The RNA export factor Gle1p is located on the cytoplasmic fibrils of the NPC and physically interacts with the FG-nucleoporin Rip1p, the DEAD-box protein Rat8p/Dbp5p and a new protein Ymr255p

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Gle1p is an essential, nuclear pore complex (NPC)-associated RNA export factor. In a screen for high copy suppressors of a GLE1 mutant strain, we identified the FG-nucleoporin Rip1p and the DEAD-box protein Rat8p/Dbp5p, both of which have roles in RNA export; we also found Ymr255p/Gfd1p, a novel inessential protein. All three high copy suppressors interact with the C-terminal domain of Gle1p; immunoelectron microscopy localizations indicate that Gle1p, Rip1p and Rat8p/Dbp5p are present on the NPC cytoplasmic fibrils; Rip1p was also found within the nucleoplasm and on the nuclear baskets. In vivo localizations support the hypothesis that Rip1p contributes to the association of Gle1p with the pore and that Gle1p, in turn, provides a binding site for Rat8p/Dbp5p at the NPC. These data are consistent with the view that Gle1p, Rip1p, Rat8p/Dbp5p and Ymr255p/Gfd1p associate on the cytoplasmic side of the NPC to act in a terminal step of RNA export. We also describe a human functional homologue of Rip1p, called hCG1, which rescues Rip1p function in yeast, consistent with the evolutionary conservation of this NPC-associated protein.

Keywords: DEAD-box protein/FG-nucleoporin/nuclear pore complex/RNA export/yeast

Introduction

Transport through nuclear pores requires a concerted interaction between the structural components of the nuclear pore complex (NPC) and the soluble transport factors that bind specific cargoes and shuttle between the nuclear and cytoplasmic compartments. Transport is a signal-mediated and energy-dependent process. Localization signals within the transported cargoes are recognized by transport receptors, which mediate either import or export. These receptors (importins or exportins) are members of a family of proteins related to the import receptor importin-β. They all share the functional characteristic of binding to phenylalanine–glycine (FG) repeat domains of a family of NPC proteins called FG-nucleoporins, as well as the small GTPase Ran/Gsp1p (reviewed in Mattaj and Englmeier, 1998). Ran acts as a molecular switch that regulates the association of transport receptors with their cargoes (reviewed in Dahlberg and Lund, 1998). The first transport receptor identified was importin-β, the import factor for nuclear proteins containing a ‘classical’ nuclear localization signal (NLS) (reviewed in Görlich, 1998).

Export receptors were identified more recently, and a major contributor to the understanding of export was the human immunodeficiency virus type 1 (HIV-1) Rev protein. Rev is a shuttling RNA-binding protein whose role is to promote the export of partially spliced or unspliced viral transcripts. Rev was the first protein in which a nuclear export signal (NES) was identified. Rev-NES was shown to function in a variety of organisms including yeast, indicating that the export machinery targeted by Rev was evolutionarily conserved (reviewed in Pollard and Malim, 1998). A yeast two-hybrid screen initially identified the yeast FG-nucleoporin Rip1p and the non-homologous mammalian protein hRip/RAB as potential targets for Rev-NES at the nuclear pore (Bogerd et al., 1995; Fritz et al., 1995; Stutz et al., 1995). However, the export factor Crm1/Xpo1p was subsequently shown to interact directly with Rev-NES in a Ran-GTP-dependent manner and to promote the export of Rev and its associated RNA, presumably through an interaction with components of the NPC (reviewed in Mattaj and Englmeier, 1998; Stutz and Rosbash, 1998). As the initially described Rev–Rip1p two-hybrid interaction was compromised in a CRM1 mutant background, it was proposed that Crm1p bridges this association; Crm1p interacts with Rip1p in vitro (Floer and Blobel, 1999) but also with many other FG-repeat domains in the two-hybrid assay (Neville et al., 1997). However, most of these domains can be deleted individually without affecting viability (Fabre and Hurt, 1997), indicating extensive functional redundancy, and it is unclear at present which FG-repeat regions are relevant to Crm1p function in vivo.

Despite the identification of Crm1p and the elucidation of its role in Rev-mediated RNA export, our understanding of cellular RNA export is still limited. Newly synthesized RNAs are exported as ribonucleoprotein complexes (RNPs), which harbour multiple signals recognized by the export machinery. The export of the different classes of RNAs is dependent on nuclear Ran-GTP, suggesting the involvement of exportins in these processes (Izaurralde et al., 1997). There is strong evidence that Crm1p exports UsnRNAs and 5S RNAs in vertebrate systems, but the exact contribution of Crm1p to mRNA export is still under debate (Neville and Rosbash, 1999; reviewed in Stutz and Rosbash, 1998).

Several shuttling hnRNP proteins (hnRNPA1, hnRNPK)
or the yeast hnRNP-like proteins, Npl3p and Hrp1p/Nab4p, have been proposed to participate in mRNA export (reviewed in Nakielny et al., 1997; Izaurralde and Adam, 1998; Stutz and Rosbash, 1998). However, no clear physical connection has yet been established between these RNA-binding proteins and a specific export factor or component of the NPC.

The understanding of RNP translocation through the nuclear pore relies in part on the functional characterization of the NPC components involved in that process. Numerous nucleoporins have been identified in yeast, a number of which assemble in discrete NPC sub-complexes. The potential role of these sub-complexes has been elucidated mainly by determining how deletion or mutation of individual members affect NPC function (e.g. RNA export, protein import) or biogenesis (reviewed in Doye and Hurt, 1997; Fabre and Hurt, 1997). Nup159p and Nup84p define two sub-complexes with primary roles in RNA export (Siniossoglou et al., 1996; Teixeira et al., 1997; Belgarè et al., 1998; Hurwitz et al., 1998; and see Discussion).

Several additional factors essential for poly(A)⁺ mRNA export have been found in association with the NPC. One consists of the yeast Mex67p/Mtr2p protein complex; the two proteins are present on both sides of the NPC, suggesting that their localization may be dynamic (Santos-Rosa et al., 1998). TAP, the human homologue of Mex67p, is the cellular factor recruited by the CTE (constitutive transport element) of type D retroviruses to export unspliced viral RNAs (Grüter et al., 1998). TAP shuttles between nucleus and cytoplasm, and both Mex67p and TAP bind RNA and interact with FG-nucleoporins (Katahira et al., 1999). TAP and Mex67p may promote mRNA export by mediating the interaction of mRNPs with FG-nucleoporins at the pore. These properties define TAP and Mex67p as new types of export receptors, not related to the importin-β family of transporters (de Castilla and Rout, 1999).

The DEAD-box protein Rat8p/Dbp5p is also an essential player in mRNA export. Rat8p/Dbp5p was detected in the cytoplasm as well as in association with NPCs. The RNA-dependent ATPase and ATP-dependent RNA-unwinding activities of Rat8p/Dbp5p were proposed to participate in RNA export by restructuring RNP complexes during translocation through and release from the NPC. In the cytoplasm, the energy generated by ATP hydrolysis may also be used to dissociate shuttling RNA-binding proteins and promote their recycling back into the nucleus (Snay-Hodge et al., 1998; Tseng et al., 1998; Schmitt et al., 1999).

Finally, Gle1p is another NPC-associated component essential for RNA export; it was identified initially in a screen for mutations synthetically lethal with a RIP1 allele, supporting a role for Rip1p in poly(A)⁺ RNA export. Rip1p contains two domains: an N-terminal FG-repeat region, which interacts with Crm1p and Rev (presumably through bridging by Crm1p) in the two-hybrid assay (Neville et al., 1997), and a unique C-terminus (referred to as the C-domain). The C-domain is sufficient to rescue synthetic lethality of GLE1 mutants, indicating a functional interaction between this region and Gle1p. Rip1p also becomes essential under heat shock conditions, where it is required for the efficient export of heat-induced transcripts; the short C-domain of Rip1p is both necessary and sufficient for this activity (Saavedra et al., 1997; Stutz et al., 1997). These data suggest that the C-domain of Rip1p performs a similar function in RNA export under stress or normal conditions.

Here we further characterize the functional relationship between Gle1p and Rip1p and show that the physical interaction between these two proteins is distinct from the Rev–Rip1p interaction. Through a high copy suppressor screen of a GLE1 mutant strain, we identified Rip1p and two additional Gle1p partners: the DEAD-box protein Rat8p/Dbp5p and an unknown protein, Ymr255p. Immuno-electron microscopy localizations indicate that these interactions are likely to take place on the cytoplasmic side of the NPC and may therefore contribute to a late step of RNA export. We also describe the identification of a human homologue of Rip1p, termed hCG1, which recapitulates several aspects of Rip1p function in yeast.

