see Section 2, data not shown) which verified the presence of this intron, but not of a second one, predicted by the A. nidulans genome database.

ShrA shows significant homology to proteins Csh3p, Sh3p and Psh3p (Fig. 1A). Similar regions are scattered throughout the length of the four proteins and the position and length of transmembrane segments are well conserved.

Using TBLASTN (http://www.ncbi.nlm.nih.gov/blast/) and the ShrA protein as a probe, many putative homologues have been found, exclusively in fungal species. The most similar to the *Aspergillus* protein (more than 50% sequence identity) are hypothetical proteins from *Magnaporthe grisea* (EAA48838), *N. crassa* (XP327689) and Gibberella zeae (EAA67851). A phylogenetic tree including all fungal homologues was constructed using the CLUSTALW algorithm (Fig. 1B).

3.2. The expression of the shrA gene is constitutive

To our knowledge, no specific data are available bearing on the regulation of expression of the SHR3 gene in *S. cerevisiae* or its homologues in *S. pombe* and *C. albicans*. However, expression of yeast genes, encoding ER resident proteins with similar function to that of Shr3p, is regulated at the transcriptional level (Lau et al., 2000; Trilla et al., 1999). The expression pattern of these “chaperones” follows the same pattern as that of the genes coding for their cognate substrates. In the present work we have examined whether the expression of the shrA gene is subjected to developmental or nutritional regulation at the transcriptional level. We have also compared the expression pattern of the shrA gene to that of the prnB gene, coding for the major proline transporter of *A. nidulans* (PrnB), a putative substrate of the ShrA protein.

Using northern blot analysis, we have studied the expression of the shrA gene in the wild-type strain during germination (0–6 h at 37 °C), i.e., the sequence of events that converts a resting conidiospore into a rapidly growing germ tube from which the mycelium will be formed by elongation and branching (d’Enfert, 1997). Though dormancy is characterized by very low metabolic rates, transition to germination activates many biochemical processes, amino acids uptake included, primarily at the transcriptional level (Amillis et al., 2004). Developmental regulation of the prnB gene expression is typical of this phenomenon (Amillis et al., 2004; Tazebay et al., 1997). Though undetectable in resting conidiospores (0 h), *sprnB* mRNA steady-state levels reach their maximum 2 h after onset of germination and decrease in the young mycelium (5 h; Tazebay et al., 1997; Fig. 2A). As is the case for *prnB*, expression of the shrA gene is also rapidly activated during germination of conidiospores (Fig. 2A). shrA mRNA steady-state levels are detectable 1 h after inoculation, but do not show significant decrease, as opposed to the *prnB* levels, in young mycelia.

Additionally, we have studied the expression of shrA in the wild-type strain under different nutritional conditions, which are known to regulate the expression of the *prnB* gene. In mycelia, *prnB* transcription is induced in the presence of proline (Fig. 2B, lane 1) and is subjected to nitrogen metabolite (ammonium or glutamine) and carbon catabolite (glucose) repression (Fig. 2B, lane R, also reviewed in Sophianopoulos and Diallinas, 1995; Tazebay et al., 1997). The expression of the shrA gene is not affected by the growth conditions tested (Fig. 2B), i.e., non-inducing (NI),...
inducing (I, presence of proline) or repressing (R, simultaneous presence of glucose and ammonium). Thus, besides the increase in mRNA steady state-levels during the isotropic phase of spore germination (1–2 h), \( shrA \) is constitutively expressed in all conditions tested.

