The nuclear pore complex (NPC) gates the only known conduit for molecular exchange between the nucleus and cytoplasm of eukaryotic cells. Macromolecular transport across the NPC is mediated by nucleocytoplasmic shuttling receptors termed karyopherins (Kaps). Kaps interact with NPC proteins (nucleoporins) that contain FG peptide repeats (FG Nups) and altogether carry hundreds of different cargoes across the NPC. Previously we described a biochemical strategy to identify proteins that interact with individual components of the nucleocytoplasmic transport machinery. We used bacterially expressed fusions of glutathione S-transferase with nucleoporins or karyopherins as bait to capture interacting proteins from yeast extracts. Forty-five distinct proteins were identified as binding to one or several FG Nups and Kaps. Most of the detected interactions were expected, such as Kap-Nup interactions, but others were unexpected, such as the interactions of the multisubunit Nup84p complex with several of the FG Nups. Also unexpected were the interactions of various FG Nups with the nucleoporins Nup2p and Nup133p, the Gsp1p-GTPase-activating protein Rna1p, and the mRNA-binding protein Pab1p. Here we resolve how these interactions occur. We show that Pab1p associates nonspecifically with immobilized baits via RNA. More interestingly, we demonstrate that the Nup84p complex contains Nup133p as a subunit and binds to the FG repeat regions of Nups directly via the Nup84p subunit. Binding of Nup85p to the GLFG region of Nup116p was quantified in vitro \((K_D = 1.5 \ \mu M)\) and was confirmed in vivo using the yeast two-hybrid assay. We also demonstrate that Nup2p and Rna1p can be tethered directly to FG Nups via the importin Kap95p-Kap60p and the exportin Crm1p, respectively. We discuss possible roles of these novel interactions in the mechanisms of nucleocytoplasmic transport.

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The study of nucleocytoplasmic trafficking in the yeast Saccharomyces cerevisiae is well suited for a proteome-scale analysis of protein interactions as more than 50 proteins (Fig. 1A) participate in the nucleocytoplasmic transport of hundreds of cargoes. The nuclear pore complex (NPC) is the site of macromolecular exchange between the nucleus and the cytoplasm of eukaryotic cells (Fig. 1A, center). In S. cerevisiae it is composed of 30 nucleoporins (Fig. 1A, left columns) that are presumed to be present in eight or more copies per NPC (1). Transport of cargoes across the NPC is facilitated by a family of mobile receptors termed karyopherins (Kaps) (Fig. 1A, right columns), which bind targeting sequences in cargo proteins (nuclear localization signals and nuclear export signals) and interact with nucleoporins that contain FG (phenylalanine-glycine) peptide repeats (FG Nups). It is generally assumed that karyopherins translocate across the NPC by facilitated diffusion whereby Kap-cargo complexes associate and dissociate with FG Nups as they transit in either direction across the NPC (2). In S. cerevisiae there are eleven importins (Kaps responsible for import of cargoes), six exportins (Kaps responsible for export of cargoes), and one transportin (Kaps that carries cargo bidirectionally across the NPC). The small GTPase Gsp1p (termed Ran in vertebrates) and its effectors (Fig. 1A, bottom) play a key role in nucleocytoplasmic transport (3, 4). When Gsp1p-GTP binds to importins it disrupts their interaction with cargo and Nups, whereas binding of Gsp1p-GTP to exportins enhances their binding to cargoes and Nups.

Knowledge of all protein interactions that occur during nucleocytoplasmic transport could greatly enhance our understanding of the transport mechanism. To obtain an extensive map of interactions that may occur at the NPC, we previously developed a glutathione S-transferase (GST)-based affinity-capture assay to identify proteins that bind to individual Nups and Kaps (4, 5). 135 proteins were identified, comprising 45 different proteins captured by one or multiple Nups and Kaps. Here we include two additional affinity-capture assays using the FG nucleoparin Nup159p and the exportin Cse1p as baits. We also extend our general experimental strategy to answer

1 The abbreviations used are: NPC, nuclear pore complex; Kap, karyopherin; Nup, nucleoporin; FG Nup, nucleoporin that contains FG peptide repeats; GLFG region, region of nucleoporin that contain GLGF peptide repeats; GST, glutathione S-transferase; CID, collision-induced dissociation; LC-MSMS, liquid chromatography-tandem mass spectrometry; MALDI, matrix-assisted laser desorption/ionization; TOF, time-of-flight; MS, mass spectrometry; AA, amino acid(s); FPLC, fast protein liquid chromatography.
FIG. 1. A, diagram of the nuclear pore complex (center) and the proteins that participate in nucleocytoplasmic transport in S. cerevisiae. Nups are the structural components of the NPC; these include the FG Nups, the non-FG Nups (which lack FG peptide repeats), and the nuclear pore membrane proteins (POMs). Kaps bind and translocate cargo across the NPC; these include the importins (Kaps dedicated to nuclear import), the exportins (Kaps dedicated to nuclear export), and the transportins (Kaps that carry cargo in both directions across the NPC). The Gsp1p GTPase (Ran in vertebrates) and its effectors (Rna1p is a GTPase-activating protein; Fpr20p and Mog1p are guanine nucleotide exchange factors; Yrb1p is a guanine nucleotide dissociation inhibitor) impart directionality to the transport process by modulating the interaction between Kaps and Nups. B, flow chart of our proteomic strategy for deciphering and characterizing networks of protein interactions at the nuclear pore complex. Cells are grown under any of a variety of conditions and are lysed. Soluble proteins are separated from insoluble proteins by differential centrifugation. Proteins in the soluble pool are mixed with Sepharose beads coated with a GST fusion (GST-nucleoporin, GST-karyopherin, etc.). After washing beads, specifically bound proteins are eluted with salt and resolved by SDS-PAGE. Individual proteins are then identified by mass spectrometric analysis. Identified proteins are produced in recombinant form and tested for their ability to bind directly to the GST fusion in the absence of other proteins or in the presence of adapter proteins. Direct interactions detected in vitro are then confirmed in vivo using a yeast two-hybrid assay.

