Differential Export Requirements for Shuttling Serine/Arginine-type mRNA-binding Proteins*

Received for publication, December 3, 2003
Published, JBC Papers in Press, December 15, 2003,
DOI 10.1074/jbc.C300522200

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Messenger RNAs are transported to the cytoplasm bound to several shuttling mRNA-binding proteins. Here, we present the characterization of Hrb1, a novel component of the transported ribonucleoprotein complex in Saccharomyces cerevisiae. The protein is similar to the other two serine/arginine (SR)-type proteins in yeast, Gbp2 and Npl3. Hrb1 is nuclear at steady state and its import is mediated by the karyopherin Mtr10. Hrb1 binds to poly(A)* RNA in vivo and its binding is significantly increased in MTR10 mutants, suggesting a role for Mtr10 in dissociating Hrb1 from the mRNAs. Interestingly, by comparing the export requirements of all three SR proteins we find similarities but also striking differences. While the export of all three proteins is dependent on the export of mRNAs in general, as no transport is observed in mutants defective in transcription (rpb1-1) or mRNA export (mex67-5), we find specific requirements for components of the THO complex, involved in transcription elongation. While both Hrb1 and Gbp2 depend on Mft1 and Hpr1 for their nuclear export, Npl3 is exported independently of both proteins. These findings suggest that Hrb1 and Gbp2, but not Npl3, might be loaded onto the growing mRNA via the THO complex components Mft1 and Hpr1.

The cellular separation of transcription and translation in eukaryotic cells, requires transport across the nuclear envelope from one compartment into the other. Although significant progress has been made in understanding nuclear transport, unraveling the export mechanisms for mRNAs remains to be a challenge. General transport requires transport receptors of the importin family, also termed karyopherins and the Ran GTPase cycle (1–3). Interestingly, neither seems to be required for the export of mRNAs (4). Instead, several shuttling mRNA-binding proteins might contribute to the export process (5–8). One of the key players is Mex67 (TAP or NXF1 in metazoans), which recognizes the mature mRNA and mediates the interaction between the messenger ribonucleoparticles (mRNPs)* and the nuclear pore complexes (9). Mex67 is recruited to the mRNA by Yra1, a component of the transcription and export complex that couples transcription with export. Transcription and export is composed of Yra1 and Sub2 and the heterotetrameric THO complex (consisting of Mft1, Hpr1, Thp2, and Tho2), which functions in transcription elongation (10–15).

In addition to Mex67, other mRNA-binding proteins like Npl3 and Nab2 might contribute to the translocation of the mRNA through the nuclear pore complex by their interaction with the nuclear pore complex component Mlp1 (16). Furthermore, Npl3 has been shown to be involved in packaging the mRNA into an export competent mRNP (17). Gbp2, a protein that is highly homologous to Npl3, has recently been identified as another component of the exported mRNP (18). Both proteins comprise a serine/arginine-rich region (SR domain), which is the target of the SR-specific protein kinase Sky1 (SRPK1 and SRPK2 in mammalia) (19, 20). In addition, the SR domain of both RNA-binding proteins is important for the nuclear re-import mediated by the import receptor Mtr10 (transportin SR (TRN-SR1 and TRN-SR2) in mammalia) (18, 21). Furthermore, the cap binding complex, the cleavage and polyadenylation factor (CF IB) Hrp1, a DEAD-box helicase D5p/Rat8 (DBP5 in metazoans), and the poly(A)-binding protein Pab1 (PABP1 in metazoans) have been shown to be part of the exported mRNP (22–28).

Here, we present data that characterize Hrb1 as a shuttling mRNA-binding protein that accompanies mRNAs to the cytoplasm and can therefore be added to the list of proteins that comprise the exported mRNP. Hrb1 is highly homologous to the SR-type shuttling mRNA-binding proteins Gbp2 and Npl3. Interestingly, although the import requirements of all three proteins are very similar, we find striking differences in their export requirements. While both Hrb1 and Gbp2 are dependent on Mft1 and Hpr1, two components of the THO complex, the export of Npl3 is independent of both components, suggesting differences in the recruitment of the SR proteins to the mRNAs.

