Cellular localization and phosphorylation of Hrb1p is independent of Sky1p

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Received 25 August 2005; received in revised form 2 January 2006; accepted 3 January 2006

Available online 25 January 2006

Abstract

Protein phosphorylation plays a major role in regulating cellular functions. We have previously demonstrated that Sky1p, the SR protein kinase of the budding yeast Saccharomyces cerevisiae, is a regulator of polyamine transport and ion homeostasis. Since its kinase activity was demonstrated essential for fulfilling these roles, we assumed that Sky1p function via substrates phosphorylation. Using an in vitro phosphorylation assay, we have identified Hrb1p as a putative Sky1p substrate. However, phosphorylation analysis in WT and sky1Δ cells and localization studies disproved Hrb1p as a true Sky1p substrate, although a segment of the RS domain is required for determining its subcellular localization. Furthermore, we demonstrate that Hrb1p and additional putative Sky1p substrates, identified by computational approach, are not involved in mediating the spermine tolerant phenotype of sky1Δ cells.

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Keywords: Polyamines; SKY1; HRB1; SR proteins

1. Introduction

Protein kinases are involved in regulating fundamental cellular processes. SR protein kinases (SRPKs) phosphorylate a group of evolutionary conserved proteins known as SR proteins (reviewed in [1]). SR proteins are essential for several cellular processes; assembly of the spliceosome and defining mRNA splice sites, regulating temporal and cell-specific selection of alternative splice sites, thus regulating gene expression [2,3]. SR proteins are also involved in regulating mRNA stability and translation, and in regulating the process of mRNA transport from the nucleus to the cytoplasm [1]. One such shuttling protein is the yeast RNA-binding protein Npl3p that is involved in mRNA export and in regulating mRNA translation [4,5]. The nuclear import of Npl3p is regulated through phosphorylation by the cytoplasmic kinase Sky1p, a conserved orthologue of mammalian SRPK1 and SRPK2 that is the only SR protein kinase of the budding yeast [4,6]. To complete the shuttling cycle, Npl3p is dephosphorylated in the nucleus by the phosphatase Gle7p [7].

We have previously identified Sky1p as a regulator of polyamine transport across the plasma membrane [8]. Kinase activity of Sky1p was required for reversing the spermine-tolerant phenotype of sky1Δ cells [8]. We therefore assumed that Sky1p’s involvement in polyamine tolerance is through phosphorylating substrate proteins. Interestingly, the known substrates of Sky1p, Npl3 and Gbp2, are not involved in the polyamine resistance phenotype [8]. Using biochemical purification methods and in vitro phosphorylation assay with purified recombinant Sky1p, we identified Hrb1p as a putative Sky1p substrate. Although its RS domain was essential for phosphorylation in vitro, Hrb1p phosphorylation in cells and its cellular localization were independent of the presence of Sky1p, or of the 8 potentially phosphorylated serine residues located within the RS segment. Nevertheless, deletion of the RS segment affected the cellular localization of Hrb1p. Finally, our analysis demonstrated that neither Hrb1p nor several additional potential Sky1p substrates mediate the effect of Sky1p on polyamine transport.