3.3. \( shrA \) is an ER resident protein

Based on its hydrophobicity profile, \( shrA \) is a membrane protein with four transmembrane segments. \( shrA \) also contains an ER retrieval motif (KKXX; \( X = \) any amino acid, Gaynor et al., 1994) for ER retrieval in the sequence of protein \( shrA \) is also underlined. Phylogenetic relationships between fungal \( shrA \) homologues were identified using TBLASTN and the \( shrA \) sequence as a probe. M. grisea (accession number EAA48838), N. crassa (accession number XP_327689), G. zeae (accession number EAA67851), Yarrowia lipolytica (accession number CAG79921), Debaryomyces hansenii (accession number CAG88836), Ustilago maydis (accession numbers EAK81799 and EAK85542), Ashbya gossypii (accession number AAS0634), Candida glabrata (accession number CAG59540), Kluyveromyces lactis (accession number XP_453431), S. cerevisiae \( shrA \) (accession number AAT93090), C. albicans \( Csh3p \) (accession number EAK92385), Cryptococcus neoformans \( var. \) neoformans (accession numbers AAW41353 and EAL17645) and S. pombe \( Psh3p \) (accession number CAB52622). The tree was created using CLUSTALW (http://www.ebi.ac.uk/clustalw/, date 05/05).

\[ \text{Fig. 1. The } shrA \text{ gene of } A. \text{nidulans. (A) Alignment of the } \text{ShrA sequence with its characterized homologues from } C. \text{albicans (Csh3p), } S. \text{cerevisiae (Shr3p)} \]

\[ \text{and } S. \text{pombe (Psh3p), using the Multalin version 5.4.1 of INRA (Corpet, 1988, date 05/05). The gap and gap length weight parameters used were 12 and 2, respectively. Letters in black box represent conserved amino acid residues in all proteins aligned, whereas gray letters represent conserved amino acid residues in two or three proteins. Underlined letters represent amino acid residues predicted to reside within transmembrane domains. The conserved carboxy-terminal dilyssine motif (KKXX; } X = \text{ any amino acid, Gaynor et al., 1994) for ER retrieval in the sequence of protein Shra is also underlined. (B) Phylogenetic relationships between fungal } shrA \text{ homologues. Putative or characterized } shrA \text{ homologues were identified using TBLASTN and the } shrA \text{ sequence as a probe. M. grisea (accession number EAA48838), N. crassa (accession number XP_327689), G. zeae (accession number EAA67851), Yarrowia lipolytica (accession number CAG79921), Debaryomyces hansenii (accession number CAG88836), Ustilago maydis (accession numbers EAK81799 and EAK85542), Ashbya gossypii (accession number AAS0634), Candida glabrata (accession number CAG59540), Kluyveromyces lactis (accession number XP_453431), S. cerevisiae } shrA \text{ (accession number AAT93090), C. albicans } Csh3p \text{ (accession number EAK92385), Cryptococcus neoformans } var. \text{ neoformans (accession numbers AAW41353 and EAL17645) and S. pombe } Psh3p \text{ (accession number CAB52622). The tree was created using CLUSTALW (http://www.ebi.ac.uk/clustalw/, date 05/05).} \]

in germinated conidiospores of \( A. \text{nidulans} \). A modified version of GFP (sGFP S65T; Chiu et al., 1996) that has been proven functional in \( A. \text{nidulans} \) was used for this purpose (Fernández-Ibaros et al., 1998; Suelmann et al., 1997; Tavoularis et al., 2001, 2003; Valdez-Taubas et al., 2000).