EXPERIMENTAL PROCEDURES

Plasmid Constructs and Yeast Strains—The plasmids expressing NLS-GFP (pHK511), HRBI-GFP from the GAL1 promoter (pHK230), MTR10 (pHK144), and Gbp2-GFP from the GAL1 promoter (pHK367) were described previously (18, 21, 33). All other plasmids were created by PCR with specific primers, subcloned in different vectors, and verified by sequencing. The complete open reading frame of HRBI and 500 bp upstream (HRBI promoter) was cloned into pHK12 (18), a CEN plasmid with a URA3 marker, resulting in pHK537. The open reading frame of NPL3 was subcloned into pHK290 resulting in GAL1-GFP-NPL3 (pHK144). The yeast strains wild type (HKY36), mtr10-7 (HKY270), skyl::TRP1 (pHK267), rpb1-1 (HKY77), hmt1::HIS3 (HKY53), thp2::KAN (HKY325), mft1::KAN (HKY329), and hpr1::HIS3 (HKY327) were described previously (10, 11, 13, 18).

Expression and GFP Localization—All procedures were essentially carried out as described previously (18). For fluorescent microscopy, strains were grown to the logarithmic growth phase (1 × 10⁶ cells/ml) at 25 °C in appropriate media and then shifted to 37 °C for the indicated times. The GFP signal was analyzed directly in fluorescent microscopy and pictures were taken from fixed cells. For this purpose 5 ml of the cultures were mixed with 350 μl of 37% formaldehyde. Cells were immediately collected by centrifugation and washed once in 0.1 M

*This work was supported by Grant SFB397 from the Deutsche Forschungsgemeinschaft (to H. K.). The costs of publication of this paper were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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The abbreviations used are: mRNP, messenger ribonucleoprotein complex; SR protein, serine/arginine-rich protein; NLS, nuclear localization signal; WT, wild type; GFP, green fluorescent protein.
K$_{2}$HPO$_{4}$-KH$_{2}$PO$_{4}$ at pH 6.5 and once in P solution (0.1 M K$_{2}$HPO$_{4}$-KH$_{2}$PO$_{4}$ at pH 6.5, 1.2 M sorbitol). For experiments with the GAL1 HRB1-GFP plasmid, strains were grown to the early log phase (5 x 10$^6$ cells/ml) in raffinose containing medium. The induction of the fusion protein was induced by addition of galactose and further incubation for 2 h. In this time the strains grew to ~1 x 10$^7$ cells/ml and were then treated as described above.

UV Cross-linking of Hrb1 to Poly(A)$^+$ RNA—Isolation of UV-cross-linked poly(A)$^+$ RNA-RNP complexes and analysis of cross-linked Hrb1-GFP by SDS-PAGE and Western blotting were done essentially as described previously (29). 1.5 liters of the wild type strain HXY36 or mtr10-7 mutant strain (HXY270) was grown to A$_{600}$ of 1.0 and if indicated shifted to 37 °C for 30 min. Cells were harvested, washed twice in phosphate-buffered saline, and split in two equal portions each. The first portion was not UV-irradiated, and the second portion was UV-irradiated for 2 x 2.5 min on ice in the Stratalinker (Stratagene). All samples were washed in phosphate-buffered saline and lysed in lysis buffer. The poly(A)$^+$ RNA was purified and the proteins analyzed as described previously (29, 30). All eluate samples were loaded normalized to the purified RNA and 10 A$_{600}$ units were loaded per lane. The Western blots were detected using anti-GFP antibodies (1:5000) and the SuperSignal® West Femto Kit (Pierce).

RESULTS AND DISCUSSION

Hrb1 Is a Nuclear SR-type Protein That Requires the Karyopherin Mtr10 and the SR-specific Protein Kinase Sky1 for Its Proper Nuclear Localization—Hrb1 is highly homologues to the SR-type mRNA-binding proteins, Gbp2 and Npl3. The protein shows an overall identity of 47% to Gbp2 and 23% to Npl3, respectively (Fig. 1A). It is comprised of three RNA recognition motifs and one serine/arginine-rich domain with 10 SR or RS motifs. This SR domain contains also two arginine/glycine/glycine (RRG) motifs, which for Npl3 have been shown previously to be substrates for arginine methyltransferase Hmt1 (31–34).

