The small GTPase Gsp1p binds to the repeat domain of the nucleoporin Nsp1p

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The small GTPase Gsp1p of *Saccharomyces cerevisiae* and its homologue Ran play essential roles in several nuclear processes, such as cell-cycle progression, nuclear organization and nucleocytoplasmic traffic of RNA and proteins. Gsp1p/Ran is an abundant nuclear protein that interacts with different cytoplasmic and nuclear factors. Several of the previously identified Ran-binding proteins located at the nuclear-pore complex carry a specific Ran-binding domain. So far, direct interactions between the GTPase and other proteins have been mostly characterized in higher eukaryotes. Here we report that the yeast protein Gsp1p can directly bind to the nucleoporin Nsp1p *in vitro*. Nsp1p does not contain a Ran-binding domain and therefore represents a

distinct type of nucleoporin that associates with Gsp1p. We demonstrate that the middle domain of Nsp1p is sufficient to mediate this interaction. Importantly, we show that a conserved cluster of positively charged amino acid residues of Gsp1p located at positions 142–144 is essential for the binding reaction. Thus we have identified Nsp1p as a new candidate protein located at the nuclear pore complex of the yeast *S. cerevisiae* that interacts directly with Gsp1p. We further demonstrate that both Gsp1p and Nsp1p are components of larger protein complexes *in vivo*, supporting the idea that the association between both proteins takes place in growing cells.

INTRODUCTION

The yeast protein Gsp1p and its homologue Ran in higher eukaryotes are involved in various nuclear processes, including protein import, mRNA export and maintenance of the nuclear organization (reviewed in [1]). In *Saccharomyces cerevisiae* *GSP1* is an essential gene [2]; expression of dominant negative mutants of *GSP1* interferes with facilitated import of proteins into the nucleus and also results in nuclear accumulation of bulk poly(A⁺) mRNA [3]. Using *in vitro* binding assays, the possible role of Ran or Gsp1p in nuclear protein import was analysed in detail. These experiments support the idea that Ran/Gsp1p interacts with the β -subunit of importin, a heteromeric protein complex, which binds nuclear localization sequences (NLSs) and is essential for nuclear protein import (reviewed in [1]). Ran-GTP and also Ran-GDP (in complex with RanBP1) bind to the N-terminal portion of importin- β ; approx. 380 N-terminal residues of importin- β are required for this association [4,5]. Binding of Ran to importin- β requires a C-terminal acidic stretch of the GTPase, and a deletion of this conserved part of the protein abolishes binding of Ran-GTP and of Ran-GDP/RanBP1 [4].

Several of the Ran-binding proteins identified so far contain Ran-binding domains that recognize Ran-GTP; such domains are present in the mammalian protein RanBP2 (also termed Nup358), and in the nucleoporin Nup2p of *S. cerevisiae* [1]. Yeast proteins Yrb1p and Yrb2p also contain Ran-binding motifs; it was proposed that Yrb2p is not involved in nucleocytoplasmic traffic of macromolecules but rather plays a role for the nuclear GTPase cycle of Gsp1p [6]. Additional Gsp1p functions that are not related to nucleocytoplasmic trafficking have been suggested by recent data describing its physical

interaction with the mitotic control protein Dis3p, a polypeptide that lacks a Ran-binding motif [7]. Interestingly, Dis3p interacts with Gsp1p in its GTP- or GDP-bound state, and binding was also obtained for the ‘empty’ state [7].

Further studies describe a Ran mutant not affected in nuclear protein transport but exhibiting detrimental effects on cell growth [8]. Thus it was shown for yeast and mammalian cells that Gsp1p/Ran participates in essential cellular functions that are independent of nuclear protein transport [7,8].

Various proteins have been identified that associate with Gsp1p, and some of the polypeptides are likely to play a role in different pathways of nucleocytoplasmic traffic [1,9,10]. However, it is presently not understood how Ran/Gsp1p is involved in distinct nuclear reactions [1]. Since Gsp1p is involved in a variety of processes, it is conceivable that additional factors, yet to be identified, interact with the GTPase. The identification of such proteins will be essential for understanding the function of Ran/Gsp1p in different nuclear reactions. Some of these interacting components could be associated with nuclear pore complexes. This hypothesis is supported by indirect evidence for Ran-binding sites at the nuclear periphery in semi-permeabilized mammalian cells that are not identified to date [11]. In these cells both Ran-GTP and Ran-GDP associated with the nuclear envelope, indicating that binding cannot solely be attributed to the Ran-binding motif of RanBP2. Importantly, the interactions were independent of importin- β , which is in line with the interpretation that Ran binds to components at the nuclear pore complex that are presently not characterized. Consistent with this interpretation are results for a derivative of Ran that carries the mutation T24N. This mutant protein does not interact with RanBP2; however, it is partially associated with the nuclear

Abbreviations used: DTT, dithiothreitol; HA, epitope derived from the influenza haemagglutinin protein; HSA, human serum albumin; MBP, maltose-binding protein; NLS, nuclear localization sequence; SV40, simian virus 40.