Protoplasts of \( A. \text{nidulans} \) strain ya2 pantoB100 argB2 were transformed with plasmid pAN:ShrAGFP carrying an in-frame-fusion of the sGFP gene to the carboxyl-terminus of the \( shrA \) cDNA clone with a linker of four amino acids, DIGG, between them (Tavoularis et al., 2001). The dilyssine ER retrieval motif at the C-terminus of the \( shrA \) sequence was maintained in the chimeric protein. The expression of the cloned sequences in this plasmid is driven by the five
regulatory sequences of the uapA gene (Argyrou et al., 2001). *uapA* codes for the uric acid/xanthine transporter of *A. nidulans* (Diallinas and Scazzocchio, 1989) and is subjected to uric acid induction and nitrogen metabolite (ammonium or glutamine) repression (Gorfinkel et al., 1993). Plasmid pAN:ShrAGFP contains also the *argB* gene (Johnstone et al., 1985) as a selection marker. *argB* transformants were selected on minimal medium lacking arginine and examined by PCR and Southern blot analysis for the integration of the *shrA-sgfp* sequences in their genomes. In all cases, multiple copies of *shrA-sgfp* sequences were introduced by heterologous recombination at the same genetic locus of transformed strains (data not shown). Conidia of transformant strains were grown for 16 h (young mycelia stage) at 25 °C in the presence of uric acid to induce the expression of the *shrA-sgfp* chimeric sequences, and were observed on a confocal laser microscope. The fluorescence staining of the mycelia responds to the signals known to regulate the *uapA* promoter (data not shown). Identical localization was seen in a single-copy transformant where the *shrA-sgfp* sequence is driven by the native promoter (data not shown). These results strongly suggest that ShrA, like its homologues from other fungi, is a component of the ER.
3.4. The ShrA protein can partially restore the amino acid transport capacity in a shr3Δ strain

In S. cerevisiae, the deletion of the SHR3 locus affects the ability of the strain to transport amino acids and amino acid analogues. As a result, a shr3Δ strain is resistant to toxic amino acid analogues (Roberg et al., 1997) or toxic amino acid concentrations (Ljungdahl et al., 1992) and grows poorly on proline as sole nitrogen source (Martinez and Ljungdahl, 2004). We assessed the function of the ShrA protein by its ability to complement the SHR3 deletion in yeast.

Yeast strain FGY145 (shr3Δ) was transformed individually with plasmids pRS202 (negative control, contains only the marker gene ura3; Connelly and Hieter, 1996), pPL250 (positive control, contains the marker gene ura3 and a 1.4 kb genomic fragment having the SHR3 gene, Gilstring et al., 1999) or pDB21 (contains the ura3 marker gene and the shrA cDNA clone driven by the adh1 yeast promoter; Becker et al., 1991; see Section 2). Transformants were selected on SD medium supplemented with 1 mM histidine. In cells transformed with plasmid pDB21, the integration of the shrA cDNA clone was verified by PCR (data not shown). shrA cDNA clone fully complements the SHR3 deletion when tested in the presence of histidine at a toxic concentration (30 mM, Fig. 4 panel 5) or proline as sole nitrogen source (Fig. 4 panel 3). Complementation is partial when assessed in the presence of L-azetidine-2-carboxylate (azetidine, panel 4), SPD supplemented with 30 mM histidine (panel 5) or SD supplemented with L-azetidine-2-carboxylate (azetidine, panel 4). Therefore, the presence of a functional ShrA protein can restore the correct topogenesis of only a subset of amino acid permeases in yeast. These findings clearly indicate that, though not entirely equivalent to Shr3p, ShrA is involved in the topogenesis of amino acid transporters.

3.5. Deletion of the shrA locus affects the amino acid transport systems of A. nidulans

The first step in understanding gene function involves generally the disruption and/or the overexpression of the target gene (Yu et al., 2004). We have assessed the function of the shrA gene in A. nidulans by replacement of its coding sequence by a marker gene, via homologous double-crossover.

Protoplasts of pabaA1 pyrG89 prnBgfp strain (see Section 2) were transformed with 2 μg of an 8 kb linear tripartite construct, composed of the upstream and downstream regions of the shrA locus flanking the pyr4 gene of N. crassa (Buxton and Radford, 1983). Strain pabaA1 pyrG89 prnBgfp expresses functional PrnB-sGFP chimeric transporters and thus is suitable for the in vivo study of putative intra- or extragenic mutations affecting PrnB transporter topogenetics (Tavoularis et al., 2001). The pyr4 sequence complements the pyrG89 mutation of A. nidulans (Balance and Turner, 1985; Oakley et al., 1987). The 8 kb construct was produced by the Double-Joint PCR (D-J PCR) technique, as described in Yu et al. (2004). pyrG expression was selected on minimal medium lacking uracil and uridine (Oakley et al., 1987).

