Purification of Protein A-tagged Yeast Ran Reveals Association with a Novel Karyopherin β Family Member, Pdr6p*

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The small GTPase Ran (encoded by GSP1 and GSP2 in yeast) plays a central role in nucleocytoplasmic transport. GSP1 and GSP2 were tagged with protein A and functionally expressed in a gsp1 null mutant. After affinity purification of protein A-tagged Gsp1p or Gsp2p by IgG-Sepharose chromatography, known karyopherin β transport receptors (e.g. Kap121p and Kap123p) and a novel member of this protein family, Pdr6p, were found to be associated with yeast Ran. Subsequent tagging of Pdr6p with green fluorescent protein revealed association with the nuclear pore complexes in vivo. Thus, functional tagging of yeast Ran allowed the study of its in vivo distribution and interaction with known and novel Ran-binding proteins.

Ran belongs to the Ras superfamily of small guanine nucleotide-binding proteins and is highly conserved within the eukaryotic kingdom (for review, see Refs. 1, 2). In Saccharomyces cerevisiae, Ran is encoded by the GSP1 and GSP2 genes (3, 4). Although the gene products are nearly identical, gsp2 disruption does not lead to any apparent phenotype. The lethal gsp1 null mutation can be complemented by overexpression of GSP2, suggesting that the two isoforms are functionally equivalent but differently regulated (3).

Mainly two unique features distinguish Ran from the other members of the Ras superfamily: its high abundance in the cell and its nuclear location. In agreement with these features, Ran was shown to play an essential role in nucleocytoplasmic transport, a hallmark of eukaryotic cells (for review, see Refs. 5–8). During transport Ran is continuously shuttling between nucleus and cytoplasm and switching between a GDP-bound and a GTP-bound state. The main accessory proteins, which assist Ran during transport, are NTF2, a RanGDP-binding protein at the nuclear envelope required for nuclear import of Ran (9), RanGAP1 in (Rna1p) in the nucleus (which are then disassembled after GTP hydrolysis on Ran by RanGAP1 (in the cytoplasm). The latter step requires in addition a cytoplasmic RanGTP-binding protein called RanBP1 (Yrb1p in S. cerevisiae; Refs. 10, 11). These nuclear transport receptors (also referred to as karyopherins), which include both import (also called importins) and export receptors (also called exportins), share structural similarity in their amino-terminal RanGTP-binding domain and constitute a protein family, the founding member of which is importin β (Kap95p in S. cerevisiae), the subunit of the heterodimere binding complex for proteins containing a classical nuclear localization sequence (for review, see Refs. 12, 13).

Based on the pleiotropic phenotypes of alterations in the Ran-GTPase cycle, it was proposed that Ran may be involved in cellular processes other than nucleocytoplasmic transport (for review, see Ref. 1). The recent identification of a centrosomal Ran-interacting protein, RanBPM (14), and the implication of Ran in microtubule organization in the absence of nuclear trafficking (15–17) suggest a direct role of Ran at least in some of these processes.

To study the dynamic intracellular localization of Ran and its interaction with partner proteins in the living cell, Gsp1p and Gsp2p were tagged with GFP* and ProtA, respectively. Despite the bulky tags, Gsp1p and Gsp2p fusion proteins were functional. These tools allowed us to study the in vivo localization of Gsp1p and to identify a novel member of the karyopherin β transport receptor family called Pdr6p.

EXPERIMENTAL PROCEDURES

Yeast Strains—Strains used in this study are listed in Table I. For construction and cultivation, standard procedures were applied. A GSP1 shuffle strain (Y1009) was constructed by transformation of diploid yeast strain YMO106 (gsp1::HIS3/GSP1; Ref. 4) with plasmid pRS316-GSP1 and subsequent sporulation and tetrad dissection. His+, Ura+ spores were selected and checked for disruption at the GSP1 locus by PCR. Shuffle strains carrying additional rna1-1 or prp20-1 mutant strains (18), respectively. For construction of a pdr6::HIS3 disruption strain, the entire open reading frame of pDR6 was replaced by the HIS3 gene. The diploid RS453 was transformed with the pdr6::HIS3 gene disruption construct which was