2. Materials and methods

2.1. Purification of in vitro Sky1p substrates

The outlines of the purification steps are shown in Fig. 1. sky1Δ yeast cells were grown in 10 l of YPD to mid-log phase, harvested and washed with cold water. The cell pellet was resuspended in 25 mM Tris pH=7.4, 0.1 mM EDTA containing protease inhibitors, and opened with glass-beads using a Bead-beater
(Biospec products). The cells extract was brought to 1 M ammonium sulfate and the precipitated proteins removed by centrifugation. The soluble fraction was loaded on Phenyl Sepharose-6 fast-flow high-sub column (Pharmacia Biotech) and the proteins were eluted in the same buffer containing 0.25 M ammonium sulfate. The 1–0.25 M ammonium sulfate fraction was dialyzed against 25 mM Tris pH=7.4, 0.1 mM EDTA and loaded on Q sepharose column (Pharmacia Biotech). Bound proteins were eluted with a stepwise increase of the NaCl concentration. Substrates were detected in the PQ flow through and PQ 100–300 mM NaCl fractions. The PQ-300 fraction was dialyzed against 50 mM MES pH=6. During dialysis protein precipitation occurred and substrates were found both in the supernatant and the pellet. For the identification of silver or Coomassie blue stained band that corresponds to phosphorylated bands, kinase reaction was preformed as described (typically with 1 μg proteins) and then a 20–100 μg portion of non-phosphorylated extract was added. The sample was electrophoresed on polyacrylamide gel, stained with silver stain or Coomassie blue, dried and autoradiographed. The bands were cut from a parallel non-dried gel and subjected to mass-spectrometry analysis.

2.2. Yeast strains and media

The S. cerevisiae strains used in the present work are listed in Table 1. The mtr10-7 strain was generously provided by Prof. E. Hurt and maintained as described [9]. These strains were routinely maintained in YPD medium (1% yeast extract, 2% peptone, 2% D-glucose). Transformed yeast cells were described [9]. These strains were routinely maintained in YPD medium

<table>
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<tr>
<th>Strain</th>
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<td>MATa, ade2, his3, leu2, trp1, ura3, mtr10::HIS3, pRS314-TRP1-mtr10-7</td>
<td>[9]</td>
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2.3. Plasmids and site-directed mutagenesis

The DNA segments containing the HRB1, NPL3 and MTR10 genes were amplified from genomic DNA by polymerase chain reaction (PCR). Site-directed mutagenesis on HRB1 was performed using PCR and verified by DNA sequencing. For expression in E. coli, the DNA was cloned into the pRSET vector (Invitrogen) yielding a six-His tagged proteins. SKY1 was also strep-tagged (IBA GmbH) at the carboxy-terminus. For expression in yeast cells, the relevant DNA fragments were cloned between the SalI/SacI sites of the high copy number yeast expression vector pAD54 [13], placing them in frame downstream to an HA-tag. For cellular localization studies, the DNA fragments were cloned between the SalI/KanHI sites of the high copy number yeast expression vector pVTU260 [14], downstream to a His-tag and to the green fluorescent protein (GFP).

2.4. Recombinant proteins

Bacterially expressed Sky1p, Npl3p and Hrb1p were purified on NiNTA agarose beads (Qiagen). Sky1p was subjected to additional purification step on Strept-Tactin Sepharose (IBA GmbH).

2.5. Protein kinase assay

The protein kinase reaction conditions were adapted from Knebel et. al [15]. Briefly, the assays were performed in 30 mM β-glycerophosphate pH=8, 2 mM MnCl2, 0.5–10 μg protein extract (3–5 μg for HRB1 and its mutants), 0.5 μl of 1 Ci 32P-orthophosphate at 30 °C with shaking. The cells were then washed, disrupted at 4 °C, and equal amounts of the proteins were immunoprecipitated with HA.11 antibody (BabCo). Immunoprecipitated material was resolved by electrophoresis in a 10% SDS-polyacrylamide gel and radioactivity determined using a phosphoimager.

2.6. In vivo phosphorylation assay

Cells were grown in SC-L medium until OD600=1.5, washed once with SC-L (-PO4), resuspended in 1 ml SC-L (-PO4) and grown for 3 h with 250 μCi 32P-orthophosphate at 30 °C with shaking. The cells were then washed, collected and resuspended in 1 ml PBS containing 1% Triton x-100, Phosphatase inhibitors (25 mM β-Glycerol phosphate) and Protease inhibitors (200 mM PMSF, 10 mM benzamide, 100 mM benzamide). The cells were disrupted at 4 °C, and equal amounts of the proteins were immunoprecipitated with HA.11 antibody (BabCo). Immunoprecipitated material was resolved by electrophoresis in a 10% SDS-polyacrylamide gel and radioactivity determined using a phosphoimager.