EXPERIMENTAL PROCEDURES

Construction of Recombinant Proteins in Escherichia coli and Their Purification—All recombinant proteins used (except Gsp1p and subunits of the Nup42p complex) were expressed as GST fusions using the vector pGEX-2TK (Amersham Biosciences), which incorporates a thrombin cleavage site at the fusion junction as well as a specific kinase site that remains with the N terminus of the fusion partner after cleavage. Genes encoding proteins of interest (or fragments thereof) were amplified from S. cerevisiae genomic DNA (Promega) using PCR with oligos engineered to contain the appropriate restriction endonuclease sites flanking the gene of interest. For the Nups we amplified mostly the FG repeat regions: codons 1–640 of Nup100p (Nup100p/H9004C), full-length Nup42p, codons 165–715 of Nup116p (Nup116p/H9004N/H9004C), codons 441–881 of Nup159p (Nup159p/H9004N), and codons 332–1076 of Nup1p (Nup1p/H9004). The PCR products were ligated into vector pGEX-2TK and transformed into the E. coli strain BL21 Codon Plus (Novagen). His-tagged Gsp1p was expressed and purified from E. coli as described previously (4). The hexameric Nup42p complex and its various subcomplexes were purified as described previously (6). The Rna1p D240A mutant (7) was generated using the QuikChange™ Site-Directed Mutagenesis kit (Stratagene) and the pGEX-2TK vector containing the RNA1 gene as template.

In general, E. coli strains containing the desired fusion protein were grown in 1 liter of 2× YT medium (16 g of tryptone, 10 g of yeast, 5 g of NaCl) with ampicillin (0.1 mg/ml) and 2% glucose at 37 °C to A600
Proteins were purified in FPLC sizing columns equilibrated with 20 mM Hepes, pH 6.8, 150 mM KOAc, 2 mM Mg(OAc)₂, and protease inhibitors (0.1 mg/ml phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 2 μg/ml aprotinin, and 2 μg/ml leupeptin) to a final volume of 40 ml. Yeast were lysed in a French press cell (SLM Instruments). Cell debris were removed by centrifugation at 30,000 × g for 15 min at 4 °C, and Tween 20 (0.1%) was added to the collected supernatant. Recombinant proteins in supernatants were purified on glutathione-coated Sepharose beads (Amersham Biosciences) according to the manufacturer's instructions. When needed, the GST moiety was cleaved off by incubation with thrombin (Calbiochem) at 25 °C (1 NIH unit of thrombin/100 μg of GST fusion) for controlled times. A molar excess of hirudin (Calbiochem) was added to neutralize thrombin, and free GST was removed using glutathione-Sepharose beads. Proteins were further purified in FPLC sizing columns equilibrated with 20 mM Hepes, pH 6.8, 150 mM KOAc, 2 mM Mg(OAc)₂. Aliquots were supplemented with 0.1% Tween 20 and 1 mM dithiothreitol, frozen in liquid nitrogen, and stored at −70 °C.

Preparation of **S. cerevisiae Extracts**—GYP60 yeast (**MATα**, his4−579, leu2−3, 112 trp1−289, ura3−52, pep1−, pep4−:URA3, gat2) was grown in 1 liter of YPD medium (50 g of yeast extract, 100 g of bactotryptone, 2% glucose) at 30 °C to an **A**₅₀₀ = 2.0. Yeast were harvested by sedimentation at 5,000 × g for 10 min and resuspended in 20 ml Hepes, pH 6.8, 150 mM KOAc, 250 mM sorbitol, 2 mM Mg(OAc)₂, and protease inhibitors (0.1 mg/ml phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 2 μg/ml aprotinin, and 2 μg/ml leupeptin) to a final volume of 40 ml. Yeast were lysed in a French press cell, and cell debris were removed by sedimentation at 30,000 × g for 30 min at 4 °C. The supernatant was desalted in a Sephadex G-25 fine column (Amersham Biosciences) pre-equilibrated in 20 ml Hepes, pH 6.8, 150 mM KOAc, 2 mM Mg(OAc)₂. Pooled fractions were supplemented with 0.1% Tween 20, 2 mM dithiothreitol, and protease inhibitors. Aliquots were frozen in liquid nitrogen and stored at −70 °C.