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1 The abbreviations used are: GFP, green fluorescent protein; ProtA, protein A; PCR, polymerase chain reaction; kb, kilobase; PAGE, polyacrylamide gel electrophoresis; TEV, tobacco etch virus.
obtained by PCR using the HIS3 gene as a template and matching primers bearing 5’ and 3’ untranslated region sequences from the PDR6 gene (5’-CTTCAACAGAATTAGCTCTTCGTACGGGAGAAA-
TCTTTAACCCTGGCCCCTCCTAG-3’ and 5’-CACAGATTTGAATGCAC-
CATAATAGCTCTTACCTTACCATGTCAGAAGACAC-3’). His+ transformants were analyzed by PCR for the correct protA::HIS3 gene disruption. All four spores of complete tetrad from such transformants were viable, showing that PDR6 is not essential.

**Plasmids**—For plasmid construction standard procedures (19) were applied. pRS16-GSP1 (2.9 kb) was constructed from Yep532-GSP2 (3) by subcloning a 2.9-kb SalI-SacI fragment into pRS16 (CEN/ARS/ URA3); pRS14-GSP1 (2.9 kb) was constructed in the same way using pRS314 (CEN/ARS/TRP1) as a recipient vector. For construction of pRS314-GSP1 (1.6 kb), pRS314-GSP1 (2.9 kb) was digested with NcoI and SacI, thereby removing a 1.3-kb NcoI-SacI fragment containing the SECT2 gene, and religated after filling in with Klenow DNA polymerase. pUN100-ProtA-GSP1 (pNOPPATA-GSP1) was described previously (20). For pUN100-GFP-GSP1, the GSP1 open reading frame was cloned as a 0.7-kb ProtA restriction fragment from pNOPPATA-GSP1 into a pUN100-NOP1::GFP:ADH1 cassette. The resulting fusion protein contains a TEV cleavage site after the GFP moiety. pNOPGPFAAGSP1 (pUN100-ADE2-NOP1::GFP:TEV:ADH1), containing a ProtA cloning site between the GFP-TEV and the ADH1 terminator in a pUN100 plasmid backbone in which the LEU2 gene was replaced by the ADE2 gene.

For pNOPPATA-PDR6, the whole open reading frame of PDR6 was amplified from pRS14-PDR6 (obtained by inserting a genomically derived SalI-SmaI PCR fragment containing the whole PDR6 gene (with 5’ and 3’ untranslated regions) into SalI-SmaI-opened pRS314) with the following primers: 5’-GGTTTTTTCATTAGCTTCCATCATCG-
GAAGTGTTG-3’ and 5’-GGGGGATCCATCTCCTTCGTAAGTTG-
GAAGTGTTG-3’). The PCR product was digested with NciI and BamHI and inserted into the corresponding sites of pNOPPATA open reading frame from pRS134-ProtA-PDR6 into the ProtA-cutter vector Yplac112-NOP1::GFP:ADH1 (2μ, TRP1). Note that in this construct the 8-carboxyl-terminal residues from Pdr6p are missing.

**Affinity Purification of ProtA-TEV-Gsp1p, ProtA-TEV-Gsp2p, ProtA-TEV-Nup85, and ProtA-TEV-Pdr6p—Affinity purification of ProtA-tagged fusion proteins by IgG-Sepharose chromatography was done as described earlier (22), with the following modifications. Strain gsp1::HIS3 complemented by ProtA-TEV-GSP1 or ProtA-TEV-GSP2 and strain pdr6::HIS3 complemented by ProtA-TEV-PDR6p were grown in YPD medium, respectively, to approximately 10^6 cells/mL. Cells were harvested by centrifugation and spheroplasted. Three grams of spheroplasts were lysed in 20 mL of lysis buffer containing 150 mM KOAc, 20 mM Hepes, pH 7.0, 2 mM Mg(OAc)2, 0.1% Tween 20, 1 mM dithiothreitol, and 1 volume of beads was mixed with 1 volume of the lysis buffer and 1 volume of IgG-Sepharose beads (Amersham Pharmacia Biotech). After washing the beads with 10 mL of the lysis buffer, the beads were transferred to spin columns, and 1 volume of MS2-PAGE, and 10 μL were used for Western blot analysis. Affinity purification of ProtA-TEV-Gsp1p from the gsp1::HIS3/nmt1-1 strain (Table I) was performed in a similar way, but cells were first grown at 23°C and shifted for 2 h to 37°C before harvesting and biochemical analysis.