**Results**

**hCG1, the human homologue of Rip1p/Nup42p**

As earlier data implicated Rip1p in RNA export, we asked whether this protein was evolutionarily conserved. Given the functional importance of the Rip1p unique C-terminus (Stutz et al., 1997), database searches were performed with the 66 C-terminal residues. A single sequence was identified (DDBJ/EMBL/GenBank accession No. U97198), corresponding to a complete human cDNA of 1778 bp named hCG1, previously described in an unrelated approach (Van Laer et al., 1997). hCG1 mRNA encodes a 423 amino acid protein. ClustalW alignment of the Rip1p and hCG1 proteins revealed a 55% homology over the entire length and a 35% identity over the C-terminal 40 amino acids. These data indicated a significant conservation of the region important for Rip1p function (Figure 1A). hCG1 also contains multiple FG-dipeptides in its N-terminal domain and therefore presents a domain organization similar to that of Rip1p. These observations together with the functional data presented below support that hCG1 corresponds to the human homologue of Rip1p/Nup42p. Further BLAST searches identified several mouse expressed sequence tags (ESTs) encoding a protein with...
extensive homology to hCG1; finally, Bombyx mori and Schizosaccharomyces pombe sequences were identified with C-terminal domains highly related to the corresponding region in Rip1p/hCG1 (Figure 1B). The comparison of the Rip1p C-terminal domains revealed a series of highly conserved residues with a spacing strictly maintained among the five species. While this manuscript was in preparation, hCG1 was also identified in two-hybrid screens with the human TAP and HIV-1 Rev proteins (Farjot et al., 1999; Katahira et al., 1999).

**hCG1 can substitute for Rip1p function in yeast**

Rip1p is not essential under normal growth conditions but is required under heat shock for the efficient export of heat-induced transcripts. Rip1p also becomes essential in the presence of mutations in the RNA export factor Gle1p, indicating a contribution of Rip1p to non-heat shock RNA export. Earlier work showed that the unique C-domain of Rip1p was sufficient to restore heat shock RNA export at 42°C in a ΔRIP1 strain and to rescue synthetic lethality in GLE1 mutant strains (Stutz et al., 1997). To test whether hCG1 was functionally homologous to Rip1p, we examined the ability of its C-terminal domain to rescue Rip1p function. For this purpose, the C-terminal regions of Rip1p and hCG1 were expressed in yeast as LexA fusions by cloning into the two-hybrid bait vector pEG202 (HIS3, 2µ). Fusion to LexA allowed good expression of these short peptides when assayed by Western blotting (data not shown), and the same fusions were used in two-hybrid interaction assays (see below).

First, we tested whether the LexA fusions restored heat shock RNA export at 42°C in a ΔRIP1 strain. Heat shock protein synthesis at 42°C was used as an assay for heat shock RNA export (Figure 2A). For comparison, [35S]methionine incorporation at 25 or 42°C was monitored in a W303 wild-type strain in which heat shock proteins are strongly induced at 42°C (Figure 2A, lanes 1 and 2). In contrast, no heat shock proteins were synthesized at 42°C in a ΔRIP1 strain transformed with an empty vector, due to the potent block of heat shock RNA export in the absence of Rip1p (compare lanes 2 and 4); heat shock protein synthesis was restored in ΔRIP1 with a plasmid expressing wild-type Rip1p (RIP1; lane 5). More importantly, heat shock protein synthesis was rescued efficiently by constructs expressing LexA fused to the last 66 or 38 amino acids of Rip1p (LexA–RIP1-C66 and LexA–RIP1-C38, lanes 8 and 9) or to the last 43 residues of hCG1 (LexA–hCG1-C43, lane 10). These results show that the
last 38 amino acids of Rip1p, which are most highly conserved, are sufficient for function, and that the C-domains of hCG1 and Rip1p have the same functional properties. Finally, plasmids expressing protein A fused to full-length Rip1p or to the last 66 amino acids of Rip1p also efficiently rescued heat shock RNA export in the ΔRIP1 strain (ProtA–RIP1–C66; lanes 12 and 14). No heat shock protein synthesis was observed with LexA alone (LexA; lane 6) nor with LexA fused to the FG-repeat domain of Rip1p (LexA–RIP1–FG; lane 7).

Secondly, we tested whether the same constructs could rescue the ΔRIP1 synthetic lethal phenotype of the gle1-8 mutant strain (Stutz et al., 1997) or with LexA fused to the FG-repeat domain of Rip1p (LexA–RIP1–FG; lane 7).

Secondly, we tested whether the same constructs could rescue the ΔRIP1 synthetic lethal phenotype of the gle1-8 mutant strain (Stutz et al., 1997). This ORF has also been identified in a screen for high copy suppressors of a GLE1 temperature-sensitive mutant. The gle1-8-rip1::KANΔ plasmid was transformed with the plasmids described in (A). Rescue of synthetic lethality was assessed by streaking the transformants on plates (Figure 3B). Overexpression of Rip1p, Rat8p/Dbp5p or Gfd1p was not able to bypass the gle1-8 null allele (data not shown).

To determine how well these three genes in high copy suppressed the growth defect of gle1-8 cells, the growth at 37°C of the gle1-8 transformants was examined in liquid cultures or on plates (Figure 3B). Overexpression of Rip1p, Rat8p/Dbp5p and Gfd1p substantially, but not completely, rescued the gle1-8 ts, since the strains transformed with high copy RIP1, RAT8/DBP5 and GFD1 plasmids did not grow as fast as that transformed with wild-type GLE1. The rescue appeared more potent on plates.

As the C-domain of Rip1p was able to complement the synthetic lethality. The LexA–hCG1-C43 and LexA–RIP1-C66 constructs complemented synthetic lethality and allowed strain FSY38 to grow on 5-fluoro-orotic acid (5-FOA) as efficiently as the wild-type RIP1 plasmid (pFS398). The LexA–RIP1-C38 construct also rescued the gle1-8 synthetic lethality, albeit less efficiently (data not shown), and no growth was detected on 5-FOA in the presence of LexA–RIP1-FG or an empty vector. Finally, the ProtA–RIP1 and ProtA–RIP1–C66 constructs similarly rescued the gle1-8 synthetic lethal mutation, showing that these fusions also function under normal growth conditions (Figure 2B and see below).

These data taken together demonstrate that the 43 C-terminal amino acids of hCG1 can substitute for the corresponding region of Rip1p under stress or normal growth conditions and support that hCG1 and Rip1p/Nup42p represent functional homologues.

Identification of extragenic high copy suppressors of a GLE1 temperature-sensitive mutant

The genetic interactions between Rip1p and Gle1p and the clear involvement of these two proteins in RNA export prompted us to explore further the genetic space around Gle1p. The gle1-8 temperature-sensitive (ts) allele, identified as one of several GLE1 alleles in an earlier ΔRIP1 synthetic lethal screen (Stutz et al., 1997), was used to screen for high copy extragenic suppressors. Gle1p comprises a non-essential N-terminal region (amino acids 1–112), an essential predicted coiled-coil domain (amino acids 113–255) and an essential C-terminal domain (amino acids 256–538), which contains the proposed Rev-like NES (Del Priore et al., 1997; Watkins et al., 1998; and Figure 3A). The gle1-8 allele contains a T21A substitution in the N-terminal domain and an E340K change (upstream of the proposed NES) in the C-terminal domain likely to be responsible for the ts phenotype. Interestingly, all the GLE1 alleles isolated in the ΔRIP1 synthetic lethal screen contained mutations within the essential C-terminal domain, indicating a role for this region in the functional interaction with Rip1p.

High copy suppressors of the gle1-8 mutant strain were identified by transformation of a yeast genomic library cloned into a 2µ-based high copy vector and selection of transformants at 37°C. Besides GLE1, the genes found most frequently and exhibiting the strongest suppression phenotype were RIP1, RAT8/DBP5, which encodes a DEAD-box protein essential for RNA export, and the uncharacterized open reading frame (ORF) YMR255w. This ORF has also been identified in a screen for high copy suppressors of a RAT8/DBP5 mutant allele and was named GFD1 (Hodge et al., 1999). High level expression of Rip1p, Rat8p/Dbp5p or Gfd1p was not able to bypass a GLE1 null allele (data not shown).

To determine how well these three genes in high copy suppressed the growth defect of gle1-8 cells, the growth at 37°C of the gle1-8 transformants was examined in liquid cultures or on plates (Figure 3B). Overexpression of Rip1p, Rat8p/Dbp5p and Gfd1p substantially, but not completely, rescued the gle1-8 ts, since the strains transformed with high copy RIP1, RAT8/DBP5 and GFD1 plasmids did not grow as fast as that transformed with wild-type GLE1. The rescue appeared more potent on plates.