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membrane [12]. These data provide further, albeit indirect, evidence that other components of the nuclear envelope bind Ran.

We now demonstrate that the yeast homologue of Ran, i.e. Gsp1p, directly interacts with the nucleoporin Nsp1p *in vitro*. We have identified the middle repeat domain of Nsp1p as a binding site for the GTPase. A conserved cluster of positively charged amino acid residues of Gsp1p is essential for this interaction. Mutation of this cluster to uncharged amino acid residues abolished binding to Nsp1p. Moreover, when the mutant derivative of Gsp1p was located in growing yeast cells, most of the protein was cytoplasmic, demonstrating that the basic cluster is essential for its accumulation in the nucleus. In addition, Gsp1p and Nsp1p are both present in high-molecular-mass complexes *in vivo*. On the basis of these results, we propose that the repetitive domain present in the nucleoporin Nsp1p provides binding sites for Gsp1p that previously have not been described. Association with Nsp1p depends on the presence of positively charged amino acid residues of the GTPase at positions 142–144.

EXPERIMENTAL PROCEDURES

Chemical cross-linking of proteins

Yeast cells were grown overnight at 30 °C in yeast nitrogen base containing 2 % (w/v) glucose and 0.25 % (w/v) casamino acids. Cells were collected by centrifugation and washed in PBS/20 % (v/v) glycerol/1 mM EDTA/0.02 % (w/v) NaN₃ (buffer A). Cells resuspended in buffer A containing 1 mM PMSF and a cocktail of protease inhibitors (aprotinin, antipain, chymostatin, leupeptin and pepstatin, each at 1 µg/ml final concentration) were lysed with glass beads. Crude lysates were centrifuged for 3 min at 14900 *g* (microfuge) and incubated with 0.2 mM *N*-succinimidyl 3-(2-pyridyldithio)propionate (Pierce, Rockford, IL, U.S.A.) for 30 min at room temperature. After addition of SDS to 1 % (final concentration), extracts were incubated with 2 mM *N*-ethylmaleimide for 10 min at room temperature. Samples were further analysed by gel filtration or immunoaffinity purification.

Immunoprecipitation of cross-linked proteins

Protein G-Sepharose (Pierce) was pre-incubated with PBS (pH 7.0)/5 mM EDTA/0.02 % (w/v) NaN₃/1 % (v/v) Triton X-100/0.1 % (w/v) SDS/1 mg/ml BSA/5 % (w/v) skim milk (buffer B) for 1 h at room temperature. Pre-incubated protein G-Sepharose was loaded with monoclonal mouse antibodies in buffer B overnight at 4 °C. Beads were collected by centrifugation and washed in buffer B. Cross-linked proteins were diluted into buffer B to give a final concentration of 1 % (v/v) Triton X-100 and 0.1 % (w/v) SDS. Samples were pre-incubated with blocked protein G-Sepharose for 1 h at room temperature. Supernatants of pre-treated samples were incubated either for 4 h at room temperature or overnight at 4 °C. Beads collected by centrifugation were washed in buffer B (three times), in buffer B without skim milk (three times) and once in PBS/5mM EDTA/0.02 % (w/v) NaN₃. Final sediments were boiled in gel-loading buffer for 10 min. Beads were removed by centrifugation (5 min at 14900 *g*), and supernatants were probed by Western blotting with rabbit polyclonal antibodies.

In vitro binding of Gsp1p to Nsp1p

Generation and purification of fusion proteins containing different portions of Nsp1p have been described previously [13]. Fusion proteins carrying *Escherichia coli* maltose-binding protein

Figure 1 Diagram of fusion proteins containing different regions of the nucleoporin Nsp1p

Fusion proteins carrying *E. coli* MBP and different parts of Nsp1p were used in the present study. Sequences of the N-terminal domain of Nsp1p are shown in grey, the repetitive middle region is represented by vertical hatching, and the C-terminal domain is depicted in black. Numbering of the amino acids denotes the positions in authentic Nsp1p.

(MBP) and regions of Nsp1p were synthesized in *E. coli* and purified by affinity chromatography. Numbers given for these proteins indicate the amino acid residues of authentic Nsp1p: MBP-Nsp1(28–798) contains MBP fused to amino acid residues 28–798 of Nsp1p. All other constructs were named accordingly. Different fusion proteins used in this study are depicted in Figure 1.