**Antibodies**—The generation of the anti-Yrb1p antisera will be described elsewhere. A specific antiserum against Gsp1p was obtained by immunization of rabbits with affinity-purified MBP-Gsp1p-G21V. The other antisera used in this study were kindly provided by the following people: anti-Gsp1p by Dr. J. Becker (Max-Planck-Institut for Molecular Physiology, Dortmund, Germany); anti-Kap95p by Dr. D. Görlich (Zentrum für Molekularbiologie Heidelberg, Heidelberg, Germany); anti-Cdc48p by P. Silver (Dana Farber Cancer Institute, Boston, MA).

**Mass Spectrometric Analysis**—The protein bands of interest were excised from the one-dimensional SDS-polyacrylamide gel and processed for mass spectrometric analysis as described (22, 25). Tryptic peptide maps were recorded on a Bruker REFLEX matrix-assisted laser desorption ionization time-of-flight mass spectrometer equipped with delayed ion extraction. Tryptic autolysis products were used for internal calibration to give an accuracy, on average, better than 30 ppm. Proteins were uniquely identified using the Peptide Search software package and a nonredundant protein sequence data base (maintained by C. Sander, European Molecular Biology Laboratory/European Bioinformatics Institute).

**Intracellular Localization of GFP-Gsp1p and GFP-Pdr6p—Intracellular localization of GFP-Gsp1p and GFP-Pdr6p was analyzed in a Zeiss Axioskop fluorescence microscope. Pictures were recorded with a Alexa Fluor 488-conjugated charge-coupled device camera. Digital pictures were sometimes processed by digital confocal imaging applying deconvolution to images, available as a module within the software program Openlab (Improvision, Coventry, UK).

**RESULTS AND DISCUSSION**

**Yeast Ran (Gsp1p and Gsp2p) Tagged with ProtA Is Functional**—The protein A (ProtA) tagging strategy was successfully applied in the past to affinity purify a single step a protein of interest from yeast and identify its interacting partners (26). We therefore intended to construct a functional yeast Ran (Gsp1p or Gsp2p) tagged with ProtA to identify novel Ran-interacting proteins. For this purpose, it was important to control whether the large ProtA tag (15 kDa) fused to Gsp1p or Gsp2p (27 kDa) did impair the in vivo function of this small GTPase. We inserted the corresponding GSP1 and GSP2 fusion genes into a centromeric (ARS/CEN/URA3) plasmid and tested for complementation of the otherwise nonviable gsp1::HIS3 mutant. pUN100-ProtA-GSP1 and pUN100-ProtA-GSP2 complemented the otherwise nonviable gsp1::HIS3 null mutant with only a slight growth retardation compared with a strain with authentic GSP1 on centromeric pRS314 (Fig. 1A). This shows that ProtA-tagged Gsp1p/Gsp2p constructs are functional in vivo. Furthermore, we tested whether GFP-tagged Gsp1p and Gsp2p are functional. Similar to ProtA-Gsp1p, GFP-Gsp1p could also complement the gsp1::HIS3 cells (Fig. 1A). As expected, this functional GFP-Gsp1p/Gsp2p exhibits a dual location in living cells, both in the nucleus and cytoplasm, with a higher concentration inside the nucleus (Fig. 1B). The same distribution was found for GFP-Pdr6p (data not shown). This intracellular GFP-Gsp1p location is consistent with previous findings using indirect immunofluorescence microscopy (28).

**Affinity Purification of ProtA-Gsp2p Reveals Association with Pdr6p, a Novel Family Member of the Karyopherin β-like Transport Factors**—GST-tagged Gsp1p has been recently affinity-purified from yeast, but no other co-isolating bands than Yrb1p were reported (27). It is possible that GST-Gsp1p is not fully functional in vivo. We therefore tested whether our ProtA-tagged versions of Gsp1p and Gsp2p, which are functionally active, allow the co-enrichment of known and new Ran-binding proteins after affinity purification. To facilitate release of native yeast Ran from the affinity matrix, the viral TEV protease

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2 M. Künzler, J. Trueheart, C. Sette, E. C. Hurt, and J. Thorner, submitted for publication.
3 B. Senger, unpublished data.
Association of Protein A-tagged Yeast Ran with Pdr6p