As the C-domain of Rip1p was able to complement the
synthetic lethality of a ΔRIP1-gle1-8 strain (Figure 2B), we tested whether this domain would also exhibit high copy suppression of the gle1-8 ts phenotype. The C-terminal 66 amino acids of Rip1p were expressed as a Gal-inducible GST fusion from a 2μ plasmid. The gle1-8 growth defect was rescued substantially at 37°C upon expression of GST–Rip1p–C66 both in liquid and on plates. This phenotype was dependent on galactose (Figure 3C).

Mutations in Gle1p induce poly(A)^+ RNA export defects. We next examined the ability of the high copy suppressors to restore poly(A)^+ RNA export in the rssl-37 (gle1) ts strain (Saavedra et al., 1997; and see below) by in situ hybridization with an oligo(dT) probe (Figure 3D). The rssl-37 strain (FSY292) transformed with an empty vector exhibited a strong nuclear poly(A)^+ RNA signal and very weak cytoplasmic staining after a 30 min incubation at 37°C (panel a). The RNA export defect was eliminated in the presence of a wild-type GLE1 plasmid (panel b). High copy plasmids expressing Rip1p and Rat8p/Dbp5p significantly rescued poly(A)^+ RNA export, as all the cells exhibited substantial cytoplasmic staining and less frequent or weaker nuclear signal (panels c and d). Overexpression of Gfd1p had a detectable, but more modest effect (panel e). The incomplete restoration of poly(A)^+ RNA export in the rssl-37 mutant at 37°C by overexpression of Rip1p, Rat8p/Dbp5p or Gfd1p parallels the partial suppression of the gle1-8 and rssl-37 growth phenotypes (Figure 3B and data not shown).

To determine whether the localization of gle1-8p was altered in the presence of the high copy suppressors, we chromosomally tagged the mutant gene with green fluorescent protein (GFP). The distribution of the gle1-8p–GFP fusion was examined in living cells at 25°C or after a shift to the non-permissive temperature in the presence of an empty vector or 2μ plasmids expressing Rip1p or Rat8p/Dbp5p (Figure 3E). The distribution of gle1-8p–GFP was compared with that of Gle1p–GFP in a chromosomally tagged wild-type strain. Like wild-type Gle1p–GFP, gle1-8p–GFP exhibited a typical nuclear rim staining. After a 4 h shift to the non-permissive temperature in the presence of an empty vector or 2μ plasmids expressing Rip1p or Rat8p/Dbp5p (Figure 3E), the three suppressors marginally increased the fraction of gle1-8p–GFP present at the nuclear envelope at 37°C (Figure 3E). Western blot analysis showed that the steady-state levels of gle1-8p–GFP at 37°C were identical to those of wild-type Gle1p–GFP (Figure 3F, compare lanes 1 and 6); the gle1-8p–GFP levels remained stable over a 6 h incubation of the cells at 37°C and were not affected by overexpression of Rip1p, Rat8p/Dbp5p or Gfd1p (compare lanes 1–5). These data indicate that the high copy suppressors do not act by stabilizing the mutant protein. As the localization of gle1-8p–GFP was only modestly affected by overexpression of Rip1p or Rat8p/Dbp5p, it is not clear whether the high copy suppressors enhance the association of gle1-8p–GFP with the NPC, or whether they suppress the ts phenotype through a different process.

**Physical interactions between Gle1p and the high copy suppressor proteins**

To investigate the relationship between Gle1p and its high copy suppressors, we tested the physical interaction between these proteins in the two-hybrid assay (Table I). As full-length Gle1p two-hybrid fusions were poorly expressed, constructs were used that expressed the C-terminal domain of Gle1p (amino acids 257–538), which contains the proposed Rev-like NES (Figure 3A).

Consistent with the genetic observations, Gle1p bait strongly interacted with preys containing full-length or the C-terminal 66 or 38 residues of Rip1p but not with preys containing the FG-repeat domain of Rip1p. An identical pattern of interaction was observed with hCG1, verifying the functional homology between Rip1p and hCG1. The reciprocal Rip1p/hCG1 bait and Gle1p prey constructs gave the same results (Table I and data not shown). The interactions between Gle1p and the C-domains of Rip1p and hCG1 were weakened by the gle1-8 mutation (E340K) and were completely abolished in the presence of the rssl-37 (gle1) ts allele. This latter GLE1 mutant, obtained by PCR mutagenesis (Saavedra et al., 1997), exhibits a stronger growth defect than gle1-8 and contains several mutations in the C-terminal domain. Western blot analysis confirmed equal expression of wild-type and mutant Gle1p two-hybrid fusions (data not shown).

As Gle1p was proposed to share functional characteristics with HIV-1 Rev, the two proteins were compared in these two-hybrid analyses. In contrast to Gle1p and consistent with our earlier data, the HIV-1 Rev bait fusion interacted with the FG-repeats of both Rip1p and hCG1 (presumably through bridging by Crm1p) but not with their C-termini (Table I). Taken together with the absence of an interaction between Gle1p and Crm1p, the data indicate that Gle1p does not behave like a bona fide NES-containing protein in this assay.

Lastly, we tested the two-hybrid interaction between Gle1p and prey fusions expressing full-length Rat8p/Dbp5p or Gfd1p prey fusions. The C-terminal domain of Gle1p strongly interacted with these two high copy suppressors, and both interactions were abolished by the gle1-8 and rssl-37 mutations. These results show that the three high-copy suppressors of the gle1-8 mutant physically interact with the C-terminal region of Gle1p. In addition, we detected an interaction between Gfd1p and the FG-repeat domain of Rip1p (Table I) and between Rat8p/Dbp5p and Gfd1p (data not shown).

**Gle1p interacts with its high copy suppressors in vitro**

To test whether the two-hybrid interactions between Gle1p and the three suppressors may represent direct associations, the same proteins were tested for binding in vitro. Full-length Gle1p and its C-terminal domain were prepared by in vitro translation in reticulocyte lysates in the presence of [35S]methionine. The [35S]-labelled Gle1p proteins were incubated with GST fusions produced in *Escherichia coli* and purified on glutathione beads (Figure 4). Full-length Gle1p specifically interacted with the C-terminal domains of Rip1p and hCG1 (GST–Rip1p–C66 and GST–hCG1–C43), as well as with full-length Gfd1p and Rat8p/Dbp5p (GST–Gfd1p and GST–Rat8p/Dbp5p) (top panel, lanes 4–7). Gle1p did not interact significantly with the FG-repeat domain of Rip1p as the binding was not greater than with GST alone (top panel, compare lanes 2 and 3). Consistent with the two-hybrid data, the same pattern was obtained...
in the presence of the truncated Gle1p protein with no substantial loss in binding efficiency, suggesting that the C-terminal region of Gle1p is responsible for these interactions (top two panels, compare Gle1p input with the amount of Gle1p bound to each GST fusion). None of the GST fusions interacted with \textit{in vitro} translated luciferase (third panel), and Coomassie Blue staining of the polyacrylamide gels before autoradiography showed comparable GST fusion input in each binding reaction (lower panel). These data strongly indicate that Gle1p interacts directly with the three high copy suppressors; however, the possibility that some of these associations are mediated by components in the reticulocyte lysate cannot be discarded.

\begin{figure}[h]
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\caption{Experimental Results}
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**Poly(A)** RNA export is dependent on Rip1p in a GLE1 mutant background

The genetic and physical relationships between Gle1p and Rip1p strongly support the participation of Rip1p in RNA export through an interaction with Gle1p. To demonstrate further the importance of Rip1p in RNA export, we compared poly(A)**+** RNA distribution in two ΔRIP1 synthetic lethal strains, FY585 and FY587, which contain the weak ts gle1-1 allele (F381S substitution) and a RIP1 disruption covered by a RIP1 plasmid. In FY585, the RIP1 plasmid (pFS398) expresses normal levels of Rip1p (Rip1p normal); in FY587, the RIP1 plasmid (pFS724) contains a truncated RIP1 promoter which results in the production of 5–10 times less Rip1p (Rip1p low) (Stutz et al., 1997). Poly(A)**+** RNA distribution was examined in FY585 and FY587 by in situ hybridization with a digoxigenin-labelled oligo(dT) probe at 25°C or after a 30 min shift to 37°C (Figure 5). Both strains exhibited an enhanced nuclear accumulation of poly(A)**+** RNA at 37°C. However, the fraction of cells presenting an export defect was significantly higher in the strain expressing low levels of Rip1p both at 25°C (Figure 5, compare panels a and e) and 37°C (compare panels c and g). The enhanced poly(A)**+** RNA export defect exhibited by the gle1-1 strain in the presence of limiting levels of Rip1p is consistent with a role for Rip1p in mRNA export.