2.7. Light microscopy

Yeast cells were grown overnight in the appropriate selective media, diluted and grown until the mid-log phase (OD600=1.1–1.3). Five hundred microliters
of cells were incubated with the nuclear stain Hoechst 33342 (Molecular probes) for 5 min, resuspended in 30 μl of PBS and placed on slides. The cells were visualized immediately using Olympus IX71 fluorescent microscope with a 100× lens and appropriate filters.

2.8. Growth assays

The growth rate of yeast strains on YPD plates containing or lacking 1 mM spermine was tested by spotting 2 μl from five fold dilutions of cultures at OD600=1.

3. Results

3.1. Identification of Hrb1p as a putative Sky1 substrate

In order to identify Sky1p substrates, recombinant Sky1p was used to phosphorylate yeast cell extract in vitro. For this purpose, crude yeast cell extract (100 μg) from sky1Δ cells was phosphorylated by recombinant Sky1p, revealing several phosphorylated bands (Fig. 1A). Autophosphorylation of Sky1p was also noted and served as a control. Next, in order to enrich for substrates and to separate substrates from potential kinase inhibitors and phosphatases, the extract was subjected to several fractionation steps prior to the phosphorylation reaction. Practically, the cellular extract was precipitated by 1M ammonium sulfate and the soluble fraction was fractionated on Phenyl Sepharose-6 column. The material eluted between 1 M and 250 mM ammonium sulfate was subjected to phosphorylation analysis (Fig. 1A). This fraction, which contained several Sky1p substrates, was further fractionated on a Q sepharose column. The bound proteins were eluted by a stepwise increase of NaCl concentration. Enrichment for putative substrates was observed in the material eluted between 100 and 300 mM NaCl (Fig. 1A). This fraction was dialyzed against 50 mM MES pH=6 forming precipitates that were collected by centrifugation. Two highly phosphorylated substrates were present in this fraction (denoted S1 and S2). These bands were cut from the gel and the proteins were identified by mass-spectrometry analysis as Hrb1p and Ydr516cp. The corresponding genes were cloned, expressed in bacteria, purified via an appended His-tag and subjected to phosphorylation in vitro by recombinant Sky1p. Hrb1p was the only protein successfully phosphorylated in vitro by Sky1p (Fig. 1B and data not shown). Interestingly, a database search for proteins with potential Sky1p and SRPK2 phosphorylation sites [16,17] identified HRB1 in addition to several other putative Sky1p substrates. Hrb1p contains a large SR domain with 8 serine residues, 3 of which are in phosphorylation relevant context (Fig. 5). As HRB1 was identified in two independent methods as a potential Sky1p substrate, it was selected for further investigation.

3.2. The RS-domain but not its serine residues is required for phosphorylation of Hrb1p by Sky1p in vitro

To further test whether Hrb1p is a true sky1p substrate we set out to characterize its phosphorylation. First, the entire RS region was cloned downstream to a 6-His tag, expressed in bacteria, purified and 3–5 μg were subjected to phosphorylation in vitro by 300 ng of recombinant Sky1p (A–C): (A) WT Hrb1p and a mutant lacking the RS region (∆RS). (B) WT Hrb1p and mutants in which the serine residues were converted to alanines. (C) WT Hrb1p, a mutant lacking the RS region (∆RS) and a mutant in which all serine residues of the RS segment were mutated to alanines (8A). Quantification of the radioactivity, relative to the WT Hrb1p is presented below. (D) HRB1 and NPL3 were cloned downstream to an HA-tag, expressed in WT and sky1Δ yeast cells and the cells were incubated with 32P orthophosphate. The proteins were immunoprecipitated from cell extracts using anti-HA antibodies, fractionated by SDS-PAGE and autoradiographed. Western blot (WB) analysis with anti-HA antibodies verified equal expression level. Quantification of the radioactivity, relative to WT cells is presented at the bottom.