Purification of ProtA-Gsp1p and ProtA-Gsp2p reveals association with Pdr6p and other karyopherin β-like transport factors. A, affinity purification of ProtA-Gsp1p and ProtA-Nup85p by IgG-Sepharose chromatography and release of Gsp1p and Nup85p, respectively, by TEV-mediated proteolytic cleavage. Lane 1, protein standard; lane 2, TEV-cleaved and -purified Gsp1p; lane 3, TEV-cleaved and -purified Nup85p. Indicated by arrowheads are Gsp1p and Nup85p, respectively, and by a square the karyopherin β-like transport factors (Kaps; also see B). Affinity purification of ProtA-Gsp2p by IgG-Sepharose chromatography and release of the Gsp2p by TEV-mediated proteolytic cleavage. Shown is a silver-stained SDS-polyacrylamide gel of the Gsp2p purification, from which bands were excised and used for the mass spectrometric analysis. The indicated bands were identified by matrix-assisted laser desorption ionization. C, affinity purification of ProtA-Gsp1p from RNA1 gsp1::HIS3 or rna1-1 gsp1::HIS3 cells shifted for 2 h to 37 °C. Gsp1p was released from the beads by TEV-protease. Supernatants and TEV eluates were analyzed by SDS-PAGE and Coomassie blue staining (left panel) and Western blot analysis using the corresponding antibodies (right panel). Lane 1, eluate from RNA1 cells; lane 2, eluate from RNA1-1 cells; lane 3, supernatant from RNA1-1 cells; lane 4, supernatant from RNA1 cells. Indicated in the Coomassie blue-stained gel (left panel) are the positions of karyopherin β-like transport factors (Kaps) Ssa1p, Yrb1p, and Gsp1p.

cleavage site was engineered between the ProtA and Gsp1 and 2 moieties (see “Experimental Procedures”). When ProtA-Gsp1p was isolated by IgG-Sepharose chromatography from the gsp1::HIS3-disrupted strain and released from the beads by TEV protease cleavage, Coomassie blue-stainable amounts of Gsp1p were recovered in the eluate. When the purified Gsp1p eluate was analyzed by SDS-PAGE and silver staining, several bands in the high molecular mass range became visible (Fig. 2A). In particular the bands at ~100–130 kDa appear to be specific, because since they are not seen in the purified Nup85p eluate, which served as a negative control and was isolated in a similar way by IgG-Sepharose chromatography and TEV proteolytic cleavage (Fig. 2A).

When ProtA-Gsp2p was affinity-purified and Gsp2p was released from the IgG-Sepharose beads by the TEV protease, the same prominent co-purifying bands in the range from 100 to 130 kDa were noticed (Fig. 2B). Some of these bands could be identified by mass spectrometric analysis and were shown to be known Ran-binding proteins (e.g. Kap123p and Kap121p, which are karyopherin β-family members involved in nuclear import of ribosomal subunits) (13). Interestingly, one of these bands in the 120-kDa range turned out to be Pdr6p, a protein previously implicated to play a possible role in pleiotropic drug resistance (Fig. 2B and Ref. 28). Although Pdr6p was reported to be distantly related to the karyopherin β-like transport receptors (29), binding to RanGTP could not be shown. Our data demonstrate that Pdr6p is indeed a bona fide member of the karyopherin β-family. According to the yeast “Kap” nomenclature, Pdr6p is called Kap122p. Additional bands in the Gsp2p eluate were Ssa1p and Erp1p (elongation factor 2). However, it is not clear whether these proteins specifically bind to Gsp2p or are contaminants. We have observed that Ssa1p tends to associate with several other ProtA-tagged fusion proteins when affinity-purified under similar conditions (data not shown). To test for the presence of other karyopherins, which were not detected by mass spectrometry, the Gsp2p eluate was analyzed by SDS-PAGE and Western blotting. This revealed that Kap95p and Kap104p, but not Mtr10p/Kap111p, are present in the eluate (data not shown). This result is consistent with the recent finding that Mtr10p has a low affinity for RanGTP (22).