**Localization of Gle1p, Rip1p and Rat8p/Dbp5p by immunoelectron microscopy**

Although Gle1p, Rip1p and Rat8p are clearly involved in RNA export, their exact contribution to this process is unknown. Defining the precise localization of these proteins within the NPC three-dimensional structure may help to assign them a role in specific steps of export. For this purpose, the location of Gle1p, Rip1p and Rat8p was determined at the ultrastructural level by performing pre-embedding labelling immunoelectron microscopy, a procedure which yields structurally intact yeast NPCs (Fahrenkrog et al., 1998). The proteins of interest were tagged with protein A and expressed in strains disrupted for the wild-type gene. The tagged proteins were detected with a colloidal gold-conjugated anti-protein A antibody. The ProtA–Gle1p and ProtA–Rat8p fusions are functional as they complement the corresponding lethal gene disruption (Murphy and Wente, 1996; Tseng et al., 1998). Two constructs that encoded protein A fused to full-length Rip1p (ProtA–Rip1p) or to the C-terminal 66 amino acids of Rip1p (ProtA–Rip1p-C66) were used to localize Rip1p.

### Table I. Gle1p two-hybrid interactions

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The indicated bait and prey constructs were transformed into the RFY206 and EGY48 strain, respectively, and containing the β-galactosidase reporter construct pSH18-34. The transformants were mated on YEPD. Diploids were replica-plated on SD-Ura-His-Trp indicator plates containing 3% galactose/1% raffinose and X-gal. The strength of two-hybrid interactions was estimated visually by the intensity of the blue colour after 2 days of reporter construct pSH18-34. The transformants were mated on YEPD. Diploids were replica-plated on SD-Ura-His-Trp indicator plates containing 3% galactose/1% raffinose and X-gal. The strength of two-hybrid interactions was estimated visually by the intensity of the blue colour after 2 days of growth at 37°C. Both strains exhibited an enhanced nuclear accumulation of poly(A)**+** RNA at 37°C. However, the fraction of cells presenting an export defect was significantly higher in the strain expressing low levels of Rip1p both at 25°C (Figure 5, compare panels a and e) and 37°C (compare panels c and g). The enhanced poly(A)**+** RNA export defect exhibited by the gle1-1 strain in the presence of limiting levels of Rip1p is consistent with a role for Rip1p in mRNA export.

### Fig. 3. Effects of the GLE high copy suppressors on growth, poly(A)**+** RNA distribution and localization of gle1-8p–GFP. (A) Gle1p contains a non-essential N-terminus followed by a coiled-coil region (amino acids 108–256) and a C-terminal domain with a putative leucine-rich (LR) NES. The amino acid changes present in the gle1-8 allele are indicated. (B) The GLE high copy suppressors partially rescue the growth defect of the gle1-8 strain at 37°C. Strain gle1-8 was transformed with control vectors (URA3 or TRP1, 2μ) or high copy plasmids (pFS998, pFS997, pFS999 or pFS952) expressing Gle1p, Rip1p, Rat8p/Dbp5p or Gfd1p, respectively. Liquid cultures of the transformed strains were diluted to OD600 = 0.03, grown for 2 h at 25°C and shifted to 37°C. Growth was followed by measuring optical density at 600 nm over time (left). The gle1-8 transformants were also compared for growth on solid medium by spotting dilution series of saturated cultures on selective plates at 25 or 37°C (right). (C) The Rip1p C-terminus partially rescues the growth defect of the gle1-8 strain at 37°C. Strain gle1-8 was transformed with the Gal-GST vector (pYGEX2T, URA3/2μ) or the galactose-inducible GST–RIP1-C66 construct (pFS961) and growth was monitored at 37°C in SD-Ura medium containing galactose (left); growth was also examined on SD-Ura plates containing galactose at 25 or 37°C (right), as described above. (D) The GLE high copy suppressors partially rescue the poly(A)**+** RNA export defect of the temperature-sensitive strain rss1-37 (gle1) at 37°C. Strain rss1-37 (FSY292) was transformed with empty vector or with high copy (2μ) plasmids expressing Gle1p, Rip1p, Rat8p/Dbp5p or Gfd1p. Transforms were grown to mid-log phase at 25°C and subjected to a 30 min incubation at 37°C before fixation and in situ hybridization with a digoxigenin-labelled oligo(dT) probe for localization of poly(A)**+** RNA. Panels a–e show the fluorescence after the cells were probed with FITC-conjugated anti-digoxigenin antibody. Panels f–j show DAPI staining of the same cells, respectively, and indicate the position of the nuclei. (E) The NPC localization of gle1-8p–GFP is modestly enhanced at 37°C by high level expression of Rip1p or Rat8p/Dbp5p. The chromosomally tagged gle1-8-GFP (FSY390) and GLE1-GFP (FSY398) strains were transformed with empty vector or high copy (2μ) plasmids expressing Rip1p or Rat8p/Dbp5p. Cells grown to mid-log phase were kept at 25°C or shifted to 37°C for 4 h and examined directly under the microscope. (F) The levels of gle1-8-GFP are stable and not affected by the high copy suppressors. The chromosomally tagged gle1-8-GFP (FSY399) and GLE1-GFP (FSY398) strains were transformed with an empty vector (V; lanes 1, 2, 6 and 7) or high copy plasmids expressing Rip1p, Rat8p/Dbp5p, Gfd1p (lanes 3–5) or Gle1p (lane 8). The transformants were grown at 25°C or incubated for 6 h at 37°C as indicated. Protein extracts from equivalent number of cells were subjected to Western blot analysis with a rabbit polyclonal antibody against Gle1p. The positions of GFP-tagged and non-tagged Gle1p are indicated. The same blot was probed with an anti-Rna15p antibody as a control for loading.
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Fig. 4. Gle1p interacts with Rip1p, hCG1, Dbp5p/Rat8p and Gfd1p in vitro. GST alone or GST fusions containing the FG-repeat or C-terminal domains of Rip1p, the C-domain of hCG1, as well as full-length Gfd1p or Rat8p/Dbp5p were produced in E. coli and purified on glutathione beads. GST fusions on beads were tested for interaction with in vitro translated and [35 S]methionine-labelled full-length Gle1p, Gle1p C-terminal region (amino acids 257–538) or luciferase. After binding and washing, beads boiled in sample buffer were analysed by SDS–PAGE and bound 35 S-labelled proteins revealed by autoradiography (three top panels, lanes 2–7). Input (lane 1) corresponds to the amount of [35 S]protein added to each binding reaction. The amounts of GST fusions in each reaction were compared by Coomassie staining of a gel before autoradiography (lower panel; each GST fusion is indicated by a dot). BSA was used as non-specific competitor in the binding reactions and co-migrates on these gels.

These ProtA fusions are functional as well, since they efficiently rescued phenotypes associated with the RIP1 deletion (Figure 2).

Gle1p is associated with NPC cytoplasmic fibrils. Consistent with earlier immunofluorescence studies (Del Priore et al., 1996; Murphy and Wente, 1996), Gle1p was detected in the cytoplasm but a significant fraction was found in association with NPCs. Interestingly, all the gold particles localized on the cytoplasmic side of the NPC, ~40–50 nm from the central plane, indicating an association with the cytoplasmic fibrils. Quantitative analysis of the labelling with respect to the centre of the 8-fold symmetry axis showed that Gle1p distributes over the whole diameter of the pore (Figure 6A and B). Given the physical interaction between Gle1p and Rip1p, we examined ProtA–Gle1p distribution in a ΔGLE1/ΔRIP1 double deletion background (Figure 6C). Surprisingly, the ProtA–Gle1p staining was mostly lost from the NPCs, and gold particles were only found in the cytoplasm. Rip1p being inessential, it is unlikely to represent a major binding site for Gle1p at the NPC. One explanation for the drastic effect of the RIP1 deletion on ProtA–Gle1p localization is that this fusion is not fully functional and is more sensitive to the absence of Rip1p; indeed, a strain expressing ProtA–Gle1p grows normally in the presence of Rip1p but is temperature sensitive when RIP1 is deleted (data not shown). The data suggest that Rip1p contributes to the association of Gle1p with the NPC.

ProtA–Rat8p is cytoplasmic and associated with the cytoplasmic fibrils. Indirect immunofluorescence experiments have localized Rat8p/Dbp5p in the cytoplasm and in association with NPCs (Snay-Hodge et al., 1998; Tseng et al., 1998). By immunogold localization, the ProtA–Rat8p fusion was detected in the cytoplasm and on the cytoplasmic fibrils, with an apparent exclusion from the nuclear compartment (Figure 7A and B). These data are consistent with an association of Rat8p/Dbp5p with NPC.