Deletion of the shrA coding sequence by introduction of the pyr4 sequence at its locus, due to homologous double-crossover events, has occurred in ~30% of the transformed strains (prnBgfpr shrAΔ), as indicated by Southern blot analysis (data not shown). By standard sexual crosses shrAΔ strains with a prnB allele were also isolated (shrAΔ; see Section 2). The fact that haploid shrAΔ strains could be generated indicates that shrA is not essential for growth on minimal medium. All shrAΔ strains isolated share identical

![Fig. 4. Growth of shr3Δ cells transformed with plasmids pRS202, pPL250 or pDB21 in the presence of toxic amino acid analogues, toxic amino acid concentrations or L-proline as sole nitrogen source. 2 μL of culture dilutions (0, −1, −2, −3) of each transformed strain were inoculated in various test media and incubated at 30 °C for 5 days. The media used were SD without nitrogen source (panel 1), SD (panel 2), SPD (proline as nitrogen source, panel 3), SD supplemented with L-azetidine-2-carboxylate (azetidine, panel 4), SPD supplemented with 30 mM histidine (panel 5) or SD supplemented with ρ-fluorod,L-phenylalanine (FPA, panel 6). pRS202 carries the selection marker ura3 and serves as a negative control in the present growth assays. pPL250 carries ura3 as well as the shr3 genomic region, providing a positive control. pDB21 carries ura3 and the shrA cDNA clone.](image-url)
growth phenotypes in complete medium (CM), similar to
the growth phenotype of a wild-type strain (shrA+, see Sec-
tion 2). Characteristics of the asexual (conidiation, timing
of conidial germination) and sexual (elestiothecia forma-
tion) life cycle of A. nidulans are not impaired by the shrAA
mutation (data not shown). Growth of a shrAA strain on
various nitrogen sources such as urea (Fig. 5A), nitrate or
ammonium is similar to that of a shrA+ strain.

Deletion of all known SHR3 homologues results in a sig-
nificant defect in the amino acid uptake systems of the
organism (Ljungdahl et al., 1992; Martinez and Ljungdahl,
2000; Martinez and Ljungdahl, 2004). A. nidulans wild-type
strain can grow on all naturally occurring amino acids-with
the exception of methionine, lysine, cysteine and histidine-as
sole nitrogen sources, and shows sensitivity to the toxic
amino acid analogues D-serine (to a
restricted range of amino acid transporter targets.

Aspartate transport across the plasma membrane is medi-
atated by a single transport activity (Apostolaki and Scac-
zucchio, personal communication). Proline uptake, on the other
hand, is mediated by the high-affinity, high-capacity PrnB
transporter (major transporter), as well as by one or more,
genetically not identified, secondary high-affinity transport
system(s). These systems are responsible for 20% of the total
transport activity at 37°C (Arst et al., 1980; Tazebay et al.,
1995; Apostolaki and Scaczzocchio, personal communication).
In order to examine the impact of the shrA deletion on the amino acid uptake systems of A. nidulans, growth tests were performed in the presence of L-amino acids (i.e., all amino acids occurring in proteins, as well as GABA and ornithine) as sole nitrogen sources or toxic amino acid analogues at 37 and 25°C. With the exception of aspartate and proline (Fig. 5A), the ability to grow on amino acids as sole nitrogen sources is not significantly affected in strain shrAA compared to strain shrA+. Strain shrAA shows no resistance to D-serine, to a final concentration ranging between 0.3 and 1 mM, nor to p-fluoro-D,L-phenylalanine. In the case of aspartate or proline (Fig. 5A) used as sole nitrogen source, strain shrAA showed a reduced growth corresponding to a partial loss-of-function phenotype for the respective transport systems. The growth defect of strain shrAA on aspartate and proline is temperature independent. Moreover, purine assimilation is not impaired in strain shrAA, since the growth phenotype on uric acid (Fig. 5A) or hypoxanthine as sole nitrogen source is similar to that of strain shrA+. Purine transport in A. nidulans is mediated by two distinct families of polytopic transmembrane transporters (Cecchetto et al., 2004; De Koning and Diallinas, 2000). Thus, within the limits of growth tests, the presence of a functional ShrA protein is important specifically for members of the amino acid transporter family. Our findings indicate also that ShrA has a restricted range of amino acid transporter targets.