Fig. 2. The RS segment but not its serine residues is essential for Hrb1p phosphorylation in vitro. WT and mutated Hrb1p were cloned downstream to a 6-His tag, expressed in bacteria, purified and 3–5 μg were subjected to phosphorylation in vitro by 300 ng of recombinant Sky1p (A–C): (A) WT Hrb1p and a mutant lacking the RS region (∆RS). (B) WT Hrb1p and mutants in which the serine residues were converted to alanines. (C) WT Hrb1p, a mutant lacking the RS region (∆RS) and a mutant in which all serine residues of the RS segment were mutated to alanines (8A). Quantification of the radioactivity, relative to the WT Hrb1p is presented below. (D) HRB1 and NPL3 were cloned downstream to an HA-tag, expressed in WT and sky1Δ yeast cells and the cells were incubated with 32P orthophosphate. The proteins were immunoprecipitated from cell extracts using anti-HA antibodies, fractionated by SDS-PAGE and autoradiographed. Western blot (WB) analysis with anti-HA antibodies verified equal expression level. Quantification of the radioactivity, relative to WT cells is presented at the bottom.
domain (amino acids 1–102) was deleted. The resulting Hrb1ΔRS mutant was expressed in bacteria with an appended His-tag, purified and phosphorylated in vitro by recombinant Sky1p. While the wild-type (WT) protein was efficiently phosphorylated, the His-Hrb1ΔRS mutant protein remained unphosphorylated (Fig. 2A), demonstrating that the RS segment is required for its phosphorylation in vitro.

The RS segment of Hrb1p contains 8 serine residues (at positions 14, 17, 33, 46, 62, 89, 92, 100). To determine which of these residues is phosphorylated by Sky1p, each of them was converted to alanine by site-directed mutagenesis. The resulting mutant proteins were expressed in bacteria, purified and phosphorylated in vitro. Each of these mutants was efficiently phosphorylated (Fig. 2B and data not shown), suggesting that more than one residue is phosphorylated in vitro by Sky1p, or that other residues are phosphorylated. We therefore prepared a mutant in which all the 8 serines were converted to alanine. Surprisingly, the resulting mutant was still phosphorylated, although less efficiently than the WT protein (~40% of WT phosphorylation — Fig. 2C).

3.3. Hrb1p is not phosphorylated by Sky1p in cells

It is possible that the forced conditions of the in vitro phosphorylation reaction do not reflect the phosphorylation status of Hrb1p in intact cells. We therefore set out to investigate Hrb1p phosphorylation in WT and in sky1Δ cells. For this purpose, HA-tagged Hrb1p and Npl3p were expressed in WT and sky1Δ cells and labeled with 32P-orthophosphate. Cellular extracts were immunoprecipitated with anti-HA antibodies. Western blot analysis demonstrated that the investigated proteins were equally expressed (Fig. 2D). No significant differences in HA-Hrb1p phosphorylation were observed when expressed in WT versus sky1Δ cells (Fig. 2D), indicating that although it is phosphorylated in vivo, the contribution of Sky1p to Hrb1p phosphorylation is minimal if at all. In contrast, HA-Npl3p phosphorylation in sky1Δ cells was ~75% of that observed in WT cells.