Fig. 5. Limiting levels of Rip1p enhance the poly(A)⁺ RNA export defect of a GLE1 mutant strain. Strains FSY58 (gle1-1, rip1::KAN⁹/pFS398) and FSY57 (gle1-1, rip1::KAN⁹/pFS724) expressing normal (wild-type) and low levels of Rip1p, respectively, were grown to mid-log phase at 25°C and either kept at 25°C or subjected to a 30 min incubation at 37°C. Cells were processed for in situ hybridization as described in Figure 3D. (a, c, e and g) The distribution of poly(A)⁺ RNA; (b, d, f and h) DAPI staining of the same cells, respectively.
proteins present on the cytoplasmic side of the pore and suggest that Rat8p/Dbp5p acts at a late step of RNA export.

Rip1p is found at two distinct sites of the NPC. The distribution of Rip1p contrasted with that of Gle1p. ProtA–Rip1p was detected in the nucleoplasm and on both sides of the NPC, in association with the nuclear baskets as well as with the cytoplasmic fibrils (Figure 8A). The quantification of the staining indicated the presence of similar amounts of Rip1p on both sides of the NPC; the distribution of Rip1p on the cytoplasmic fibrils was comparable with that of Gle1p, with a slightly higher concentration at the centre of the pore (Figure 8B). The detection of both Rip1p and Gle1p on the cytoplasmic fibrils, their physical interaction together with the enhanced RNA export defect in a GLE1 mutant when Rip1p is limiting support that Rip1p contributes to a late step of RNA export through an interaction with Gle1p on the cytoplasmic fibrils. The overall distribution of Rip1p was unchanged in a gle1-8 mutant background, suggesting that the localization of Rip1p is not dependent on a strong interaction with Gle1p (data not shown).

To examine the role of the FG-repeat versus the C-terminal domain of Rip1p in this distribution, we next localized a ProtA fusion containing only the unique C-terminus of Rip1p (ProtA–Rip1p-C66). Like full-length ProtA–Rip1p, ProtA–Rip1p-C66 was found within the nucleoplasm and on both sides of the NPC, indicating that the unique C-domain is sufficient to accumulate the fusion within the nucleus and to anchor Rip1p on both sides of the pore. In contrast to full-length Rip1p, the ProtA–Rip1p-C66 fusion was detected at a higher frequency on the cytoplasmic fibrils than on the nuclear baskets (Figure 8C and D). These observations could suggest that the FG-repeat domain of Rip1p stimulates
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Fig. 7. Rat8p/Dbp5p is located in the cytoplasm and on the cytoplasmic fibrils of the NPC. (A) Triton X-100-extracted spheroplasts from cells expressing ProtA–Rat8p/Dbp5p in a ΔRAT8/DBP5 background (FSY342) were prepared as described in Figure 6. Shown is a view of a cross-sectioned NE stretch with labelled NPCs (arrows) and a gallery of selected gold-labelled NPCs. c, cytoplasm; n, nucleus. (B) Distribution of the gold particles associated with the NPCs. Fifty gold particles were scored. Bars, 100 nm.

the recruitment of Rip1p to the nuclear baskets. Alternatively, if Rip1p was trafficking between the two sides of the pore, the FG-repeats could increase the recycling of Rip1p from the cytoplasmic to the nuclear side of the NPC. Finally, given the essential role of Rip1p under heat shock conditions, we examined the localization of ProtA–Rip1p in a ΔRIP1 background after a 10 min shift to 42°C. The overall distribution of Rip1p at the NPCs was not substantially affected under these stress conditions (data not shown).

Since through pre-embedding labelling, some cytoplasmic epitopes can be lost (the cells are treated with Triton X-100 to facilitate labelling on both sides of the NPC), we cannot exclude that a small fraction of Rip1p is also present in the cytoplasm.

In vivo localization of Gle1p–GFP and Rat8p/Dbp5p–GFP in mutant backgrounds

Immunoelectron microscopy localization of ProtA–Gle1p showed that Gle1p was lost from the NPC in an RIP1 deletion strain, suggesting a contribution of Rip1p to the association of Gle1p with the NPC (Figure 6C). To substantiate this observation, we examined the distribution of a Gle1p–GFP fusion in living cells in a ΔGLE1 strain or a ΔGLE1ΔRIP1 double deletion strain (Figure 9A). Similarly to the ProtA–Gle1p fusion, the Gle1p–GFP fusion conferred a ts phenotype in the absence of Rip1p, indicating that Gle1p–GFP was not fully functional (data not shown). Consistent with the immunoelectron microscopy data, the amount of Gle1p–GFP detected in association with the nuclear envelope after growing the cells at 37°C for 4 h was considerably lower in the absence of Rip1p. The disappearance of Gle1p–GFP from the NPC did not result from increased turnover or decreased synthesis, since the steady-state levels of Gle1p–GFP in the ΔRIP1 strain were stable at 37°C over at least 6 h (data not shown). The localization of Gle1p appeared to be affected more drastically by the absence of Rip1p when examined by immunoelectron microscopy. This difference may be due to the fact that the electron microscopy data are from the quantitation of gold particles in cell sections, while the intensity of GFP fluorescence is derived from whole cells.

An in vivo interaction between Gle1p and Rat8p/Dbp5p was suggested by the high copy suppression and protein interaction assays. To determine whether Gle1p contributes to the association of Rat8p/Dbp5p with the NPC, a Rat8p/Dbp5p–GFP fusion was localized in a strain containing the rss1-37 (gle1) mutant allele (Figure 9B). The rss1-37 mutation induces a strong poly(A)+ RNA export defect and abolishes the two-hybrid interaction between Gle1p and Rat8p/Dbp5p (Figure 3D and Table I). Two strains were examined, both of which contained the rss1-37 allele and expressed a Rat8p/Dbp5p–GFP fusion. In one case, the RAT8/DBP5 gene was tagged with GFP on the chromosome (rss1-37 RAT8/DBP5-GFP); in the other, genomic RAT8/DBP5 was deleted and Rat8p/Dbp5p–GFP expressed from a plasmid (rss1-37 ΔRAT8/DBP5 pRAT8/DBP5-GFP; Figure 9B, right). Both strains behaved similarly. At 25°C, Rat8p/Dbp5p–GFP was detected in the cytoplasm as well as in association with the NPC, resulting in a strong nuclear rim staining as described (Snay-Hodge et al., 1998). After 2 h at 37°C, the GFP staining at the rim became weaker and more diffuse but did not disappear (even after prolonged exposure to 37°C); there was a concomitant modest increase in the cytoplasmic signal. These data are consistent with the notion that Gle1p represents a binding site for Rat8p/Dbp5p at the nuclear pore, but that other interactions are involved in the recruitment of this DEAD-box protein to the NPC.

Discussion

In this study, we used a high copy suppressor approach to identify proteins functionally or physically related to Gle1p. The screen identified the NPC-associated nucleoporin Rip1p, the DEAD-box protein Rat8p/Dbp5p and a new non-essential protein Ymr255p. This protein was
Gle1p initially was proposed to contain a Rev-like NES in its essential C-terminal domain and to promote poly(A)+ RNA export through an NES-dependent export pathway involving an interaction with Rip1p, analogous to that described between Rev-NES and the FG-repeat domain of Rip1p (Murphy and Wente, 1996). However, the NES is not conserved in the human homologue of Gle1p (Watkins et al., 1998), and the localization of Gle1p–GFP at the nuclear rim was not affected in the xpo1-1 (crm1)
et al. (1998), neither we nor others were able to detect an interaction between Gle1p and Crm1p (Watkins et al., 1997); the physical interaction between Gle1p and Rip1p further shows that Gle1p does not behave like a Rev-NES-containing protein. Indeed, the C-terminal domain of Gle1p may undergo sequential interactions with Rip1p, Rat8p/Dbp5p and Gfd1p, which may be part of a series of events contributing to the release of an RNP from the NPC.

Based on the localization and interaction data, the synthetic lethality between a RIP1 deletion and GLE1 mutations is probably due to the absence of the RIP1 C-terminal domain from the cytoplasmic fibrils. This is because synthetic lethality is rescued by the Rip1p C-terminus alone, and overexpression of this domain is able to suppress a temperature-sensitive GLE1 mutation (Figures 2 and 3). A contribution of Rip1p to Gle1p function is also supported by the enhanced RNA export defect in a GLE1 mutant strain when Rip1p becomes limiting (Figure 5). One interpretation of these observations is that the binding of the C-domain of Rip1p to Gle1p stimulates Gle1p activity, perhaps by optimizing the association of Gle1p with some of its partners (e.g. Rat8p/Dbp5p or Gfd1p). This function of Rip1p becomes essential in a GLE1 mutant background or under stress conditions, where the overall efficiency of RNA export is reduced.