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transport activity at 37°C (Arst et al., 1980; Tazebay et al.,
1995; Apostolaki and Scaczzocchio, personal communication).
In order to examine the impact of the shrA deletion on the major and secondary proline transport systems inde-
dependently, strain prnB377 shrAA was isolated (see Section 2). In
this strain, only the secondary proline transport activities are functional, due to an internal deletion at the prnB coding se-
quence (Tazebay et al., 1995). Growth of strain prnB377
shrAA on proline is considerably lower than that of strain
prnB377 shrA+ (Fig. 5B), indicating that the function of the
secondary proline transporter(s) is ShrA-dependent.

Fig. 5. Growth of shrAA strains of A. nidulans on different nitrogen sources. (A) Conidiospores of strains shrAA, shrA+, accA (strains details see Section 2) were inoculated on minimal media supplemented with 5 mM urea, 595 µM uric acid, 5 mM aspartate, 5 mM proline, 5 mM phenylalanine or 5 mM leucine as sole nitrogen source and incubated at 37°C for 48 h. In strains accA, prnBC397 and agtAA the ability to grow on uric acid, proline and aspartate, respectively, is impaired due to total loss-of-function mutations in the respective transport activities (Tavoularis et al., 2001; Apostolaki and Scaczzocchio, personal communication). (B) Effect of the shrAA mutation on the ability to grow on proline as sole nitrogen source of strains carrying different prnB alleles. Conidiospores of strains prnB377 shrAA, prnB377 shrA+, prnBC397, prnBgfp shrAA, shrAA, shrA+ and prnBgfp shrAA were inoculated on minimal media supplemented with 5 mM urea or proline as sole nitrogen source and incubated at 37°C for 48 h. Growth on proline of strains carrying a prnBgfp allele is reduced compared to prnB+ strains, due to the presence of the sGFP tag (Tavoularis et al., 2001, 2003).
A kinetic analysis was carried out of aspartate, proline and xanthine uptake in germinating conidia of strains shrA+, shrAA, prnB377 shrA+ and prnB377 shrAA, at 37°C (see Section 2). In all cases, no significant differences were observed in the affinity for the substrate parameters (Km) between the shrA+ and shrAA strains (data not shown). Velocity parameters (Vmax) are summarized in Table 1. Uptake rate for aspartate shows a 50% reduction in strain shrAA as compared to strain shrA+. Proline uptake rate is also reduced by the shrAA mutation. In strain shrAA, PrnB transport capacity is reduced by 30%, whereas the uptake rate due to the secondary proline transporter(s) is reduced by 50%, as compared to strain shrA+. No significant effect on transport capacity for xanthine (incorporated by the same uptake systems as uric acid, De Koning and Diallinas, 2000) is noticed in strain shrAA. These results are in agreement with the growth phenotypes observed on aspartate, proline and uric acid (Fig. 5A).