3.4. The RS segment is involved in determination of the cellular localization of Hrb1p

The cellular localization of Npl3p and Gbp2 is regulated by Sky1p phosphorylation [4,18]. Even though we could not detect directly Hrb1p phosphorylation by Sky1p, it may still affect its cellular localization. We therefore set out to examine whether Sky1p affects the subcellular localization of Hrb1p, as this may be a more sensitive assay than the direct phosphorylation assay. For this purpose Hrb1p, Npl3p and Gbp2p were fused to GFP and their localization was examined in WT and sky1Δ cells. As reported previously [4], GFP-Npl3p was localized solely to the nucleus of WT cells, while in sky1Δ cells it was also cytoplasmic (Fig. 3A, B). GFP-Gbp2p and GFP-Hrb1p were observed both in the nucleus and in the cytoplasm of WT cells (Fig. 3E, I). However, while GFP-Gbp2p shifted to the cytoplasm in sky1Δ cells, the distribution of GFP-Hrb1p remained unchanged (Fig. 3F, J). Identical results were obtained when GFP was fused to the C’-terminus of Hrb1p, and when expressed from a low copy number plasmid (data not shown). As previously reported, GFP-Hrb1p was mislocalized to the cytoplasm in MTR10 mutant cells (mtr10-7) [5] (data not shown), further demonstrating that the chimerical protein behaves as the endogenous Hrb1p. Furthermore, similar results
were obtained when these experiments were repeated in hrb1Δ and hrb1Δsky1Δ cells, ruling out the possibility that endogenous Hrb1p competed with the expressed GFP-Hrb1p on the transport receptors or Sky1p phosphorylation (data not shown). These results further support our conclusion that Hrb1p is not an in vivo substrate of Sky1p.

As the RS domain was demonstrated to be essential for the in vitro phosphorylation of Hrb1p by Sky1p, we set out to determine whether the RS domain as such is involved in determining the cellular localization of Hrb1p. To this end, the Hrb1ΔRS mutant protein was fused to GFP and expressed in WT or sky1Δ cells. In both cell lines, GFP-Hrb1ΔRS showed altered localization and was detected mainly in the cytoplasm (Fig. 3M, N), indicating that the RS segment is important for determining the correct cellular localization of Hrb1p. Interestingly, similar analysis revealed that the cellular distribution of the mutant in which all 8 serines were converted to alanine (GFP-Hrb1-8A) was identical to that of the WT protein (Fig. 4A, compare with 3I), further supporting the notion that phosphorylation of serine residues within the RS domain is not involved in regulating the nuclear/cytoplasmic distribution of Hrb1p. Similar to WT Hrb1p, Mtr10p is essential for the correct localization of GFP-Hrb1-8A, as in mtr10-7 cells it is mislocalized (Fig. 4B). Reintroduction of WT Mtr10p (expressed from the high copy number construct, pAD-MTR10) restores the correct nuclear/cytoplasmic distribution (Fig. 4C).

In order to further map sequences of the RS domain that are involved in determining the cellular localization of Hrb1p, the RS region was divided by generating deletions of increasing number of amino acids (HrbΔ1–25, HrbΔ1–50, HrbΔ1–75), and the resulting mutants as fusions to GFP were expressed in WT cells. While the localization of GFP-HrbΔ1–25 was identical to that of the WT protein (Fig. 4D), GFP-HrbΔ1–50 and GFP-HrbΔ1–75 localized mostly to the cytoplasm, as was the GFP-Hrb1ΔRS mutant (Fig. 4E, compare with 3M-N, and data not shown). This mislocalization was not corrected by overexpressed Mtr10p (Fig. 4F), suggesting that GFP-Hrb1Δ1–50 does not bind Mtr10p. These data indicate that the segment encompassing amino acids 25–50 is important for the nuclear localization of Hrb1p, possibly by affecting its binding to Mtr10p.

3.5. Deletion of Hrb1p or of additional putative Sky1p substrates does not affect spermine tolerance

Although Sky1p kinase activity was demonstrated essential for conferring polyamine toxicity [8], it does not fulfill this role through phosphorylation of its known substrates Npl3p or Gbp2p [8]. To determine whether Hrb1p is involved in mediating polyamine tolerance, the growth of hrb1Δ cells was tested on YPD supplemented with toxic spermine concentration. Similar to npl3Δ and gbp2Δ, hrb1Δ cells were as sensitive to spermine as were WT cells (data not shown).

A database search for proteins that contain potential Sky1p phosphorylation sites [16,17] revealed several additional proteins as potential Sky1p substrates (Fig. 5), some of which were phosphorylated by Sky1p in vitro (unpublished observations). Since deletion of these genes did not alter the growth rate in the presence of toxic spermine concentrations (data not shown), we concluded that none of them is directly involved in mediating spermine tolerance.