For the conjugation of synthetic peptides to human serum albumin (HSA), peptides CSKPAFSFGAK containing the nonapeptide repeat of Nsp1p (underlined residues), CTPPKKKRKV [simian virus 40 (SV40)-NLS] and CTPPKTKRKV (SV40*-NLS) were covalently coupled to HSA as described previously [14].

Generation of a triple mutation in GSP1

The triple mutation of *GSP1* at positions 142–144 changing Arg-Lys-Lys to Gly-Met-Ile (R142G K143M K144I, termed gsp1^{GMI}) was created with the U.S.E. Mutagenesis Kit (Pharmacia Biotech, Baie d'Urfé, PQ, Canada) (P. Belhumeur, G. Barbès-Morin, M. Hájazi and F. Fortin, unpublished work).

Creation of histidine-tagged versions of Gsp1p and gsp1p^{GMI}

Histidine-tagged versions of Gsp1p and gsp1p^{GMI} were generated by cloning PCR products beginning with codon 4 of the coding sequence. Amplified fragments were digested with *Bam*HI and *Pst*I, which cleave within the primer sequences, and were cloned into pQE11 (Qiagen, Chatsworth CA, U.S.A.), which was digested with *Bam*HI and *Pst*I. Production and purification of histidine-tagged proteins was carried out according to the manufacturer's instructions. Renaturation of histidine-tagged derivatives of Gsp1p was carried out by extensive dialysis against PBS/1 mM EDTA/0.5 mM dithiothreitol (DTT)/protease inhibitors (0.1 µg/ml each aprotinin, antipain, chymostatin, leupeptin and pepstatin). The concentration of urea was stepwise reduced, i.e. dialysis buffers contained 1 M, 0.1 M, 10 mM and 0 mM urea. The final dialysis was against PBS with several changes of the buffer. Dialysed samples were spun for 15 min at 25700 *g* (4 °C) to remove aggregates. Purified proteins in the supernatant fraction were radioiodinated with IodoBeads (Pierce) as described previously [14].

Dot-blot analysis of Gsp1p binding to Nsp1p

Equal molar amounts of purified fusion proteins or protein conjugates were immobilized on nitrocellulose. Non-specific

binding sites were blocked in 5 % (w/v) BSA in 25 mM Tris (pH 7.5)/25 mM KCl/50 mM NaCl (blocking buffer) for 1 h at room temperature. Blocked filters were incubated overnight at 4 °C with 1 μ Ci of radioiodinated Gsp1p in blocking buffer followed by two washes at room temperature (10 min and 5 min) in blocking buffer without BSA. Dried filters were exposed to Kodak X-OMAT AR films. For competition of Gsp1p binding to Nsp1p, blocked filters were preincubated with 100 μ g/ml SV40-HSA in blocking buffer (4 h at room temperature) before addition of the radio-iodinated proteins. Controls were preincubated in the presence of 100 μ g/ml HSA under identical conditions. Further treatment of filters followed the procedure described above.

Gel filtration

Protein samples (500 μ l each) were loaded on to Superose 12HR (Pharmacia) equilibrated with PBS/1 mM EDTA/0.02 % (w/v) NaN₃. The column was eluted in the same buffer at 0.3 ml/min, and 1 ml fractions were collected. Aliquots of each fraction were probed with different antibodies in dot blots or analysed by SDS/PAGE and staining of the gel. Marker proteins for gel filtration (Sigma, Oakville, ON, Canada) run under identical conditions were bovine thyroglobulin (molecular mass 669 kDa), horse spleen apoferritin (443 kDa), sweet potato β -amylase (200 kDa), yeast alcohol dehydrogenase (150 kDa), BSA (66 kDa) and carbonic anhydrase (29 kDa).

SDS/PAGE and Western blotting

Proteins were separated by SDS/PAGE in 12 % gels and blotted to nitrocellulose. Filters were blocked with 5 % (w/v) skim milk in PBS (1 h at room temperature) and incubated with primary antibodies overnight at 4 °C.

Filters were washed for 1 h in PBS/0.1 % (v/v) Tween-20 and incubated in PBS/5 % (w/v) skim milk with secondary antibodies coupled to horseradish peroxidase (1 h, room temperature). Affinity-purified secondary antibodies (Jackson Immuno-Research, Mississauga, ON, Canada) to mouse IgG were pre-adsorbed against rabbit IgG, and antibodies to rabbit IgG were pre-adsorbed against mouse IgG. After treatment with secondary antibodies, filters were washed for 1 h in PBS/0.1 % (v/v) Tween-20; bound antibodies were detected with chemiluminescence reagent (NEN, Boston, MA, U.S.A.).