**Affinity-Capture and Solution Binding Assays**—All assays were performed in binding buffer (20 mM Hepes, pH 6.8, 150 mM KOAc, 2 mM Mg(OAc)₂, 1 mM dithiothreitol, 0.1% Tween 20). For each experiment, glutathione-coated Sepharose beads (Amersham Biosciences) and an **E. coli** extract containing the desired GST fusion were incubated for 15 min at 4 °C (1–5 μg of GST fusion/10 μl of packed beads). Beads were then collected at 2,000 × g for 30 s and washed repeat-
edly by six cycles of resuspension and sedimentation. Equal aliquots of beads were then incubated with yeast extracts (affinity-capture assay) or purified proteins (solution binding assay) for various times at 4°C. In the affinity-capture assay, beads were sedimented and washed six times as before, and bound proteins were eluted with 1 M NaCl. Eluted proteins were precipitated using trichloroacetic acid (7.2%) and sodium deoxycholate (0.015%) (trichloroacetic acid–sodium deoxycholate), and pellets were resuspended in 2× Laemmli sample buffer. Proteins that resisted salt extraction from the beads were eluted with Laemmli sample buffer. In the solution binding assay, beads were sedimented, and the unbound proteins were collected. Beads were subsequently washed twice, and the bound proteins were eluted with Laemmli sample buffer. All proteins were resolved by SDS-PAGE and visualized with Coomassie Blue.

Size Fractionation of Proteins Captured by Nup100pC—An affinity-capture experiment was performed as above, using GST-Nup100pC as bait, except that the reaction was scaled up 10-fold (i.e. 100 mg of protein in yeast extracts and 50 μg of GST-Nup100pC in 100 μl of packed beads). Captured proteins were eluted with 1 ml of 1 M NaCl, and the salt concentration was adjusted to 150 mM by dilution. The protein sample was concentrated in a Centricon-10 unit (Millipore) and injected into a Superdex 200 FPLC sizing column (Amersham Biosciences). Fractions were collected and used in subsequent immunoprecipitation experiments or were analyzed by SDS-PAGE.

Sample Preparation for Mass Spectrometric Analysis—Protein bands were excised from gels and digested either robotically using a ProPrep Investigator (Genomic Solutions) (8) or manually (9) using trypsin in 25 mM NH₄HCO₃ for 4 h. Robotically digested samples were desalted automatically by the ProPrep using micro C18 ziptips (Millipore) and eluted directly onto the MALDI target using 1.8 μl of matrix solution (1:5 dilution of a saturated solution of α-cyano-4-hydroxycinnamic acid in 60% acetonitrile, 0.2% trifluoroacetic acid). As for manually digested samples, the peptides were extracted first with H₂O followed by organic extraction with 50% acetonitrile, 5% formic acid. The combined extracts were concentrated by vacuum centrifugation to ~5 μl (9). A portion was used for analysis by LC-MSMS.

Protein Identification by MALDI-TOFTOF MS—All MS and MSMS data were acquired in an automated mode using a 4700 Proteomics Analyzer (Applied Biosystems). First, a MALDI-TOF MS spectrum was
acquired of each spot (2000 shots/spectrum). The five most abundant peaks in the spectrum of each spot were automatically selected for CID analysis (7500 shots/spectrum). The 4700 system was fitted with a neodymium:yttrium aluminum garnet (Nd:YAG) frequency tripled laser operating at a wavelength of 354 nm and a laser repetition rate of 200 Hz. For CID data, 1 keV collision energy was used with air as the collision gas. Postacquisition, data are automatically base line-corrected and smoothed, and peaks are stored in an Oracle™ data base. Peak lists from all MS and MSMS spectra were automatically extracted out of the Oracle™ data base and submitted for batch MS-Fit and batch MS-Tag data base searching (prospector.ucsf.edu) (9).

**Protein Identification by Nano-LC-MSMS—** High pressure liquid chromatography separation was performed on an Ultimate instrument equipped with Famos Autosampler ( Dionex) at a flow rate of 300 nl/min utilizing a Pepmap column (75-μm inner diameter × 150 mm). The solvent system consisted of solvent A (water, 0.1% formic acid) and solvent B (acetontitrile, 0.1% formic acid). Peptides were separated by a gradient of 5–35% B over 30 min. MS data were acquired using a QSTAR Pulsar LC-MSMS system (Applied Biosystems) in information-dependent acquisition mode. The most intense multiply charged ion observed in each 1-s survey MS spectrum was automatically selected for a 4-s MSMS acquisition. All MSMS spectra were submitted for automated data base searching using Mascot (www.matrixscience.com). MSMS spectra with ambiguous identifications from the Mascot search were further submitted to MS-Tag (prospector.ucsf.edu) for unambiguous identification.

**Immunoprecipitation Experiments—** Protein A-coated Sepharose beads (50 μl) were washed in phosphate-buffered saline and incubated with affinity-purified anti-Nup85p antibodies for 1 h at room temperature. Beads were washed twice and resuspended in 200 μl sodium borate, pH 9.0 and 0.1% Tween 20. To cross-link the antibody to protein A, beads were mixed with 20 μl dimethyl pimelimidate and incubated for 30 min at room temperature. Beads were washed, and the reaction was stopped by incubation with 1 M Tris-Cl, pH 8.0, 0.1% Tween 20 for 2 h at room temperature. Beads were stored in phosphate-buffered saline containing 0.5% sodium azide at 4 °C. Immunoprecipitation experiments were conducted by incubating the anti-Nup85p-coated beads (5 μl) with Nup84p complexes either purified or in Superox 200 fractions. After 2 h at 4 °C unbound proteins were collected and precipitated with trichloroacetic acid–sodium deoxycholate. Beads were then washed, and bound proteins were extracted with Laemmli sample buffer. Proteins were resolved by 8% SDS-PAGE and visualized by silver staining.