Fig. 9. (A) The localization of Gle1p–GFP at the NPC is weakened in the absence of Rip1p. ΔGLE1 (FSY297) or ΔGLE1–ΔRIP1 (FSY298) strains expressing Gle1p–GFP from a centromeric plasmid (pFS1030) were grown at 35°C in selective medium to mid-log phase, shifted to 37°C for 4 h and examined under the microscope. (B) Rat8p/Dbp5p–GFP is partially lost from the NPC in the rsl1-37 (gle1) mutant background. Rat8p/Dbp5p–GFP was localized in two strains containing the rsl1-37 allele. The strain on the left (rsl1-37 ΔRAT8/DBP5-GFP; FSY400) contains a RAT8/DBP5 gene chromosomally tagged with GFP. The strain on the right (rsl1-37 ΔRAT8/DBP5 pRAT8/DBP5-GFP; FSY401) contains a RAT8/DBP5 deletion covered by a plasmid (pCS835) expressing a Rat8p/Dbp5p–GFP fusion. The strains were grown in SD complete medium to mid-log phase at 25°C. They were then kept at 25°C or shifted to 37°C for 2 h and examined under the microscope. Two fields are shown for each strain at 25 and 37°C.

Two biochemically defined NPC sub-complexes with primary roles in poly(A)+ RNA export have been described. One is composed of the essential nucleoporins Nup159p/Nup82p/Nsp1p (Belgareh et al., 1998; Hurwitz et al., 1998) and the other consists of Nup84p/Nup85p/Nup120p/C–Nup145/Sec13p (Siniossoglou et al., 1996; Teixeira et al., 1997). Members of both sub-complexes have been localized on the NPC cytoplasmic fibrils (Kraemer et al., 1995; Fahrenkrog et al., 1998; Hurwitz et al., 1998; Katahira et al., 1999; Stoffler et al., 1999). It is noteworthy that these distributions may not all be evolutionarily conserved, as components of a human NPC sub-complex, homologous to the Nup84p sub-complex, have been located at the nuclear basket (Fontoura et al., 1999). The exact function of these complexes in pore structure versus export function is still poorly defined. More specific roles have been attributed to individual components. Nup82p, for example, anchors the corresponding complex within the NPC (Belgareh et al., 1998), whereas Nup159p or components of the Nup84p complex provide binding sites for shuttling proteins involved in more dynamic aspects of export (Santos-Rosa et al., 1998; Schmitt et al., 1999; and see below).

No physical connections have yet been established between the Nup84p and Nup159p sub-complexes, but...
The identification of the DEAD-box protein Rat8p/Dbp5 as a partner for Gle1p and the substantial decrease of Rat8p/Dbp5p-GFP rim staining in the rsl1-37 (gle1) temperature-sensitive mutant background suggest that Gle1p participates in the binding of Rat8p/Dbp5p to the nuclear pore (Figure 9B). However, the partial retention of Rat8p/Dbp5p–GFP at the NPC in the mutant background at 37°C is consistent with the existence of alternative binding sites. Indeed, a direct interaction between Rat8p/Dbp5p and the N-terminal domain of Rat7p/Nup159p has been identified (Hodge et al., 1999; Schmitt et al., 1999). These observations could explain the suppression of the rat7-1/nup159-1 temperature-sensitive mutation by high level expression of Gle1p (Del Priore et al., 1996), i.e. an excess of Gle1p may provide additional binding sites for Rat8p/Dbp5p when the mutant rat7-1/nup159-1p is degraded, and thereby partially restore poly(A)+RNA export.

The ProtA–Gle1p and Gle1p–GFP fusions are substantially lost from the NPC in the absence of Rip1p (Figures 6C and 9A); these strains are also temperature sensitive for growth indicating that Rip1p contributes to a functionally relevant association of Gle1p with the NPC. As Rip1p is inessential, it is unlikely to represent a major binding site for Gle1p. Consistently, such a role for Rip1p is revealed only in the presence of the ProtA–Gle1p and Gle1p–GFP fusions, which are not fully functional proteins (see Results). Rip1p may therefore strengthen the association of Gle1p with the nuclear pore, presumably by stabilizing Gle1p within a complex. The major anchoring site for Gle1p at the pore is not known, but it may involve the essential coiled-coil domain of Gle1p, by analogy to other NPC proteins (Belgareh et al., 1998).

The immunoelectron microscopic localizations detected Rip1p within the nucleoplasm and the NPC nuclear baskets and cytoplasmic fibrils, a distribution distinct from that observed for Gle1p and Rat8p/Dbp5p. The presence of Rip1p on both sides of the NPC suggests that this FG-nucleoporin may have a role in late as well as in early steps of export, i.e. the recruitment of export complexes from the nucleoplasm towards the baskets. Rip1p may then move from one side to the other, perhaps in association with cargoes, and participate in their release on the cytoplasmic side of the NPC. The accumulation of Rip1p at two distinct NPC sites may reflect rate-limiting steps during that process. The vertebrate NPC-associated FG-nucleoporins Nup98 and Nup153 have recently been proposed to move between the nuclear and cytoplasmic compartments in association with exported cargoes (Nakiely et al., 1999; Zolotukhin and Felber, 1999).

An association of Rip1p with RNP cargo was suggested by the identification of an interaction between the human RNA export factor TAP and the FG-nucleoporins CAN/Nup214 and hCG1, the functional homologues of Nup159p and Rip1p, respectively (Katahira et al., 1999; E.Izaurralde, personal communication; and Figure 2). These data support the view that TAP and its yeast homologue Mex67p promote the export of bound RNPs through an interaction with one or more FG-nucleoporins (de Castilla and Rout, 1999). Consistent with the TAP–hCG1 interaction, we observed a homologous two-hybrid interaction between Mex67p and the FG-repeat domain of Rip1p (D.Zenklusen, unpublished results).

The association of Mex67p with the NPC is essential for RNA export, and this interaction is mediated by Mtr2p. Nup85p, a component of the Nup84p sub-complex, represents one target of the Mex67p/Mtr2p complex at the NPC (Santos-Rosa et al., 1998). Interestingly, we identified several NUP85 alleles in the ΔRIP1 synthetic lethal screen that were only rescued by full-length Rip1p (our unpublished data). These genetic observations together with the Mex67p/Rip1p-FG two-hybrid interaction suggest that the FG-repeat domain of Rip1p may complement Nup85p by contributing to the association of Mex67p with the pore. As the immunoelectron microscopic distributions of Rip1p and Mex67p are comparable (Santos-Rosa et al., 1998; and Figure 8), Rip1p could associate with Mex67p within the nucleus and travel along with the RNP complex through the NPC. On the cytoplasmic side, Rip1p is likely to interact with Gle1p through its C-terminus; this interaction may in turn stimulate the association of Gle1p with the DEAD-box protein Rat8p/Dbp5p. ATP binding and hydrolysis may drive conformational rearrangements within associated RNPs, which ultimately will result in the release of the RNP from the NPC and/or the recycling of RNP components towards the nucleus. As the RNA-unwinding activity of Rat8p/Dbp5p in vitro is dependent on a co-factor (Tseng et al., 1998), Gle1p and/or its associated proteins could be candidates for such a function.

In summary, our data support the notion that Gle1p acts in a terminal step of RNA export through an interaction with multiple partners. Further studies will address the molecular details of these interactions and possibly reveal aspects of the dynamic rearrangements underlying export. The ability of hCG1 to rescue a RIP1 deletion is consistent with the functional conservation of Rip1p during evolution. Our data support that hCG1 represents the true homologue of Rip1p, which is distinct from the earlier described hRIP/RAB1 protein (Bogerd et al., 1995; Fritz et al., 1995); the extent of conservation between all of the partners of hCG1 in yeast and in vertebrate systems will be investigated in the future.

Materials and methods

The DNA manipulations were performed according to standard methods. Yeast media and yeast transformations were performed with established procedures (Ausubel et al., 1994). The strains and plasmids used in this study are listed in Tables II and III.

Yeast plasmid constructions

ProtA–RIP1 (pFS829) was obtained by introducing a SalI site after the ATG of RIP1 in pFS398 to generate pFS800. A 400 bp SalI PCR product containing two IgG-binding domains from protein A was introduced into the SalI site of pFS800 to generate pFS829. ProtA–RIP1-C66 (pFS923) was generated by deleting the FG-repeat domain in pFS829; briefly, a 1.6 kb HindIII–XhoI PCR fragment extending from the 5′
HindIII site to the 3' end of the two protein A moieties of pFS829 was ligated to a 1.5 kb XhoI–HindIII PCR fragment containing the last 66 codons of RIP1 and 3' sequences down to the natural HindIII site in-frame with the GST sequence of pYGEX2T (URA3/2). The pGLe1-GFP plasmid (LEU2/CEN) was obtained by cloning the GLe1 gene as a SalI fragment with 500 bp upstream and downstream sequences into YCPLac112 (LEU2/CEN) to generate pFS802. PCR was used to introduce a NotI site upstream of the GLe1 stop codon in pFS802 to generate pFS1029. GFP (S65T) was cloned as a NotI fragment into pFS1029 to generate pFS1030.