4. Discussion

We demonstrate here that despite containing an RS segment and although being phosphorylated by Sky1p in vitro, Hrb1p does not appear to be a physiological substrate of Sky1p. We
have identified Hrb1p as a potential Sky1p substrate using bioinformatic tools, biochemical methods and mass-spectrometry analysis. Our results demonstrate that Sky1p phosphorylates Hrb1p in vitro, and that the RS-domain is necessary for this phosphorylation. The RS domain contains 8 serine residues; at least 3 of them are located within sequences that conform to the Sky1p/SRPK phosphorylation sequence ([16,17], Fig. 5). Surprisingly, however, these 8 serine residues were not required for phosphorylation, as their conversion to alanines did not abolish phosphorylation in vitro. It is possible that under the in vitro conditions other residues are phosphorylated (the RS domain contain 3 threonine residues), or perhaps residues located outside the RS segment. It would be therefore interesting to identify the phosphorylated residues of Hrb1p by phosphoamino acid analysis, as performed in the case of NPL3 [4,18]. It is also possible that the RS segment contains the Mtr10p binding site. Deletion of this region might therefore prevent Hrb1p binding to Mtr10p and its recycling to the nucleus, thus leading to its cytoplasmatic accumulation. This is further supported by our observation that overexpression of Mtr10p did not restore the nuclear localization of the truncated Hrb1p (Fig. 4F), in contrast to Hrb1p overexpression [19] or transport-defective mutants of Npl3 and Gbp2 [5,18]. The observation that the Hrb1–8A mutant requires functional Mtr10p for its correct localization (Fig. 4A–C), provides additional validation to our conclusion that the subcellular localization of Hrb1p requires amino acids 25–50 that might facilitate Mtr10p binding, however it is not determined by phosphorylation of serine residues within the RS segment.

Gbp2 is 47% identical to Hrb1p, and they share many common features; both contain 3 RNA recognition motifs, an SR/RRG domain, both bind poly (A)+ mRNA, and their cellular localization is dependent on Mtr10p [5,18]. It is therefore interesting that even though both contain potential Sky1p phosphorylation sequence, only Gbp2p seems to be an in vivo substrate. The phosphorylated serine residue of Npl3p and Gbp2p is located both in the nucleus and in the cytoplasm ([4,18] and Fig. 3). While in sky1Δ cells Npl3p and Gbp2p were shifted towards the cytoplasm [4,18], the distribution of Hrb1p remained unchanged (Fig. 3), suggesting that the cellular localization of Hrb1p is not regulated by Sky1p phosphorylation. This conclusion is further supported by the observation that the Hrb1p mutant in which all 8 serines of the RS segment were converted to alanine, is localized correctly in WT cells (Fig. 4). This result also rules out the possibility that these residues mediate cellular localization by being phosphorylated by another kinase. In contrast, an Hrb1p mutant lacking the entire RS segment was mislocalized and observed mainly in the cytoplasm (Fig. 3). More careful mapping demonstrated that a subsegment of the RS region encompassing amino acids 25–50 is required for nuclear localization (Fig. 4).

Hrb1p was recently identified as a shuttling mRNA binding protein [5]. Its nuclear localization was shown to require Hmt1p, Mtr10p and Sky1p. However, in contrast to the distinct effect of Mtr10, lack of Sky1p exerted only marginal effect on Hrb1p localization [5]. Our present results actually argue that if at all, the effect of Sky1p on the cellular localization of Hrb1p is minimal. It is possible that differences in experimental methods contribute to this discrepancy, for example imaging live cells rather than fixed ones. In the absence of functional Mtr10p, Hrb1p was localized to the cytoplasm, similarly to our observation for the Hrb1ΔRS mutant. It is therefore tempting to speculate that the RS segment contains the Mtr10p binding site. Deletion of this region might therefore prevent Hrb1p binding to Mtr10p and its recycling to the nucleus, thus leading to its cytoplasmatic accumulation. This is further supported by our observation that overexpression of Mtr10p did not restore the nuclear localization of the truncated Hrb1p (Fig. 4F), in contrast to Hrb1p overexpression [19] or transport-defective mutants of Npl3 and Gbp2 [5,18]. The observation that the Hrb1–8A mutant requires functional Mtr10p for its correct localization (Fig. 4A–C), provides additional validation to our conclusion that the subcellular localization of Hrb1p requires amino acids 25–50 that might facilitate Mtr10p binding, however it is not determined by phosphorylation of serine residues within the RS segment.