**Protein Affinity Measurements—** Affinities were calculated using purified radiolabeled Nup85p in a solution binding assay essentially as described previously (10). Purified recombinant Nup85p was phosphorylated at an engineered site in its N terminus using bovine heart kinase and [γ-32P]ATP (PerkinElmer Life Sciences) as described in the Amersham Biosciences GST Handbook. GST-Nup116pΔNΔC (the GLFG region; AA 165–715) was immobilized on beads and incubated with increasing amounts of radiolabeled Nup85p at 4 °C in 20 mM Hepes, pH 6.8, 150 μM KOAc, 2 mM Mg(OAc)2, 1 mM diithiothreitol, 0.1% Tween 20, protease inhibitors, and 1 μg/ml bovine serum albumin. Following incubation, beads were sedimented and washed, and bound Nup85p was quantified by scintillation counting. Binding curves were fit to the data using GraphPad Prism™ software (Biosoft) as described previously (10).

**Yeast Two-hybrid Assay—** NUP85 was amplified using PCR from S. cerevisiae genomic DNA and was ligated into the GAL4 activation domain vector pADT7 of a two-hybrid kit (Clontech). The gene fragment encoding the GLFG repeats of Nup116p (codons 165–715) was ligated into the GAL4 DNA binding domain vector pGBK7T7 (Clontech). The plasmids were then transformed into yeast strain AH109 (Clontech) and plated on drop-out culture medium lacking Leu and Trp (to maintain the two-hybrid plasmids). The resulting colonies were replated onto drop-out medium lacking the amino acids Leu, Trp, and His and adenine to select for two-hybrid interaction (yeast strain AH109 expresses HIS and ADE genes in response to two-hybrid interaction). Growth was examined after 5 days.

**METHODOLOGY**

A Simple Biochemical Strategy for Deciphering Networks of Protein Interactions—The experimental strategy featured in our proteomic analysis of nucleocytoplasmic transport is the use of *E. coli*-produced GST fusions of Nups and Kaps immobilized on Sepharose beads to capture proteins in yeast extracts that exhibit an affinity toward the immobilized “bait” protein. We refer to this technique as the “affinity-capture assay.” After a captured protein is identified by mass spectrometry, it is produced in recombinant form, purified, and tested for its ability to bind directly (or not) to the GST fusion bait it was originally isolated on. Subsequently the identified protein can be used as bait itself to capture its interacting proteins from cell extracts, repeating the cycle. In that way, a web of protein interactions can be pursued in any direction, and a distinction can be made between direct and indirect protein interactions.

The Affinity-Capture Assay (Revisited)—We have previously described results obtained with the affinity-capture assay (4, 5, 8) but omitted details regarding the development, advantages, and disadvantages of the assay itself. Here we discuss
this methodology in more detail and include as examples two additional affinity-capture experiments with the FG region of the nucleoporin Nup159p and the exportin Cse1p as baits.

To produce the recombinant GST-fusions we used the pGEX-2TK vector (Amersham Biosciences) that encodes a thrombin cleavage site and a kinase site at the linker region between GST and the fusion partner. These two features are essential for subsequent analyses of protein interactions detected (see below). The extended linker containing the thrombin site allows for efficient cleavage of GST from the fusion protein via thrombin treatment. In this respect, the pGEX-2TK vector is superior to other pGEX vectors, which have shorter linker sequences. Conveniently the kinase site remains with the fusion protein after thrombin cleavage and can be easily phosphorylated using bovine heart kinase and γ-labeled ATP (11). The radiolabeled protein is then useful in quantitative assays such as calculation of binding affinity constants ($K_D$) (see Fig. 7C, for example) and the determination of dissociation rates (10). Alternatively it can be used directly as a probe in membrane overlay blots (data not shown).