In S. cerevisiae, the reduction of the uptake rate for amino acids in strain shr3A has been linked to the ER block of the respective permeases (Kota and Ljungdahl, 2005; Ljungdahl et al., 1992). Since V-values for transport activities directly depend on the amount of transporter present at the plasma membrane, we studied the localization of the PrnB transporter in strain prnBsgfp shrAA. The use of the GFP technology have been proven successful to study in vivo the cellular expression both of wild-type and mutant prnB alleles (Tavoularis et al., 2001, 2003). The effect of the shrAA mutation on the prnBsgfp allele is similar to that on the prnB+ allele, based on the growth phenotype on proline (Fig. 5A and B) and the uptake rate for this substrate (data not shown). Conidiospores of strains prnBsgfp shrA+ (see Section 2) and prnBsgfp shrAA were grown at 25°C for 16 h in the presence of proline to induce the expression of prnB-sgfp sequences (Tavoularis et al., 2001), and observed on an epifluorescence microscope. Whereas in a shrA+ background PrnB-sGFP molecules stain mainly the membrane of the germlings, the fluorescence pattern in a shrAA background is mainly intracellular (Fig. 6). The intracellular, instead of peripheral, fluorescence pattern observed in the shrAA germlings suggests that a reduced number of PrnB-sGFP transporter molecules is allowed to be incorporated into the plasma membrane.

4. Discussion

In the present work, we report the cloning and functional characterization of the shrA gene of A. nidulans, coding for a non-essential, ER membrane protein of the fungus, ShrA. Expression of the shrA cDNA clone partially complements the shr3A growth phenotypes on amino acids or toxic amino acid analogues in S. cerevisiae. Deletion of the shrA locus in A. nidulans affects significantly the transport capacity for aspartate and proline. Additionally, the localization to the plasma membrane of the major proline transporter PrnB of the fungus is impaired by the shrAA mutation. The subcellular localization and the involvement of protein ShrA in the function and topogenesis of amino

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**Table 1**

<table>
<thead>
<tr>
<th>Transport systems</th>
<th>pmol min⁻¹ (10⁸ viable conidiospores)⁻¹</th>
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<tbody>
<tr>
<td>PrnB</td>
<td>6.00 ± 0.32</td>
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<tr>
<td>Secondary proline uptake system(s)</td>
<td>1.46 ± 0.06</td>
</tr>
<tr>
<td>Aspartate uptake system</td>
<td>0.32 ± 0.02</td>
</tr>
<tr>
<td>Xanthine uptake systems</td>
<td>0.43 ± 0.09</td>
</tr>
</tbody>
</table>

Transport capacity (Vmax) of the major proline transporter PrnB, the secondary proline transport system(s), the aspartate transport system and the xanthine transport systems in strains shrA+ and shrAA. Velocity values are expressed in pmol min⁻¹ (10⁸ viable conidiospores)⁻¹. Secondary proline transporter(s) velocity values were estimated in a prnB377 genetic background. The transport capacity for PrnB was estimated after taking in account the background due to activity of the secondary proline system(s).

**Fig. 6.** PrnB topogenesis in strain shrAA of A. nidulans. Conidiospores of strains prnBsgfp shrA+ and prnBsgfp shrAA (for strains details see Section 2) were grown at 25°C for 16 h on minimal media supplemented with 5 mM urea as nitrogen source. L-proline at a final concentration of 20 mM was added for the last 2 h in order to induce the expression of the prnB-sgfp sequences. Young mycelia were observed on a fluorescence microscope.
acid transporters clearly indicate that this protein is an orthologue of the previously characterized Shr3p family of ER chaperones.

Expression of the shrA gene is constitutive, with the exception of the stage of resting conidiospores. After the breaking of dormancy, shrA transcription is rapidly activated in the fungal cells. Transcriptional activation upon onset of germination is common for genes involved in many biochemical processes, such as nutrient “sensing” or biosynthesis genes, and mRNA steady-state levels of these genes reach their maximum earlier than housekeeping genes, such as actin (Amillis et al., 2004). This is the case of amino acid biosynthetic genes of N. crassa (d’Enfert, 1997), as well as purine (Amillis et al., 2004), amino acid (Tazebay et al., 1997; Apostolaki and Sezzocchio, unpublished data), uracil (Amillis and Diallinas, unpublished data), ammonium (Cook and Anthony, 1978) and nitrate (Brownlee and Arst, 1983) transporters of A. nidulans. In the case of amino acid transporters, these upregulation may be linked in part to the fact that amino acid pools, especially proline pools, decrease dramatically during conidial germination (Cook and Anthony, 1978; Sachs and Yanoysky, 1991; Tazebay et al., 1997). The dependence of the aspartate and proline transport systems of A. nidulans on the presence of a functional ShrA protein may be linked to the transcriptional activation of the shrA locus in germinating conidiospores.