Further support for the notion that Hrb1p is not an in vivo substrate of Sky1p comes from studying its cellular localization. In WT cells, Npl3p is localized in the nucleus, while Hrb1p and Gbp2p are located both in the nucleus and in the cytoplasm ([4,18] and Fig. 3). While in sky1Δ cells Npl3p and Gbp2p were shifted towards the cytoplasm [4,18], the distribution of Hrb1p remained unchanged (Fig. 3), suggesting that the cellular localization of Hrb1p is not regulated by Sky1p phosphorylation. This conclusion is further supported by the observation that the Hrb1p mutant in which all 8 serines of the RS segment were converted to alanine, is localized correctly in WT cells (Fig. 4). This result also rules out the possibility that these residues mediate cellular localization by being phosphorylated by another kinase. In contrast, an Hrb1p mutant lacking the entire RS segment was mislocalized and observed mainly in the cytoplasm (Fig. 3). More careful mapping demonstrated that a subsegment of the RS region encompassing amino acids 25–50 is required for nuclear localization (Fig. 4).

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compared with Hrb1p. Interestingly, despite their structural and functional similarity, cells tolerate Hrb1p overexpression, while overexpression of Npl3p and Gbp2 is toxic [19].

Sky1p kinase activity was shown to regulate polyamine transport across the plasma membrane [8]. Cells lacking Sky1p tolerate otherwise toxic levels of spermine and salt ions [8]. It is therefore reasonable to assume that Sky1p functions via phosphorylation of substrates. However, we have shown previously that deletions of the known Sky1p substrates, NPL3 and GBP2, does not result in a spermine resistant phenotype [8]. Here we show that deletion of HRB1, as well as deletion of additional potential Sky1p substrates does not result in altered spermine tolerance. We identified 19 proteins containing potential Sky1p phosphorylation sequences (Fig. 5). Although several of them were phosphorylated by Sky1p in vitro, deletion of none of them has demonstrated altered polyamine resistance or salt tolerance unlike sky1Δ cells (data not shown). It is therefore intriguing to understand how polyamine tolerance is mediated by Sky1p. One possibility is that Sky1p phosphorylates additional substrates, perhaps with slightly different sequence specificity. Alternatively, it is possible that phosphorylation of more than one substrate is required to confer spermine tolerance, perhaps by more than one mechanism.

Another possibility is that polyamine tolerance is conferred indirectly by Sky1p-phosphorylated substrates through their effect on mRNA metabolism. In addition to its involvement in mRNA shuttling, Npl3p was demonstrated as a translational repressor [5]. It was also suggested that phosphorylation of SR proteins is necessary to prevent unspecific binding to mRNAs [20]. It is therefore possible that deletion of SKY1 leads to unspecific binding of several under-phosphorylated SR proteins, interfering with the translatability of some mRNAs, thus leading to the polyamine tolerant phenotype. In line with this possibility is the demonstration that deletion of FES1, the nucleotide exchange factor of the Hsp70 Ssa1p, results in a general translation defect [21], and in polyamine tolerant phenotype [22]. It is therefore possible that deletion of SKY1 or FES1 will result in altered expression of several proteins, the final outcome being polyamine tolerance.

Acknowledgements

We thank Prof. E. Hurt for kindly providing mtr10-7 cells. C. Kahana is the incumbent of the Jules J. Mallon Professorial chair in Biochemistry.

References