Immunoaffinity chromatography

IgG fractions of rabbit antibodies directed against Nsp1p or pre-immune serum were coupled to CarboLink (Pierce) according to the manufacturer's instructions. Columns (1 ml volume) were incubated with cross-linked proteins in buffer A containing 1 % (v/v) Triton X-100 and 0.1 % (w/v) SDS for 1 h at room temperature. Columns were washed with 8 ml of buffer A/1 % (v/v) Triton X-100/0.1 % (w/v) SDS and 8 ml of buffer A. Proteins were eluted in 7 ml of 0.1 M glycine, pH 2.8, and neutralized with 1 M Tris/HCl, pH 9.4. Aliquots of eluted material were precipitated with 10 % (w/v) tricarboxylic acid for 40 min on ice and analysed by SDS/PAGE and Western blotting.

Binding of guanosine nucleotides to wild-type and mutant HA-tagged Gsp1p

Protein G-Sepharose was loaded overnight at 4 °C with anti-HA antibodies in buffer B without skim milk but containing 5 mg/ml BSA and 10 % (v/v) glycerol (buffer C). All incubations were carried out with gentle agitation. Beads were washed twice in

buffer C before addition of yeast lysates. Controls were treated identically with omission of antibodies or by loading with unrelated monoclonal antibodies directed against β -galactosidase. Yeast cells were lysed with glass beads in buffer C/1 mM DTT as described above. Lysates were diluted 1:5 in buffer C, preincubated with protein G-Sepharose for 15 min on ice and subsequently incubated with protein G-Sepharose preloaded with or without antibodies overnight at 4 °C. For these experiments, an excess of antigen was used to ensure that the same amounts of wild-type and mutant protein were bound to the resin. Beads were washed three times in buffer C and three times in 25 mM Tris/HCl (pH 7.4)/150 mM potassium acetate/5 mM MgCl₂/10 % (v/v) glycerol/1 mM DTT/5 mg/ml BSA (buffer D). Immunoprecipitates were incubated in buffer D containing 2.5 μ Ci of [γ -³²P]GTP for 30 min at room temperature. Samples were washed three times in buffer D containing 100 μ M ATP, and radioactivity bound to the resin was determined. All samples were run in duplicate. Binding of [³H]GDP to immunopurified HA-Gsp1p or HA-gsp1p^{GMI} was analysed essentially as described previously [7].

Filter-binding assays for analysis of GTP and GDP binding to wild-type and mutant forms of Gsp1p

Wild-type and mutant HA-Gsp1p were immunopurified from yeast cell extracts as described above. Final sediments were resuspended in gel-loading buffer and incubated for 1 h at room temperature. Samples were centrifuged to remove Sepharose beads and loaded on 12 % gels. Proteins were transferred to nitrocellulose, and filters were treated essentially as described previously [7], except that washes were carried out in binding buffer containing 100 μ M ATP. Filters were exposed to Reflection Autoradiography film (NEN) for [γ -³²P]GTP. For [³H]GDP binding, bands corresponding to the position of Gsp1p were cut out, and radioactivity bound to the filter was determined by scintillation counting.

RESULTS

The small GTPase Gsp1p associates with the nuclear envelope, and this interaction is in part mediated by nucleoporins that contain Ran-binding sites or indirectly by association with other factors that dock at the nuclear envelope. Several analyses provided indirect evidence for the presence of additional factors that bind Ran/Gsp1p, which are located at the nuclear envelope and not characterized. We now demonstrate that for *S. cerevisiae* the nucleoporin Nsp1p associates with Gsp1p. The use of purified components enabled us to show that Nsp1p can directly interact with the small GTPase.

Wild-type Gsp1p binds to the nucleoporin Nsp1p *in vitro*

We have used immobilized fusion proteins carrying *E. coli* MBP and different regions of the yeast nucleoporin Nsp1p to test their capacities to bind wild-type Gsp1p and the mutant derivative termed gsp1p^{GMI} *in vitro*. To this end, gene fusions encoding different regions of Nsp1p were expressed in bacteria and purified by affinity chromatography. Purified fusion proteins carrying different regions of Nsp1p migrated to the expected position when analysed by SDS/PAGE [13]. A schematic representation of fusion proteins used in this study is shown in Figure 1.

To determine whether Gsp1p interacts with Nsp1p, we generated a histidine-tagged version of the GTPase, termed His6-Gsp1p. His6-Gsp1p was synthesized in bacteria and purified by metal-affinity chromatography. Purified protein was renatured by extensive dialysis against buffer containing EDTA, conditions

Figure 2 Gsp1p binds to the repetitive domain of Nsp1p

Equal molar amounts of purified fusion proteins, protein conjugates or control proteins were immobilized on nitrocellulose. Each polypeptide was applied as duplicates. Before the addition of radio-iodinated Gsp1p, filters were pre-incubated with unlabelled HSA, SV40-HSA or SV40*-HSA, each at 100 μ g/ml.

that remove GDP from GDP-Ran complexes [7]. Dialysed protein was radio-iodinated for the subsequent binding studies.