A functional GFP fusion of the protein is localized to the nucleus at steady state, and its import is mediated by the import receptor Mtr10 (Fig. 1, B and C). Strains with mutations in genes encoding different import receptors were grown to log phase before they were shifted to 37 °C for various times. Hrb1 keeps its nuclear localization in kap104-16, kap123A, and pse1-1 strains (data not shown). In contrast, it is localized to the cytoplasm of a mtr10-7 mutant strain at the non permissive temperature (Fig. 1C). Thus, Mtr10 is necessary for proper import of Hrb1. These data are further supported by affinity purification experiments that identified Hrb1 as a co-purifying protein of ProtA-tagged Mtr10 (21). Interestingly, the localization of Hrb1 in a knock-out strain of SKY1, encoding the only SR-specific protein kinase in yeast, shifted slightly to the cytoplasm, indicating that phosphorylation of Hrb1 might be involved in localizing Hrb1 properly to the nucleus (Fig. 1C). The observed mislocalization phenotype is very similar to the Npl3 localization in SKY1 deletion strains, and it has been discussed that phosphorylation of Npl3 is necessary for Mtr10 binding (19, 20). Thus, cytoplasmic accumulation has been explained by a slowed import rate. It has also been discussed that phosphorylation increases the affinity of the SR proteins for specific mRNA targets (35, 36). Thus, in a different model phosphorylation could result in an unspecific binding of the SR proteins to any mRNA, which also could lead to an increased cytoplasmic localization, observed in SKY1 knock-out strains.

Hrb1 Binds to Poly(A)$^+$ RNA in Vivo—The overall structural features of Hrb1 indicate that it is an RNA-binding protein. We therefore tested its ability to bind to poly(A)$^+$ RNA in vivo by UV cross-linking experiments. Yeast cell lysates of log phase cells were purified using oligo(dT)-cellulose columns and compared with the resulting eluates on Western blots (Fig. 2), clearly demonstrating that Hrb1 binds to poly(A)$^+$ RNA in vivo. Interestingly, similar to what was shown for Npl3 and Gbp2 before (18, 20), binding of Hrb1 to poly(A)$^+$ RNA is significantly enhanced in mtr10 mutant strains, shifted to 37 °C for 30 min prior to cross-linking, indicating that Mtr10 might be involved in dissociation of Hrb1 from the mRNA in the cytoplasm (Fig. 2). In fact, since all three SR proteins show an increased binding to poly(A)$^+$ RNA when Mtr10 is not functional (Fig. 2 and Refs. 18 and 20), Mtr10-induced dissociation of the SR proteins seems to be a general mechanism for this type of proteins.

Overexpression of HRB1 Saturates Its Import Receptor—In expectation of Hrb1 to be a shuttling protein (33), we searched for conditions in which the export rate of the protein exceeds its import rate and in this way would enable us to study the export requirements of Hrb1. To possibly saturate its import receptor Mtr10, we overexpressed HRB1-GFP from the strong GAL1 promoter, shifted the strain to 37 °C, and analyzed the cellular localization of the protein. Indeed, we found that high level Hrb1 results in a cytoplasmic localization phenotype (Fig. 3A). Furthermore, as expected, simultaneous overexpression of
**MTR10** rescues the cytoplasmic mislocalization (Fig. 3A). These data indicate that the steady state localization of Hrb1 can be shifted from one cellular compartment to the other by tuning the protein level, and moreover, Mtr10 seems to be a bottleneck for Hrb1 import.

**Overexpression of HRB1 is not toxic to cells (Fig. 3B).** Wild type strains carrying either an empty vector or **HRB1-GFP**, **GBP2-GFP**, or **NPL3-GFP** under the strong **GAL1** promoter were grown to log phase before 10-fold serial dilutions were spotted on either a glucose or galactose plate (induced). In contrast to overexpression of **GBP2** and **NPL3** under these conditions, **HRB1** was not toxic to cells, because it is cytoplasmic (Fig. 3A) and cytoplasmic versions of **Hrb1** (by overexpression from the **GAL1** promoter) or **Gbp2** and **Npl3** (by mutations **gbp2-S15A** and **npl3-S411A**, respectively) were expressed as GFP fusions in WT or different mutant strains. Localization is shown in an RNA-polymerase II mutant strain, **rpb1-1**, in a strain mutated in the mRNA export receptor, **mex67-5**, in a knock-out of the arginine methyltransferase **Hmt1** and in deletion strains of components of the THO complex, **thp1::KanMX**, **hpr1::KanMX**, and **mft1::KanMX**. All strains were grown to log phase and shifted to 37 °C for 1 h prior to fluorescent microscopy.