Because after biochemical isolation of ProtA-tagged yeast Ran from wild-type cells, Ran-GTP may be largely hydrolyzed to Ran-GDP by Ran1p, we sought to stabilize the Gsp1p-GTP form by isolating ProtA-Gsp1p from rna1-1 mutant cells. Under these conditions, GTP hydrolysis by Ran GAP (Rna1p) should be inhibited, and a higher amount of RanGTP-binding proteins may co-purify with Gsp1p. A haploid rna1-1 gsp1::HIS3 strain was constructed, which was complemented by plasmid-borne ProtA-GSP1 (Table I). Before ProtA-Gsp1p affinity purification, this strain was shifted for 2 h to the nonpermissive temperature. Purified Gsp1p was analyzed by SDS-PAGE and silver staining (Fig. 2C). As anticipated, Gsp1p when isolated from rna1-1 cells contained a higher amount of karyopherin β members. This was confirmed by Western blot analysis using antibodies against the exportins Los1p and Cse1p and the importin Kap95p (Fig. 2C). The strongest enrichment was observed for Cse1p. In addition, the RanGTP-binding protein Yrb1p was found to be significantly enriched with purified Gsp1p-GTP (Fig. 2C). In contrast, Gsp1p isolated from prp20-1 cells did not show an enhanced binding of these RanGTP-binding proteins (data not shown). Thus, biochemical purification of Gsp1p and Gsp2p from yeast allows the co-enrichment of several karyopherin β members and other Ran-binding proteins.

**GFP-Pdr6p Localizes Predominantly to the Nuclear Envelope**—To study the in vivo role of Pdr6p, which we found to be physically associated with yeast Ran, we disrupted the PDR6 gene. Haploid pdr6::HIS3 progeny are viable, showing that PDR6 is not essential for cell growth. No apparent growth defect was observed in pdr6Δ cells when grown at various temperatures (e.g. 23, 30, and 37 °C; data not shown). Because Pdr6p was implicated to be involved in pleiotropic drug resistance (for review, see Ref. 28), we analyzed whether drugs such as cycloheximide cause different growth properties of pdr6Δ cells compared with PDR6Δ cells. However, this was not the case (data not shown). When other stress pathways were tested in pdr6Δ cells (e.g. thermotolerance, oxidative stress such as H₂O₂ and amino acid analogues), the cells also appeared to be normal compared with PDR6Δ cells (data not shown). Therefore, it remains to be shown whether Pdr6p is at all involved in pleiotropic multidrug resistance or other stress pathways. To find out whether Pdr6p not only binds to Gsp1p but also associates, like other karyopherin β-like transport receptors, with nuclear pore proteins, we examined its subcellular location. Pdr6p was tagged with GFP and analyzed by fluorescence microscopy. GFP-Pdr6p exhibits a punctate nuclear envelope staining typical for a nuclear pore complex association (Fig. 3, upper panel). This is in agreement with the finding that GFP-Pdr6p co-clusters with nuclear pores in nup133Δ cells (Fig. 3, lower panel). In addition, some GFP-Pdr6p can also be detected
mutants were constructed between the pdr6::HIS3 strain and several mutant or knockout karyopherin alleles (e.g., pdr6::HIS3/los1::HIS3, pdr6::HIS3/mtr10-7, pdr6::HIS3/kap121-1, pdr6::HIS3/kap123::HIS3, pdr6::HIS3/snm5::HIS3, pdr6::HIS3/cse1-1, pdr6::HIS3/xpo1-1, and pdr6::HIS3/msn5::TRP1) and found to be viable.\textsuperscript{4} This suggests that Pdr6p either fulfills a completely nonessential function in yeast (at least under laboratory growth conditions), or that Pdr6p is highly redundant with other karyopherin \(\beta\)-family members. It could also be possible that Pdr6p is involved in a regulated nuclear import or export pathway and thus only required under special growth or stress conditions. This was recently found for some of the uncharacterized yeast \(\beta\)-like karyopherins, which were shown to be involved in regulated nuclear import and export mechanisms under conditions of stress or under distinct metabolic stages (30–33).

In conclusion, yeast Ran was functionally modified with both the GFP and ProtA tag, which allowed the study of the steady state in vivo location and its interaction with partner proteins. This enabled us to identify a novel karyopherin \(\beta\)-like transport receptor, and this system will serve in the future as a useful tool to further study the cell biological and biochemical aspects of this essential regulator of eukaryotic nucleocytoplasmic transport.

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