Comparable amounts of purified proteins carrying different regions of Nsp1p were immobilized on nitrocellulose filters and probed with radiolabelled His6-Gsp1p. As shown in Figure 2, MBP-Nsp1(28–798), i.e. the MBP fusion carrying amino acid residues 28–798 of Nsp1p, bound His6-Nsp1p. Binding was also detected for MBP-Nsp1(110–592), a fusion protein containing a small portion of the N-terminal domain of Nsp1p and the middle repetitive portion of Nsp1p. By contrast, neither MBP alone nor fusion proteins harbouring the amino acids 28–166, 110–166 or 609–823 associated with His6-Gsp1p. From these data we conclude that His6-Gsp1p interacts specifically with the repetitive region of Nsp1p.

We tested also whether nonapeptide repeats present in the middle domain of Nsp1p supported the association of Gsp1p. To achieve this, the peptide CSKPAFSFGAK was covalently coupled to HSA (HSA-peptide) and immobilized to nitrocellulose. However, no association of His6-Gsp1p with these repetitive elements of Nsp1p could be detected.

A basic cluster of Gsp1p is important for binding to Nsp1p

It has previously been shown that the middle domain of Nsp1p can associate with clusters of basic amino acid residues. Specifically, a peptide carrying the NLS of SV40 T-antigen (TPPKKKRKV) binds to Nsp1p with high affinity [13]. By contrast, a mutant derivative of this peptide, termed SV40*-NLS (TPPKTKRKV, where the bold T is the mutation) does not bind to Nsp1p.

To determine whether positively charged amino acid residues present in Gsp1p could play a role for its interaction with Nsp1p, we analysed the effect of different competitors on the binding reaction. SV40-HSA represents the wild-type NLS of SV40 T-antigen covalently coupled to HSA; SV40*-HSA carries a mutant derivative of the NLS described above. SV40-HSA competed efficiently for binding of Gsp1p to Nsp1p or the middle repeat domain. By contrast, SV40*-HSA was an ineffective competitor

Figure 3 The mutant gsp1p^{GMI} does not bind to Nsp1p

Immobilized fusion proteins containing different regions of Nsp1p, control proteins and protein conjugates were incubated with comparable amounts of radio-iodinated Gsp1p (wild-type) or gsp1p^{GMI} (mutant) as detailed in the Experimental procedures section.

(Figure 2). These results are consistent with the idea that Gsp1p and Nsp1p associate via ionic interactions and that positively charged amino acid residues play an important role for this interaction.

To characterize further the association between Gsp1p and Nsp1p, we took advantage of a mutant derivative of Gsp1p that carries the triple mutation R142G, K143M, K144I, termed gsp1p^{GMI}. A histidine-tagged version of the mutant protein was generated, synthesized in bacteria, affinity purified and radio-iodinated as described for His6-Gsp1p (see above). When analysed in the dot-blot assay, the mutant gsp1p^{GMI} failed to associate with Nsp1p or its middle domain (Figure 3). This lack of binding does not simply reflect the instability and degradation of purified gsp1p^{GMI}; analysis of the radiolabelled protein by denaturing gel electrophoresis, and autoradiography revealed that the mutant protein migrated at the expected position.

From these data we conclude that mutation of the basic cluster of Gsp1p prevented its interaction with Nsp1p. Results presented in Figures 2 and 3 support the hypothesis that Gsp1p can associate with the nucleoporin Nsp1p; the middle domain containing nonapeptide repeats is sufficient for this interaction. A triple mutation that destroys a conserved basic cluster of Gsp1p abolishes this interaction. Since purified components have been used to test the association between Gsp1p and Nsp1p, it can be assumed that Gsp1p directly binds to Nsp1p.

Gsp1p and Nsp1p are present in high-molecular-mass complexes *in vivo*

To determine whether the interaction between Gsp1p and Nsp1p observed for purified proteins reflects their possible association *in vivo*, we used two different approaches, i.e. gel filtration of crude yeast extracts and immunopurification of protein complexes containing Gsp1p or Nsp1p respectively.

To analyse the presence of Gsp1p and Nsp1p in high-molecular-mass complexes, yeast cells harbouring HA-tagged

Figure 4 Gsp1p and Nsp1p are components of high-molecular-mass complexes *in vivo*

Top: distribution of Gsp1p, Nsp1p, Srp1p and Prp20p during gel filtration. Yeast cells synthesizing HA-Gsp1p were lysed under native conditions, and extracts were separated by gel filtration as detailed in the Experimental procedures section. Aliquots of each fraction were tested in duplicates with different antibodies as indicated. Molecular masses of gel-filtration markers (kDa) are shown at the top of the panel. Bottom: protein profile for fractions of the gel filtration. Aliquots of different fractions shown in the top panel were separated by SDS/PAGE and revealed with a combined Coomassie Brilliant Blue/silver stain. Positions of marker proteins (kDa) are given at the left-hand side. Numbers at the top of the panel indicate the position of gel-filtration markers (kDa).