**Overexpression of Hrb1 and Gbp2, but not Npl3, are dependent on Hrp1 and Mft1, two components of the THO complex.** Cytoplasmic versions of Hrb1 (by overexpression from the **GAL1** promoter) or Gbp2 and Npl3 (by mutations **gbp2-S15A** and **npl3-S411A**, respectively) were expressed as GFP fusions in WT or different mutant strains. Localization is shown in an RNA-polymerase II mutant strain, **rpb1-1**, in a strain mutated in the mRNA export receptor, **mex67-5**, in a knock-out of the arginine methyltransferase **Hmt1** and in deletion strains of components of the THO complex, **thp1::KanMX**, **hpr1::KanMX**, and **mft1::KanMX**. All strains were grown to log phase and shifted to 37 °C for 1 h prior to fluorescent microscopy.

**Fig. 2. Hrb1 is a poly(A)* RNA-binding protein, and its binding is significantly enhanced in mtr10 mutants.** UV cross-linking of Hrb1 to poly(A)* RNA is shown. Wild type cells and mtr10-7 cells that express Hrb1-GFP were grown to log phase; shifted for 30 min to 37 °C, and exposed to UV light for cross-linking (+ or − UV irradiation). The lysates were taken before the material was applied to an oligo(dT)-cellulose column. The eluates containing the cross-linked poly(A)* RNA, and bound proteins were treated with RNases. Proteins were analyzed by SDS-polyacrylamide gel electrophoresis followed by Western blotting using anti-GFP antibodies.

**Fig. 3. Overexpression of Hrb1 saturates its import receptor Mtr10 and leads to a predominantly cytoplasmic localization.** A, subcellular localization of Hrb1-GFP, expressed from the strong **GAL1** promoter, is shown in a wild type strain (WT) either in the absence or in the presence of high copy Mtr10. The strains were grown in raffinose-containing medium to the early log phase before the expression of **HRB1** was induced for 50 min by adding galactose. Finally, expression was stopped by adding glucose, and the strains were incubated at 37 °C for 30 min prior to analysis. B, overexpression of **HRB1** is not toxic to cells, as determined by the growth of equal amounts of wild type cells carrying either an empty vector or a **GAL1 HRB1-GFP**-containing vector spotted in 10-fold serial dilutions on glucose or galactose plates. In comparison, both overexpression of **GBP2** and **Npl3** is toxic to cells under the same conditions (bottom).

**Fig. 4. The export of Hrb1 and Gbp2, but not Npl3, are dependent on Hrp1 and Mft1, two components of the THO complex.** Cytoplasmic versions of Hrb1 (by overexpression from the **GAL1** promoter) or Gbp2 and Npl3 (by mutations **gbp2-S15A** and **npl3-S411A**, respectively) were expressed as GFP fusions in WT or different mutant strains. Localization is shown in an RNA-polymerase II mutant strain, **rpb1-1**, in a strain mutated in the mRNA export receptor, **mex67-5**, in a knock-out of the arginine methyltransferase **Hmt1** and in deletion strains of components of the THO complex, **thp1::KanMX**, **hpr1::KanMX**, and **mft1::KanMX**. All strains were grown to log phase and shifted to 37 °C for 1 h prior to fluorescent microscopy.

**Table:**

| Condition       | Lysate | Eluate |
|-----------------|--------|--------|
| **UV**          | −      | −      |
| **WT**          | +      | +      |
| **mtr10-7**     | +      | +      |

**Fig. 5.** The export of Hrb1 and Gbp2, but not Npl3, are dependent on Hrp1 and Mft1, two components of the THO complex. Cytoplasmic versions of Hrb1 (by overexpression from the **GAL1** promoter) or Gbp2 and Npl3 (by mutations **gbp2-S15A** and **npl3-S411A**, respectively) were expressed as GFP fusions in WT or different mutant strains. Localization is shown in an RNA-polymerase II mutant strain, **rpb1-1**, in a strain mutated in the mRNA export receptor, **mex67-5**, in a knock-out of the arginine methyltransferase **Hmt1** and in deletion strains of components of the THO complex, **thp1::KanMX**, **hpr1::KanMX**, and **mft1::KanMX**. All strains were grown to log phase and shifted to 37 °C for 1 h prior to fluorescent microscopy.