For each affinity-capture assay, glutathione-Sepharose beads (10 μl of packed beads) are first coated with a GST fusion protein (GST-Nup, GST-Kap, etc.) for 15 min at 4 °C in physiological buffer (binding buffer) containing 0.1% Tween 20. Tween 20 prevents beads from sticking to Eppendorf tubes and reduces nonspecific protein interactions. The GST fusions are immobilized on beads directly from E. coli extracts or are provided in purified form. GST forms very stable dimers, so the fusion protein will also be dimeric. The concentration of immobilized GST fusion should ideally represent the physiological concentration of the protein in its cellular location, but the concentration can be increased to boost signal for weak interactions or decreased to reduce nonspecific interactions. The immobilization step is followed by six washes of the beads with binding buffer with the third and fourth washes containing 1 mM ATP. The ATP wash reduces binding of bacterial chaperones to the recombinant GST fusion proteins. Each wash (10 μl of beads diluted into 500 μl of buffer) constitutes a 50-fold dilution. Washed beads are then mixed with “desalted” yeast extracts containing 5–10 mg/ml protein solution. Cell extracts can be prepared by multiple methods; however, soluble proteins must be separated from insoluble proteins (and cell debris) by differential centrifugation. Desalting the soluble extract through a G-25 Sephadex column removes endogenous glutathione, which otherwise competes for binding of the GST fusions to the glutathione-Sepharose beads during the affinity-capture assay. The mixture of beads with cell extracts is incubated (15 min to overnight) at 4 °C, and at the end of incubations, the beads are washed by repeated cycles (as before) to remove nonspecifically bound proteins. We determined that six washes (15 billion-fold dilution) was the minimum number of washes needed to select proteins from yeast extracts that display specific interactions with the immobilized GST fusion. Additional washes do not reduce significantly the amount or diversity of proteins captured (data not shown). Interacting proteins are eluted from the GST fusion baits with 500 μl of buffer with 1 M NaCl during a 10-min incubation at room temperature. Conveniently the GST fusion is rarely eluted from the glutathione-coated beads with 1 M NaCl. In cases where interacting proteins resist 1 M NaCl extraction, they can be eluted from the bait (and beads) with 250 mM to 1 M MgCl₂ in binding buffer; however, we have observed that variable amounts of the GST fusions are lost from the beads under such conditions. Eluted proteins are then concentrated by trichloroacetic acid-sodium deoxycholate precipitation, resolved by SDS-PAGE, and stained with Coomassie Blue. Using this assay we have identified proteins that bind to the immobilized GST fusion protein with affinities ranging from $K_D \approx 50$ pM to 2 μM. We estimate that proteins exhibiting weaker binding affinities toward the bait ($K_D > 2$ μM) are not detected in the assay as these interactions are unlikely to withstand the extensive washings of the immobilized proteins.

**RESULTS**

Identification of Nup159p- and Cse1p-interacting Proteins

Affinity-capture assays using as bait the exportin Cse1p and a portion of the FG region of Nup159p were performed as part of our ongoing effort to identify FG Nup- and Kap-interacting proteins. Glutathione-coated beads (10 μl of packed beads) were coated with 5 μg of GST-Cse1p or GST-Nup159pΔN1C (AA 441–881) (equivalent to 5–10 μM Cse1p or Nup159p within the porous Sepharose beads). The concentration of Nup159p at the NPC is estimated to be 30 μM, assuming there are eight molecules of Nup159p protruding from the cytoplasmic face of the NPC, occupying an estimated volume of 3.9 x 10⁻¹⁹ liters (1, 12). The concentration of Cse1p in nuclei has been estimated to be 12 μM (14). As we previously noted that interactions between karyopherins and nucleoporins are modulated in response to Gsp1p-GTP in cell extracts (4), we also included incubations with Gsp1p-GTP here. A high concentration of Gsp1p-GTP mimics a nucleoplasmic environment and is used to stimulate formation of exportin-cargo interactions as well as exportin-Nup interactions. A mutant version of Gsp1p (Q71L) that cannot hydrolyze bound GTP is used in these experiments because wild-type Gsp1p-GTP is rapidly converted to its GDP form in cell extracts due to the presence of Rna1p (the Gsp1p GTPase-activating protein).

The results of the Cse1p and Nup159p affinity-capture experiments are shown in Fig. 2, A and B. As expected, the addition of Gsp1p-GTP changed the pattern of proteins captured (Fig. 2, A and B, compare lanes 1 and 3) The most abundant proteins were identified using specific antibodies and Western blots and/or mass spectrometry (see “Experimental Procedures”). The less abundant proteins were not identified because their presence can be variable. We found that Cse1p captures mostly Kap60p and Gsp1p in cell extracts supplemented with Gsp1p-GTP (Fig. 2A). This is con-
sistent with results showing that Gsp1p, Cse1p, and Kap60p form an exportin-cargo complex (13–15), which presumably translocates across the NPC by interacting with FG Nups. The FG region of Nup159p captured Crm1p and Rna1p in a Gsp1p-GTP-dependent manner and captured heat shock proteins Ssa1/2p and Ssb1/2p in a Gsp1p-independent manner (Fig. 2, B and C). Peptide sequencing using either MALDI-TOFTOF MS or LC-MSMS identified these proteins unambiguously. As an example, Fig. 2C displays MALDI-TOFTOF CID spectra of two tryptic peptides from proteins captured by Nup159p. The monoisotopic masses of these two peptides are 1332.72 (Fig. 2C, top panel) and 2341.15 (bottom panel), respectively. In addition, due to the side chain