versions of Gsp1p were lysed under native conditions and subjected to gel filtration. The HA-tagged version of Gsp1p can functionally replace the wild-type protein *in vivo*. Each fraction was tested for the presence of HA-Gsp1p and Nsp1p by probing with antibodies recognizing the HA epitope or Nsp1p. Gsp1p was detected in fractions representing complexes of 500–700 kDa, indicating that most of the cellular HA-Gsp1p is not a free monomeric protein but is associated with other cellular components (Figure 4, top). All of the fractions positive for Gsp1p also contained Nsp1p. The nucleoporin Nsp1p was present in additional fractions of lower molecular masses that were not enriched for Gsp1p (Figure 4, top). Similar results were obtained for cells synthesizing the untagged wild-type version of Gsp1p (not shown).

We further examined the distribution of Srp1p and Prp20p during gel filtration, other components involved in nucleocytoplasmic traffic of macromolecules. Although there was some

Figure 5 Immunopurification of protein complexes containing Gsp1p and Nsp1p

Top: cross-linked protein complexes were immunopurified with immobilized rabbit polyclonal antibodies against Nsp1p (lane 1) or pre-immune serum (lane 2). Cross-links of purified complexes were cleaved with DTT, and comparable volumes were analysed by Western blotting with mouse monoclonal antibodies recognizing the HA tag (lanes 1 and 2). Alternatively, cross-linked proteins were immunoprecipitated with antibodies to the HA tag (lane 3) or with unrelated mouse monoclonal antibodies against β -galactosidase (lane 4). Immunoprecipitates were probed in Western blots with polyclonal rabbit antibodies against Nsp1p (lanes 3 and 4). Positions of marker proteins (kDa) are given at the left-hand side. Bottom: cross-linked protein complexes prepared from strains expressing HA-Gsp1p (wild type) or HA-gsp1p^{GMI} (mutant) were incubated with immobilized antibodies against Nsp1p (AS) or pre-immune serum (PS). Immunopurified protein complexes were cleaved, and comparable amounts of material were analysed in parallel. Western blotting was carried out with mouse monoclonal antibodies directed against the HA tag. Positions of marker proteins (kDa) are given at the left-hand side.

overlap with the presence of these polypeptides and Gsp1p, the distribution was clearly different. Peak fractions for Srp1p and Prp20p eluted at about 150–200 kDa, i.e. at lower molecular masses than observed for fractions containing the bulk of Gsp1p (Figure 4, top).

Analysis of the protein profile obtained for gel filtration revealed multiple protein bands in each fraction (Figure 4, bottom). Since the presence of both Gsp1p and Nsp1p in complexes of the same molecular mass does not necessarily reflect a functional interaction between these proteins, it was important to obtain more direct evidence for the potential association between Gsp1p and Nsp1p. Prolonged incubation of cell extracts resulted in dissociation of high-molecular-mass complexes containing Nsp1p and Gsp1p, and we stabilized protein interactions by reversible cross-linking with the cleavable cross-linker *N*-succinimidyl 3-(2-pyridyldithio)propionate. Cross-linked proteins were subjected to immunopurification with antibodies against Nsp1p or the HA tag. Cross-links were cleaved, and samples were analysed by Western blotting with antibodies to the HA tag or Nsp1p. Protein complexes purified with antibodies against Nsp1p contained wild-type Gsp1p and vice versa (Figure 5, top). The specificity of these immunopurifications was demonstrated by use of pre-immune serum or unrelated antibodies against β -galactosidase. As expected, for these control

Table 1 Binding of guanine nucleotides to HA-Gsp1p and HA-gsp1p^{GMI}

HA-tagged Gsp1p and gsp1p^{GMI} were immunopurified from yeast and incubated with radiolabelled guanine nucleotides as described in the Experimental procedures section. Radioactivity associated with immobilized HA-Gsp1p, HA-gsp1p^{GMI} (Immunopurified) or non-specific binding in control experiments (Non-specific) is shown in c.p.m. for [γ -³²P]GTP and in d.p.m. for [³H]GDP. Specific binding was defined as the difference between binding to immunopurified proteins and non-specific binding. Numbers given for bound radioactivity are means of duplicate determinations.

GTPase ...	Radioactivity	
	HA-Gsp1p	HA-gsp1p ^{GMI}
[γ - ³² P]GTP binding		
Non-specific	846	1103
Immunopurified	4951	4108
Specific	4105	3005
[³ H]GDP binding		
Non-specific	1369	1907
Immunopurified	2250	2809
Specific	881	902

experiments precipitation of Nsp1p or Gsp1p was not observed (Figure 5, top).