**Fig. 6.** Overexpression of Hrb1 and Gbp2, but not Npl3, are dependent on Hrp1 and Mft1, two components of the THO complex. Cytoplasmic versions of Hrb1 (by overexpression from the **GAL1** promoter) or Gbp2 and Npl3 (by mutations **gbp2-S15A** and **npl3-S411A**, respectively) were expressed as GFP fusions in WT or different mutant strains. Localization is shown in an RNA-polymerase II mutant strain, **rpb1-1**, in a strain mutated in the mRNA export receptor, **mex67-5**, in a knock-out of the arginine methyltransferase **Hmt1** and in deletion strains of components of the THO complex, **thp1::KanMX**, **hpr1::KanMX**, and **mft1::KanMX**. All strains were grown to log phase and shifted to 37 °C for 1 h prior to fluorescent microscopy.

**Fig. 7.** Overexpression of Hrb1 and Gbp2, but not Npl3, are dependent on Hrp1 and Mft1, two components of the THO complex. Cytoplasmic versions of Hrb1 (by overexpression from the **GAL1** promoter) or Gbp2 and Npl3 (by mutations **gbp2-S15A** and **npl3-S411A**, respectively) were expressed as GFP fusions in WT or different mutant strains. Localization is shown in an RNA-polymerase II mutant strain, **rpb1-1**, in a strain mutated in the mRNA export receptor, **mex67-5**, in a knock-out of the arginine methyltransferase **Hmt1** and in deletion strains of components of the THO complex, **thp1::KanMX**, **hpr1::KanMX**, and **mft1::KanMX**. All strains were grown to log phase and shifted to 37 °C for 1 h prior to fluorescent microscopy.
nuclear localization of the otherwise cytoplasmic protein, we have established a method to shift the steady state localization of the protein allowing us to investigate the export requirements of Hrb1.

**Different Export Requirements for the Three Shuttling SR-type Proteins Hrb1, Gbp2, and Npl3**—All three shuttling SR proteins in yeast are localized to the nucleus at steady state but shifted to the cytoplasm when either mutated in the SR domain (18, 20) or overexpressed (Fig. 3A). Single amino acid residue exchanges in Npl3 and GBP2 (npl3-S411A and gbp2-S15A) alter the localization of each protein (Fig. 4, top row), and it has been discussed that either lack of phosphorylation and/or reduced binding to Mtr10 causes the observed phenotype (18, 20).

Since the cytoplasmic presence of the SR proteins at steady state neither influence the growth rate of the yeast strains nor the intracellular localization of the mRNA (data not shown), we were able to compare the export requirements of all three SR proteins. All strains carrying plasmids with either GAL1-HRB1-GFP, gbp2-S15A-GFP, or npl3-S411A-GFP were shifted to 37°C for 1 h prior to analysis. As expected, the nuclear export of all three SR proteins is dependent on RNA polymerase II transcription as shown in rpb1-1 mutants (Fig. 4). Similarly, when the mRNA export is blocked in mutants of the nuclear export receptor MEX67, mex67-5, the export of Hrb1, Gbp2, and Npl3 is inhibited (Fig. 4). At the same time, the localization of a control reporter protein is not affected under these conditions (data not shown and (18)). Thus, all three SR proteins function similarly in that ongoing transcription and a functional mRNA export machinery are required for their cytoplasmic entry. However, differences are indicated by analysis of their localization in hmt1 knock-out strains. Cells deleted for the arginine methyltransferase retain Npl3 and Hrb1 but not Gbp2 in the nucleus (Fig. 4). These findings are consistent with **in vitro** arginine methylation studies, in which Npl3 and Hrb1 have established a method to shift the steady state localization of the otherwise cytoplasmic protein, allowing us to investigate the export requirements of Hrb1.

Together, we have shown the characterization of Hrb1 as the third shuttling SR-type mRNA-binding protein in yeast, which accompanies the transported mRNAs to the cytoplasm. While the export requirements of all three SR proteins are similar, we reveal striking differences in the export requirements of these proteins and thus provide novel insights into the different mechanisms by which the SR proteins might be recruited to the growing mRNAs.

Acknowledgments—We are grateful to A. Aguilera, C. Guthrie, E. Hurt, and P. A. Silver and K. Straßer for strains and plasmids. We thank H. Bastians for discussion and critically reading the manuscript.

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K. Straßer, personal communication.