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<td>RATS-GFP fusion cloned in YCPlac111 (LEU2/CEN)</td>
<td>Murphy et al. (1996)</td>
</tr>
<tr>
<td>pVDP29</td>
<td>prsl1-37 (g1el)</td>
<td>rssl1-37 temperature-sensitive GLe1 allele in YCP111 (LEU2/CEN)</td>
<td>Saavedra et al. (1997)</td>
</tr>
<tr>
<td>pYS410</td>
<td>GST–RIP1-FG</td>
<td>RPI-1 codons 121–230 cloned as an EcoRI–XhoI PCR fragment into pGex4T1</td>
<td>this study</td>
</tr>
<tr>
<td>pFS507</td>
<td>GST–RIP1-C66</td>
<td>RPI-1 codons 364–430 cloned as an EcoRI–XhoI PCR fragment into pGex4T1</td>
<td>this study</td>
</tr>
<tr>
<td>pFS883</td>
<td>GST–hCIG1-C43</td>
<td>hCIG1 codons 380–423 cloned as an EcoRI–XhoI PCR fragment into pGex4T1</td>
<td>this study</td>
</tr>
<tr>
<td>pFS955</td>
<td>GFP–DGF1</td>
<td>GFP1 coding region clones as an EcoRI–XhoI PCR fragment into pGex4T1</td>
<td>this study</td>
</tr>
<tr>
<td>pFS956</td>
<td>GST–RAT8/DBP5</td>
<td>RATS/DBP5 coding region cloned as an EcoRI fragment into pGex4T1</td>
<td>this study</td>
</tr>
</tbody>
</table>

*See Materials and methods for two-hybrid constructs.

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### Table III. Plasmids used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pFS652</td>
<td>RIP1 HindIII genomic 3.5 kb fragment into SacI of pCH1122 (URA3/CEN) with SacI linkers</td>
<td>Stutz et al. (1997)</td>
</tr>
<tr>
<td>pFS398</td>
<td>RPI-1</td>
<td>YCP50-based vector in which URA3 was replaced with LEU2</td>
</tr>
<tr>
<td>pFS724</td>
<td>RPI-1 low</td>
<td>RIP1 HindIII genomic 3.5 kb fragment inserted into the HindIII site of p366; contains 0.38 kb RPI 5’-flanking sequences</td>
</tr>
<tr>
<td>pFS800</td>
<td></td>
<td>pFS398 with engineered SacI site after the ATG</td>
</tr>
<tr>
<td>pFS829</td>
<td>ProtA–RIP1</td>
<td>pFS800 with two IgG-binding domains (protein A) in the 5’ SacI site</td>
</tr>
<tr>
<td>pFS923</td>
<td>ProtA–RIP1-C66</td>
<td>pFS829 with a deletion of the FG-domain (codons 2-364)</td>
</tr>
<tr>
<td>pCA503</td>
<td>GLe1-A–GFP</td>
<td>GLe1 with five IgG-binding domains inserted at the 5’ end (TRP1/CEN)</td>
</tr>
<tr>
<td>pFS925</td>
<td>GLe1-GFP</td>
<td>YMR255G/GFP cloned in the Eag1 site of YEp24 (URA3/2/µ)</td>
</tr>
<tr>
<td>pFS997</td>
<td>RPI-1 HindIII genomic 3.5 kb fragment cloned in YEPlac112 (TRP1/2a)</td>
<td>this study</td>
</tr>
<tr>
<td>pFS998</td>
<td>GLe1 cloned as a SacI fragment from pFS802 into YEPlac112</td>
<td>this study</td>
</tr>
<tr>
<td>pFS999</td>
<td>RAT8 cloned as a Spht-BamHI fragment into YEPlac112</td>
<td>this study</td>
</tr>
<tr>
<td>pYGEX2T</td>
<td>Galactose-inducible GST fusion yeast expression vector (URA3/2/µ)</td>
<td>P.Silver laboratory</td>
</tr>
<tr>
<td>pFS961</td>
<td>yGST–RIP1-C66</td>
<td>RPI-1 C-terminal codons 365–430 cloned as a BamHI–HindIII fragment into pYGEX2T</td>
</tr>
<tr>
<td>pFS802</td>
<td>GLe1 cloned as a SalI fragment into YCPLac111 (LEU2/CEN)</td>
<td>this study</td>
</tr>
<tr>
<td>pFS1029</td>
<td>GLe1 3’NotI</td>
<td>pFS802 with engineered NotI site upstream of GLe1 stop codon</td>
</tr>
<tr>
<td>pFS1030</td>
<td>GLe1 GFP</td>
<td>GLe1 SacI fragment with GFP inserted into the engineered NotI site of pFS1029</td>
</tr>
<tr>
<td>pCA503</td>
<td>RATS-GFP</td>
<td>RATS-GFP fusion cloned in YCPlac111 (LEU2/CEN)</td>
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</tr>
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<td>RATS/DBP5 coding region cloned as an EcoRI fragment into pGex4T1</td>
</tr>
</tbody>
</table>

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The high copy plasmid pFS997 was obtained by cloning the genomic 3.5 kb RPI1 HindIII fragment into YEpLac112 (TRP1/2). pFS998 was obtained by cloning the GLEI SalI fragment from pFS802 into YEpLac112. pFS999 was obtained by cloning the RI–SalI fragment as an SpH1–BamHI fragment from pCS830 into the corresponding sites of YEpLac112. The RIP1-C66 bait and prey constructs were made by cloning a 500 bp genomic DNA as a SalI fragment from pCS830 into the corresponding sites of YEpLac112. pFS992 was made by cloning the coding region of YMR255w/GFD1 fragment from pCS830 by 300 bp upstream and downstream sequences into vector YepE24 (URA3/2) as an EagI PCR fragment (Cole Laboratory, Dartmouth, VT).

**Two-hybrid LexA bait and prey cloning, strains and interaction assays**

All baits were expressed as LexA fusions by cloning into vector pEG202 (His3/2µ); the prey constructs were obtained by cloning into pG4-5 (TRP1/2µ) (Ausubel et al., 1994). The GLEI bait or prey constructs were obtained by inserting a BamHI–SalI or an EcoRI–SalI PCR fragment corresponding to codons 257–383 of GLEI into pEG202 cut with BamHI–Xhol or pG4-5 cut with EcoRI–Xhol to generate pFS795 and pFS935, respectively. The RIP1-C66 bait and prey constructs were made by cloning a 500 bp EcoRI–SalI PCR fragment corresponding to codons 365–430 and following 3’ sequences of RPI1 into pG4-5 or pG4-5 cut with EcoRI–Xhol to generate pFS1031 and pFS1032. The RIP1-C38 bait and prey constructs were made by ligating a shorter EcoRI–SalI fragment containing the last 38 codons of RPI1 into pEG202 or pG4-5 digested with EcoRI–Xhol to generate pFS1033 and pFS1034. The hCG1-C43 bait and prey constructs were obtained by inserting a 255 bp EcoRI–Xhol PCR fragment containing the last 43 codons followed by codons 121–230 and 364–430 of RI to generate pFS1035 and pFS1036. The hCG1 and hCG1-FG prey constructs were made by cloning EcoRI–Xhol PCR fragments of the whole coding region and codons 1–380, respectively, into pG4-5 cut with EcoRI–Xhol to generate pFS1037 and pFS1038. The RIP1-FG bait construct pFS476 contains codons 121–130 of Rip1 as an EcoRI–Xhol fragment in pG4-5. The YMR255w/GFD1 prey construct pFS1039 contains the whole coding region as an EcoRI–Xhol PCR fragment in pG4-5 EcoRI–Xhol. The DBPS/RAT8 prey plasmid pFS1040 contains the whole coding region as an EcoRI PCR fragment in pG4-5 EcoRI. The CRM1 and Rev bait as well as the Rev and RIP1-FG prey constructs have been described (Stutz et al., 1995; Neville et al., 1997).