Fig. 4. Size fractionation and identification of proteins and protein complexes eluted from Nup100pΔC-coated beads. Beads (100 μl packed) containing immobilized GST-Nup100pΔC (AA 1–640) (50 μg) were incubated with 10 ml of yeast extracts (100 mg of protein total) for 3 h at 4 °C. After washing the beads six times, bound proteins were eluted with 1 M NaCl, and the salt concentration was reduced to 150 mM. Proteins in the sample (shown in A) were concentrated and resolved in a Superdex 200 gel filtration column equilibrated in physiological buffer (B). Size standards across the top of the gel correspond to the migration of thyroglobulin (85 Å, 669 kDa), ferritin (61 Å, 440 kDa), catalase (52 Å, 240 kDa), and bovine serum albumin (35.5 Å, 66 kDa). C–E, identity of proteins in Superdex 200 column fractions. Proteins were identified by mass spectrometry and/or by Western blotting using specific antibodies. The data depict the various protein complexes present in the column fractions. The presence of Mex67p in fraction 2 was variable.
fragmentation during CID analysis, a few high energy CID ions, \textit{w}_{a5}, \textit{w}_{a8}, \textit{w}_{a9}, \textit{w}_{a10}, \textit{v}_{10}, \textit{v}_{11}, \textit{v}_{12}, and \textit{v}_{13}, were also observed. In the CID spectrum of the larger peptide (Fig. 2C, bottom panel), one dominant immonium ion \textit{m} / \textit{z} 110 (His) and most of the sequence ions (\textit{b} and \textit{y} ion series) are clearly observed. Thus, both CID spectra gave definitive fragment ion information, allowing the MS-Tag\textsuperscript{TM} search program to identify the peptide sequences unambiguously as ESEPFIQTIIR (MH\textsuperscript{+} 1332.72) from the yeast protein Crm1p and (−acetyl-ATLHFVPQHEEQYSISGK (MH\textsuperscript{+} 2341.15) from the yeast protein Rna1p. The latter peptide reveals that Rna1p is acetylated at its N terminus.

Deciphering Networks of Protein Interactions

The data obtained in affinity-capture experiments were not of sufficient resolution to differentiate between direct and indirect protein interactions, particularly in cases where many interacting proteins are identified. For example, binding of a soluble protein to an immobilized GST fusion protein could be mediated indirectly by an adapter protein or RNA (see Fig. 5). Alternatively the protein in question may be part of a larger protein complex where only a single subunit binds to the immobilized protein (see Fig. 6B). To differentiate between these possibilities, we tested individual interactions in a solution binding assay (2). This assay is very similar to the affinity-capture assay except it utilizes purified proteins instead of cell extracts. This technique provides indisputable evidence regarding direct or indirect binding and serves as an excellent platform for biochemical characterization of observed interactions (e.g. mapping of binding domains (5), calculation of interaction affinities (10), and determination of molecular association and dissociation rates (10)).

We used the solution binding assay to examine the putative interaction between Rna1p and Nup159p as this has not been previously described in the literature. Crm1p is an exportin and binds many FG Nups (4), including Nup159p (16). A genetic interaction between Crm1p and Rna1p has been observed, and cells defective in Crm1p function accumulate Rna1p in the nucleoplasm (17). Thus, we tested whether Crm1p can mediate the interaction between Rna1p and Nup159p in a Gsp1p-GTP-dependent manner. Gsp1p-GTP stimulates Crm1p ability to bind cargo as well as nucleoporins by directly binding to Crm1p (4, 18). A mutant version of Rna1p (D240A) that cannot interact with Gsp1p-GTP to stimulate hydrolysis of GTP (7) was used in these experiments to preserve the levels of Gsp1p-GTP during the incubation. Wild-type Gsp1p was used here because the Q71L mutant version forms a stable interaction with Rna1p (not shown). Sepharose beads coated with GST-Nup159p were incubated with purified Rna1p in the presence or absence of Crm1p and Gsp1p-GTP for 1 h at 4°C. Following the incubations, the “unbound” proteins were collected, the beads were washed twice, and the “bound” proteins (including the bait) were eluted from the beads with SDS. Gsp1p-GTP stimulated binding of Crm1p to the FG region of Nup159p as expected for an exportin (Fig. 3, compare lanes 2 and 5). Truncations of the bait interfered with the visualization of Gsp1p in the bound fraction (data not shown). More interestingly we found that Crm1p tethers Rna1p to Nup159p only when Gsp1p-GTP is present (Fig. 3, compare lanes 4 and 8). Also Rna1p had a stimulatory effect on Crm1p binding to Nup159p when Gsp1p-GTP was present (Fig. 3, compare lanes 5 and 8). Crm1p also tethered Rna1p to Nup42p and Nup100p in a Gsp1p-GTP-dependent manner in similar reconstitution experiments (data not shown).

Size Fractionation of Proteins Eluted from Nup100p-coated Beads

Not all patterns of proteins captured in affinity-capture assays are as simple as those shown here for Cse1p or Nup159p. In some cases, up to 31 different proteins are captured specifically by the immobilized GST fusion protein (see Fig. 8). To identify discrete protein complexes bound to
Nup100p, we size-fractionated the salt eluate in a Superdex 200 gel filtration column. After eluting proteins from Nup100p/H9004C-coated beads with 1 M NaCl, the salt concentration was adjusted to physiological levels (150 mM), and the protein sample was concentrated in a Centricon 10 unit to 200 l. The concentrated eluate was then applied to a Superdex 200 sizing column equilibrated in physiological buffer (Fig. 4B). Proteins in each fraction were resolved by SDS-PAGE and stained with Coomassie Blue. Proteins in the gel were subsequently identified using mass spectrometry as before or using Western blots with specific antibodies (Fig. 4, B–E).

Among the proteins extracted from Nup100pΔC-coated beads there were examples of large protein complexes with Stokes radii ($R_s$) of 85 Å (Complexes I–III) (Fig. 4C, fractions 2–4). There were also small protein complexes ($R_s = 61$ Å) (Complexes IV and V) (Fig. 4D, fractions 8–10) and some apparent monomers (52 Å) (Fig. 4E, fractions 11–13). The 200-kDa protein band in fraction 2 was identified as Fas1p and Fas2p; these two proteins are involved in fatty acid biosynthesis and were among a few captured nonspecifically by several baits (data not shown). Complexes I–III have been described previously (19–21), but characterization of their association with FG repeat regions is novel to this report. Below we show how these complexes bind to the FG repeat region of Nup100p and/or other FG Nups and identify the subunit of each complex responsible for physical contact with the FG Nup.