We have also generated extracts from cells expressing HA-gsp1p^{GMI} followed by cross-linking and immunopurification; co-purification of the mutant GTPase and Nsp1p was not obtained (Figure 5, bottom).

In summary, results described for the association between Gsp1p and Nsp1p are consistent with those obtained for *in vitro* studies and verify that the small GTPase Gsp1p and the nucleoporin Nsp1p are components of the same protein complexes *in vivo*.

The mutant protein gsp1p^{GMI} binds GTP and GDP

To begin to define the defect of the mutant gsp1p^{GMI}, we tested its ability to bind GTP and GDP. When wild-type and mutant proteins synthesized in *E. coli* were tested for the association with radiolabelled nucleotides, we found that the loading efficiency was very low. A similar observation has been reported previously by others [7]. In contrast, HA-tagged versions of the wild-type and mutant protein purified from crude yeast extracts bound radiolabelled nucleotides. Since gsp1p^{GMI} does not cover a deletion of the wild-type copy of Gsp1p, we transformed cells harbouring the chromosomal wild-type allele with genes encoding either HA-Gsp1p or HA-gsp1p^{GMI}. Crude extracts prepared from these cells were subjected to immunopurification with antibodies specific for the HA epitope. An excess of antigen was used to ensure that similar amounts of wild-type and mutant proteins are purified. Immunoprecipitates were incubated with [γ -³²P]GTP or [³H]GDP as detailed in the Experimental section; control experiments were carried out under identical conditions without antibodies or with unrelated monoclonal antibodies. Specific binding of radioactive nucleotides was defined as the difference between radioactivity obtained for samples containing anti-HA antibodies and for control experiments. Examples of results for these experiments are shown in Table 1.

Immunopurified HA-Gsp1p as well as HA-gsp1p^{GMI} bound [γ -³²P]GTP, and binding to the mutant protein was approx. 70 % of the association observed for HA-Gsp1p (Table 1). Immunoprecipitated HA-Gsp1p and HA-gsp1p^{GMI} also interacted with radiolabelled GTP in filter-binding assays (results not shown).

Moreover, both immunopurified HA-Gsp1p and HA-gsp1p^{GMI} bound radiolabelled GDP when tested by filter-binding assays. It should be noted that binding of [³H]GDP was somewhat variable, and some of the experiments gave higher binding to HA-gsp1p^{GMI} than to wild-type HA-Gsp1p. Taken together, our data demonstrate that neither GTP nor GDP binding was abolished for the mutant protein HA-gsp1p^{GMI}.

DISCUSSION

Gsp1p is an essential protein in *S. cerevisiae* that plays a major role in nucleocytoplasmic traffic of macromolecules and also in the structural organization of the nucleus. Previous analyses in higher eukaryotes have indicated that the homologous protein Ran can associate with the nuclear envelope via different binding partners, including RanBP2, importin- β , NTF2 (nuclear transport factor 2) and other factors [1]. Several novel factors that may mediate direct or indirect association of Ran/Gsp1p with the nucleus have recently been described [9,10].

In our present study we have demonstrated an interaction between Gsp1p and the nucleoporin Nsp1p. Thus we have identified a yeast protein located at the nuclear pore complex that does not carry a Ran-binding domain but associates with Gsp1p.

From our data we conclude that (a) Gsp1p can directly bind to the nucleoporin Nsp1p. (b) The repetitive domain of Nsp1p mediates this association. (c) Binding to Nsp1p is dependent on positively charged amino acid residues provided by Gsp1p. Our results indicate that this cluster of basic residues is also critical for the nucleocytoplasmic distribution of Gsp1p *in vivo* (P. Belhumeur, G. Barbès-Morin, M. Hájazi and F. Fortin, unpublished work).

Nsp1p belongs to the FXF family of nucleoporins, which contain multiple copies of such sequence elements (reviewed in [15]); this protein is organized into three domains. The essential C-terminal portion comprising amino acids 604–823 contains heptad repeats that are involved in interactions with other nucleoporins [16]. In contrast, the middle portion of Nsp1p is expected to be accessible to binding of other factors. The middle domain (amino acids 166–603) carries 22 copies of nonapeptide repeats that are separated by highly charged spacer regions. We have recently shown this part of Nsp1p to recognize sequence elements that contain clusters of positively charged amino acid residues [13].

In vitro binding assays described here demonstrate that Gsp1p and the repeat domain of Nsp1p interact directly. This interaction does not require any cofactors, and it is abolished by the destruction of a positively charged cluster of Gsp1p at positions 142–144 of Gsp1p. It is conceivable that this binding reaction is supported by ionic interactions between Gsp1p and Nsp1p. Spacer regions within the repeat domain are highly charged and contain clusters of acidic residues that could contribute to this binding. In contrast, repeat elements contain uncharged or basic amino acid residues that may not participate in the association. Our results showing that repeat elements of Nsp1p conjugated to HSA are not sufficient to bind Gsp1p support this idea.