Both the heterologous expression and the inactivation approaches imply that ShrA is an orthologue of Shr3p, Psh3p and Csh3p, though clearly not completely equivalent to these proteins. ShrA can restore the correct localization of only a subset of amino acid permeases in a shr3Δ strain, and this, in some cases, is probably accomplished in a limited degree. Partial complementation was also reported in the case of the psh3 gene of S. pombe and may be due to the multiplicity of protein interactions in which Shr3p is involved (Martínez and Ljungdahl, 2000). Deletion of the “SHR3” loci in S. cerevisiae, S. pombe or C. albicans impairs pleiotropically and often drastically the amino acid transport systems of these organisms (Ljungdahl et al., 1992; Martínez and Ljungdahl, 2000; Martínez and Ljungdahl, 2004). In A. nidulans, however, at least three distinct amino acid permeases are dependent on ShrA for their function: the aspartate transporter, the major (PrnB) and secondary proline transporters. Thus, ShrA appears to exert a greater degree of specificity in the amino acid transporter family, compared to the other fungal homologues.

Interestingly, the ShrA-dependent transport activities identified in this work coincide with the amino acid permeases that are mostly affected by “SHR3” mutations in other fungi. More precisely, proline permeases are highly affected in S. cerevisiae and C. albicans deleted strains. Transport of glutamate is severely impaired in S. cerevisiae and S. pombe deleted strains. Glutamate is also a substrate of the aspartate transporter of A. nidulans. However, the glutamate uptake was shown to be ShrA-independent, by the means of the growth test analysis, due to the redundancy of the transport activities in this organism (Sezzocchio, personal communication). The shrA cDNA clone, on the other hand, can restore proline transport in a shr3Δ background. These findings imply that ShrA can interact with the same transporters in yeast, as it does in A. nidulans, probably by recognizing the same motifs. Though Shr3p physically associates with amino acid permease Gap1p, mainly via its hydrophobic domain (Gilstring et al., 1999; Kota and Ljungdahl, 2005), the region(s) of the permease, involved in this interaction, remain unknown.

Since no other “SHR3” homologues have been identified in the Aspergillus database, the restricted range of ShrA on amino acid transporter membrane localization cannot be attributed to the presence of overlapping ShrA-like activities. In S. cerevisiae, proteins Gsf2 and Pho86 are necessary for the ER to Golgi exit of specific members of the Hexose Transporters family (HXT; Lau et al., 2000; Sherwood and Carlson, 1999). They are ER membrane proteins, preventing the aggregation of their substrates in the ER membrane, like Shr3p. Gsf2p and Pho86p share no sequence or structural homology with each other or Shr3p. gsf2Δ mutation affects the topogenesis of the low-affinity glucose transporter Hxt1p and the galactose transporter Gal1p, but not the topogenesis of the high-affinity glucose transporter Hxt2p (Sherwood and Carlson, 1999), pho86Δ mutation results to the ER block of the phosphate transporter Pho84p (Lau et al., 2000).

The restricted range of amino acid transporters affected by the shrAΔ mutation, as well as the degree to which these permeases depend on ShrA for their function, indicate the existence of a different and maybe more complex system regulating the topogenesis of amino acid transporters in A. nidulans. It is possible that other proteins may regulate the topogenesis of amino acid transporters in A. nidulans at the same stage as ShrA. These putative proteins may be functional but not structural homologues to protein ShrA.

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