**Complex I: Pab1p-RNA-Mex67p**

Mex67p and Pab1p were among the proteins captured by Nup100pΔC (4) (Fig. 4). Pab1p was also captured by additional FG Nups (4). Mex67p promotes mRNA export from the nucleus and binds directly to mRNA and to FG Nups (22–24), whereas Pab1p is an abundant poly(A) RNA-binding protein required for the initiation of translation (25, 26). After salt elution from Nup100pΔC-coated beads, these two proteins co-fractionated in the void volume of the Superdex 200 gel filtration column (Fig. 4C, fraction 2), suggesting that they may exist as part of a large ($\geq$1 MDa) complex. Because both Mex67p and Pab1p bind mRNA, the large size of the putative Mex67p-Pab1p-containing complex may be due to mRNA being part of the complex. Indeed, RNase A treatment of the Nup100p eluate prior to its fractionation disrupted this com-

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**Fig. 5. Pab1p associates indirectly with FG Nups via RNA.** A, the diagram depicts the interactions tested; the gray underline marks the Nup fragment used in the experiment. GST-Nup100pΔC- (AA 1–640) and GST-Nup1pΔN (AA 300–1076)-coated beads (2 μg in 10 μl of beads) were incubated with purified recombinant Pab1p (2 μg each) in the absence and presence of RNase A (25 μg) to degrade E. coli RNAs. After 1 h at room temperature, beads were concentrated by centrifugation, unbound proteins were collected, beads were washed twice, and the bound proteins were eluted with SDS. Bound and unbound proteins were resolved by SDS-PAGE and visualized with Coomassie Blue. B, Pab1p associates indirectly with the exportin Mex67p via RNA. GST-Mex67p-coated beads (1 μg in 10 μl of beads) were incubated with purified recombinant Pab1p (1 μg) in the absence and presence of RNase A (25 μg). After 1 h at room temperature, beads were collected, and samples were processed as in A. Bound and unbound proteins were resolved by SDS-PAGE and visualized with Coomassie Blue.
plex (data not shown). RNase A treatment also prevented interaction between purified recombinant Pab1p and GST-Nup100p/C, GST-Nup1p3N, or GST-Mex67p (Fig. 5, A and B); presumably, the RNAs responsible for the interaction were captured by the immobilized GST fusion proteins, which were isolated from E. coli extracts. Thus, we conclude that Pab1p associates indirectly (nonspecifically) with Mex67p, Nup100p, and several other FG Nups via RNA molecules.

**Complex II: the Nup84p Complex**

*The Nup84p Complex Binds Directly to FG Nups—*Subunits of the Nup84p complex were among the proteins captured by several FG Nups including Nup100p (Fig. 4) (4). To test whether the Nup84p complex binds directly or indirectly to FG regions of Nups, we mixed purified recombinant Nup84p complex and immobilized GST-FG Nups in solution binding assays. Instead of using Nup100p/C, we used full-length Nup49p and the FG region of Nup116p for this experiment because the size of the GST-Nup100p/C in SDS-PAGE is similar to the size of Nup85p, Nup49p, and cNup145p, preventing detection of these proteins in the bound fraction (data not shown). Nup100p and Nup116p bind many of the same proteins in affinity-capture experiments (4) and display redundant functions in vivo (27). We found that the purified Nup84p complex binds directly to the immobilized GLFG region of Nup116p (AA 165–715) and to Nup49p (Fig. 6A). RNA did not mediate these interactions as treatment with RNase A as above had no effect on the observed binding (data not shown). We also observed direct binding of the purified Nup84p complex to the FG repeat regions of Nup42p and Nup57p (data not shown). In contrast, the FXFG Nups, Nup1p and Nup2p, did not bind to the purified Nup84p complex (data not shown) in agreement with experiments using yeast extracts as a source of the Nup84p complex (see Fig. 8) (4).

*Nup85p Mediates the Interaction between the Nup84p Complex and the FG Regions of Nups—*To determine which subunit of the Nup84p complex contacts the FG Nups, we tested individually purified subunits of the complex for direct binding to the FG region of Nup116p. We found that Nup85p monomers and Nup120p-Nup85p-Seh1p complexes bind directly to the FG region of Nup116p (Fig. 6B). In contrast, the cNup145p-Sec13p complex (Fig. 6B, lane 3), Nup84p monomers (lane 1), and Seh1p monomers (not shown) did not bind. Addition of excess recombinant Nup85p monomers fully prevented binding of the hexameric Nup84p complex to Nup116p, whereas addition of excess monomeric Nup84p did not interfere with binding (data not shown). Altogether these experiments suggest that Nup85p tethers the Nup84p complex directly to the FG regions of Nups excluding FXFG Nups (data not shown).