**High copy suppressor screen**

The gle1-8 mutant allele had been identified earlier in a screen for mutants synthetically lethal with a RPI1 deletion. The mutant allele was amplified by PCR (High Fidelity Expand PCR, Boehringer MA) from yeast cultures grown at 25°C or shifted to 42°C were labelled with [35 S]Methionine-labelled full-length Gle1p and Gle1p C-terminus were obtained by coupled T7 transcription–translation in reticulocyte lysates (TrtT kit, Promega). T7 templates were generated by PCR. The template for full-length Gle1p was obtained by amplifying wild-type GLE1 with the 5’ primer OFS296 5'-GGGCCGAAATTTACAGCTACTAT-AGGGACACCATGAGTTGTTCGAGGTGTTT-3’, containing the T7 RNA polymerase promoter and sequences complementary to the 5’ end of the GLE1 coding region, and the 3’ primer OFS138 5’-CGATTTTCTGCACATCCCTTGATCGAGCCG-3’ complementary to a region of GLE1 downstream of the stop codon. The template for the Gle1p C-terminus was generated with the 5’ primer OFS385 5'-GGGCCGAAATTTACAGCTACTATAGGGACACCATGAGTTGTTCGAGGTGTTT-3’, containing the T7 promoter and sequences complementary to GLE1 starting from codon 257, and with the OFS138 3’ primer. A 2 µg aliquot of each T7 PCR template was used per 25 µl transcription–translation reaction.

**In vivo translation**

[15S]Metone-labelled full-length Gle1p and Gle1p C-terminus were obtained by cloning PCR fragments into vector pGex-4T-1 (Pharmacia Biotech). The constructs used to produce GST fusions in E.coli were obtained by cloning PCR fragments into vector pGex-4T-1 (Pharmacia Biotech). GST–RIP1-FG and GST–RIP1-C66 were made by inserting EcoRI–Xhol PCR fragments, corresponding to codons 121–230 and 364–430 of RPI1, respectively, into pGex-4T-1 EcoRI–Xhol to generate pFS410 and pFS507. GST–hCG1-C43 was obtained by inserting a 255 bp EcoRI–Xhol PCR fragment corresponding to the last 43 codons and 3’-untranslated sequences of hCG1 into pGex-4T-1 EcoRI–Xhol to generate pFS883. The complete coding sequences of DBPS/RAT8 and YMR255w/GFD1 were amplified as EcoRI and EcoRI-FXhol fragments, respectively, and cloned into pGex-4T-1 cut with the corresponding enzymes to generate pFS955 and pFS956.

**GST fusions and in vitro binding assay**

The constructs used to produce GST fusions in E.coli were obtained by cloning PCR fragments into vector pGex-4T-1 (Pharmacia Biotech). GST–RPI1–FG and GST–RPI1–C66 were made by inserting EcoRI–Xhol PCR fragments, corresponding to codons 121–230 and 364–430 of RPI1, respectively, into pGex-4T-1 EcoRI–Xhol to generate pFS410 and pFS507. GST–hCG1–C43 was obtained by inserting a 255 bp EcoRI–Xhol PCR fragment corresponding to the last 43 codons and 3’-untranslated sequences of hCG1 into pGex-4T-1 EcoRI–Xhol to generate pFS883. The complete coding sequences of DBPS/RAT8 and YMR255w/GFD1 were amplified as EcoRI and EcoRI-FXhol fragments, respectively, and cloned into pGex-4T-1 cut with the corresponding enzymes to generate pFS955 and pFS956.

The GST fusion constructs were transformed into E.coli strain BL21 (DE3) and fusion protein synthesis was induced overnight at 16°C in the presence of 0.5 mM isopropyl-β-thiogalactopyranoside (IPTG). After cell lysis, GST fusions were affinity purified on glutathione–agarose beads (Pharmacia) as described by the manufacturer. In vitro binding reactions contained 10 µg of GST fusion protein immobilized on 25 µl of packed glutathione–agarose beads and 1/10 of an in vitro transcription–translation reaction (2.5 µl) in 100 µl of universal binding buffer [20 mM HEPES pH 7, 10% glycerol, 0.1% bovine serum albumin (BSA), 0.1% Tween, 100 mM KAOc, 2 mM MgOAc, 5mM β-mercaptoethanol and 1 tablet/50 ml of protease inhibitors (Boehringer)]. Binding reactions were incubated for 1 h at 4°C on a turning wheel and washed three times with 500 µl of binding buffer. The beads were resuspended in 20 µl of 2X sample buffer, boiled and 1/4 of the binding reaction (10 µl) was fractionated on 10% polyacrylamide gels. To evaluate binding efficiency, 1/4 of the in vitro translated protein input (0.6 µl) was loaded in parallel. After Coomassie Blue staining, gels were dried and autoradiographed.

**In situ hybridization**

Strain FSY292 (rss1-37) was obtained by transforming the GLEI shuffle strain FSY197 with pVPD29 (pRSS1-37, LEU2/CEN) followed by selection on 5-FOA. Strains FSY57 (gle1-1, Rip1p low) and FSY58 (gle1-1, Rip1p normal) have been described (Stutz et al., 1997). Yeast strains were grown to OD600 = 0.5 at 25°C. Half of each culture was shifted to 37°C by the addition of 1 vol. of medium pre-heated to 49°C and incubated for 30 min at 37°C. Cells were fixed and processed for in situ hybridization with digoxigenin-labelled oligo(GT) probes on multi-well slides as described (Neville et al., 1997). Pictures were taken.
on a Zeiss axioplan 2 fluorescence microscope equipped with a cooled CCD camera and 100× and 63× objective lenses. Identical exposure times were used for comparable images, and composites were prepared using Adobe Photoshop.

**Immuon Electron microscopy localizations**

The ProA–Gle1p strains FSY251 and FSY252 were obtained by transforming pSW464 (Murphy and Wente, 1996) into the GLE1 shuffle strains FSY195 and FSY201 followed by selection on 5-FOA. The ProtA–Rip1p/Dbp5p strain FSY342 was obtained by transforming pCA5032 into the RAT8/DBP5 shuffle strain CSY512 followed by selection on 5-FOA. The ProtA–Rip1p strain FSY221 and FSY329 were obtained by transforming pFS829 and pFS923 into FSY17 (W303 ΔRIP1). The localization of the ProA–fusions was determined by using a pre-embedding labelling protocol essentially as described (Fahrenkrog et al., 1998). Epon embedded and pre-embedded for electron microscopy as described. The pre-embedding labelling conditions were adapted for each strain analysed. ProtA–Rip1p in ΔRIP1: 15 min zymolysate digest, 0.025% Triton X-100 extraction, 2.5 h antibody labelling; ProtA–Rip1p/C66 in ΔRIP1: 15 min zymolysate digest, 0.025% Triton X-100 extraction, 2.5 h labelling; ProtA–Rip1p in ΔRIP1 at 42°C: 10 min heat shock, 10 min zymolysate digest, 0.025% Triton X-100, 1 h labelling. ProtA–Gle1p in ΔGLE1 or ΔGLE1/ΔRIP1 background: 20 min zymolysate digest, 0.025% Triton X-100, 2.5 h labelling; ProtA–Rip1p in ΔRAT8 background: 15 min zymolysate, 0.025% Triton, 2.5 h labelling.

**In vivo localizations**

Strains FSY297 (ΔGLE1 pGLE1-GFP) and FSY298 (ΔGLE1/ΔRIP1 pGLE1-GFP) were obtained by transforming pFS1030 into the GLE1 shuffle strains FSY195 (gle1::HIS3, pGLE1 URA/CEN) or FSY201 (gle1::HIS3, ripl1::KAN8 pGLE1 URA/CEN), followed by selection on 5-FOA. Cells were transformed with a pRSAD2 plasmid to minimize vacuolar fluorescence. The gle1-8 allele in FSY216 and the wild-type GLE1 gene in W303 were chromosomally tagged with GFP using the pFA6a-GFP/S65T-ManMX6 module as described (Longtine et al., 1998) to generate FSY399 and FSY398, respectively. These strains were transformed with high copy plasmids and pRSAD2E. The temperature-sensitive rss1-37 (gle1) strain FSY249 was obtained by replacing wild-type GLE1 by the rss1-37 mutant allele (Saavedra et al., 1997) in strain CH1462 (Holm, 1993); the strategy was the same as the one used to generate FSY216 (see above). The rss1-37 allele of strain FSY249 was tagged with GFP using the pFA6a-GFP/S65T-ManMX6 module (Longtine et al., 1998) to generate FSY400. FSY249 was also crossed with strain CSY835 (rat/dhp5::HIS3, pCS835) (Snay-Hodge et al., 1998) to generate the haploid strain FSY401, a rss1-37 rat8/dbp5::HIS3 double mutant strain expressing Rat8p/Dbp5p–GFP from plasmid pCS835 (LEU2/CEN). All the strains were grown in selective or SD complete medium to early logarithmic phase, shifted for various times to 37°C, spun for short time and examined directly under a Zeiss axioplan 2 fluorescence microscope equipped with a 100× objective lens and a cooled CCD camera (Kappa). Pictures were taken as described for in situ hybridizations.

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**References**