We have searched *S. cerevisiae* databases with different spacer sequences derived from the middle repeat domain of Nsp1p. Yeast proteins that contain similarities to one of the spacer sequences include nucleoporins Nup1p, Nup49p and Nup145p. Like Nsp1p, Nup2p belongs to the FXF family of nucleoporins, whereas Nup49p and Nup145p are members of the GLFG family [15]. It has to be determined whether these nucleoporins also provide binding sites for Gsp1p that are distinct from Ran-binding domains.

Previous experiments have demonstrated the importance of the conserved C-terminal stretch of acidic residues of Gsp1p/Ran for interactions with importin- β [5]. We now show that a basic cluster of Gsp1p plays an essential role in the interaction of Gsp1p with Nsp1p. Importantly, a derivative carrying mutations in this cluster accumulates in the cytoplasm *in vivo* (P. Belhumeur, G. Barbès-Morin, M. Héjazi and F. Fortin, unpublished work). Sequence analysis of homologues of Gsp1p, such as Gsp2p, Spi1p or TC4, shows that this basic cluster has been conserved during evolution, indicating its importance for function of the GTPase. According to the crystal structure determined for Ran-GDP, this conserved sequence RKK is part of an α -helical region [17]. Introduction of the helix-breaking residue glycine and the β -sheet-promoting residue isoleucine are expected to modify the secondary structure of gsp1p^{GMI} in this region as compared with the wild-type protein.

The interaction of Nsp1p with clusters of positively charged amino acid residues was previously shown to be dependent on both charge and additional structural elements [13]. These requirements explain why the mutant GTPase with altered charge and secondary structure fails to interact with Nsp1p. Importantly, although RKK in α -helix 4 is conserved between Gsp1p and its homologues in higher eukaryotes, this sequence element is absent from the small GTPase Ras. Therefore, it is not required for the general function of small GTPases but is likely to be necessary for more specific interactions of Ran/Gsp1p with other factors.

When immunopurified from yeast extracts, HA-Gsp1p and HA-gsp1p^{GMI} bound radiolabelled GTP and GDP, demonstrating that removal of the basic cluster at residues 142–144 of Gsp1p does not abolish binding of guanine nucleotides. This result is consistent with the prediction that the α -helical region of Ran containing the RKK sequence is not involved in binding of GDP-Mg²⁺ [17].

Immunoprecipitation of protein complexes demonstrated that wild-type Gsp1p and Nsp1p can be copurified and therefore are components of the same high-molecular-mass complexes *in vivo*. The mutant protein gsp1p^{GMI}, however, could not be copurified with Nsp1p. These results are consistent with the interpretation that wild-type Gsp1p interacts with Nsp1p, whereas gsp1p^{GMI} cannot perform this association. However, we cannot rule out the possibility that a reduced stability of gsp1p^{GMI} *in vivo* prevents us from detecting such complexes.

Release of immunopurified yeast HA-Gsp1p from immobilized protein G did not yield sufficient quantities of protein to detect binding of nucleotide-loaded HA-Gsp1p to Nsp1p. This prevented us from directly testing whether GTP- and GDP-bound Gsp1p show different affinities for Nsp1p. However, the characteristics for binding of Gsp1p to Nsp1p resembled those for the interaction between Gsp1p and Dis3p [7]. Thus the association of Gsp1p with Nsp1p seems to occur even under conditions that favour an 'empty' state of Gsp1p and therefore may be independent of nucleotide binding.

During the revision of this manuscript it was reported that an interaction between Gsp1p and Nsp1p was observed in using a two-hybrid screen [18]. This provides further support for the idea

that Gsp1p and Nsp1p can interact. Taken together, different independent lines of evidence point to the fact that Gsp1p and Nsp1p are components of protein complexes *in vivo*. It should be noted, however, that data obtained from co-immunopurification or from two-hybrid screens do not demonstrate the direct interaction of these proteins (see for example [10]). In contrast, results reported here for binding of Gsp1p and Nsp1p *in vitro* show that direct binding between these two proteins can occur.

Our results do not address the question of whether the interaction between Gsp1p and Nsp1p are directly involved in nucleocytoplasmic transport of proteins or RNA. Unique effectors of Ran/Gsp1p, as exemplified by Yrb2p, may play a role in different nuclear functions of the small GTPase. It is therefore conceivable that direct binding of Gsp1p to Nsp1p is relevant to nuclear functions unrelated to transport across the nuclear pore. Future experiments will have to dissect these reactions and the role for binding between Gsp1p and Nsp1p for different nuclear functions.

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