**Affinity of Interaction between Nup85p and the GLFG Region of Nup116p—**The binding affinity of Nup85p toward the GLFG region of Nup116p was measured using radiolabeled Nup85p and GST-Nup116pΔNΔC immobilized on Sepharose beads. Nup85p was labeled with [γ-32P]ATP at a specific site in the N-terminal tag (provided by the pGEX-2TK vector used to produce the recombinant Nup85p). After 1 h of incubation at 4 °C, beads containing GST-Nup116p-Nup85p complexes were sedimented, and the amount of radiolabeled Nup85p was quantified by liquid scintillation. From the binding curve obtained we calculated that Nup85p binds the GLFG region of Nup116p with an affinity of ~1.5 μM (Fig. 6C). This is physiologically significant considering that the concentration of Nup85p and Nup116p within the NPC are estimated at 200 μM. This is based on the copy number of Nup85p and Nup116p within the NPC (1) and assuming that most Nup85p and Nup116p are concentrated in the central transporter region, which has a diameter of 100 nm and a height of 33 nm (12).

*Nup85p Can Interact with the GLFG Repeat Region of Nup116p in Vivo—*Having provided biochemical data that Nup116p and Nup85p interact directly, our next goal was to determine whether this interaction can occur in vivo. For this purpose, a yeast two-hybrid assay was set up using the GLFG repeat region of Nup116p (AA 165–715) fused to the Gal4p DNA binding domain as bait and Nup85p fused to the activation domain of Gal4p. Indeed, Nup85p and the GLFG region of Nup116p interact in vivo as indicated by robust growth of the two-hybrid yeast strains carrying both proteins (Fig. 6D). Cells carrying plasmids without inserts did not survive as expected. Because this positive “two-hybrid” interaction is presumed to occur in the nucleoplasm, it remains to be determined whether binding between Nup85p and Nup116p and other centrally located FG Nups also occurs in situ at the NPC.

*Nup133p Is a Member of the Nup84p Complex—**The Nup84p complex includes at least six subunits: Nup84p, Nup85p, Nup120p, the C terminus of Nup145p (cNup145p), Sec13p, and Sec13p (20), and recent evidence shows that recombinant Nup133p can bind the Nup84p complex and may represent a seventh subunit (6). We found that Nup133p co-fractionates with the hexameric Nup84p complex when bound to various FG Nups (4) and in a Superdex 200 sizing column after salt elution from Nup100p-coated beads (Fig. 4, B and C), suggesting that Nup133p is indeed a member of the Nup84p complex in vivo. Nup133p interacts with Nup84p by two-hybrid assay (28, 29), and in the mammalian homologue of the Nup84p complex (the Nup107 complex), Nup133 is a subunit (29). In yeast, the Nup84p complex performs essential roles in NPC biogenesis and mutants in Nup84p, Nup85p, Nup120p, and Nup145p display defects in the export of mRNA as well as drastic structural abnormalities of the nuclear envelope (20, 30, 31). Nup133 mutants display the same phenotypes and are lethal when combined with mutant members of the Nup84p complex (32–35). Despite all the evidence, it has not been possible to formally demonstrate that Nup133p forms part of the endogenous Nup84p complex in *S. cerevisiae.*

To establish that Nup133p is an integral part of the endog-
**A.** The Nup84p complex binds directly to the GLFG region of Nup116p and to Nup49p. GST-Nup116pΔNJC (AA 165–715) and Nup49p (full length) were immobilized separately on glutathione-coated Sepharose beads (2 μg each in 10 μl of packed beads) and were mixed (or not) with purified hexameric Nup84p complex (1 μg) for 1 h at 4 °C. Bound and unbound proteins were collected and proteins were resolved by SDS-PAGE and stained with Coomassie Blue. Not shown are the Nup84p complex subunits Seh1p and Sec13p because their visualization was obstructed by truncations of the GST fusion protein (data not shown).

**B.** Nup85p (a subunit of the Nup84p complex) binds directly to the GLFG region of Nup116p. GST-Nup116pΔNJC was immobilized on Sepharose beads (1 μg in 10 μl of beads) and was mixed with purified subcomplexes or subunits of the Nup84p complex (1–4 μg each) as indicated. After 1 h at 4 °C, bound and unbound proteins were collected, and proteins were resolved by SDS-PAGE and stained with Coomassie Blue.

**C.** Affinity of Nup85p toward the GLFG region of Nup116p. GST-Nup116pΔNJC-coated beads (1 μm within the volume of the beads) were incubated with increasing concentrations of radiolabeled Nup85p for 1 h at 4 °C in binding buffer with 1 mg/ml bovine serum albumin and protease inhibitors. At the end of incubations, bound Nup85p was collected and quantified in a scintillation counter as described under “Experimental Procedures.” The dissociation constant \(K_D\) of the Nup116p–Nup85p complex was calculated as described previously (5, 10). Each data point was performed in duplicate, and error bars represent S.E. D, Nup116p binds the GLFG region of Nup116p in vivo in a yeast two-hybrid assay. Bait plasmid (B) encoding GAL4BD-Nup116p (NUP116 codons 165–715) was co-transformed with the “activation” plasmid (A) encoding GAL4AD-Nup85p into the two-hybrid reporter yeast strain AH109 (Clontech). Cell growth was scored after incubation at 30 °C for 5 days. E, Nup133p co-immunoprecipitates with the Nup84p complex. The diagram depicts the interaction being tested. Affinity-purified anti-Nup85p antibodies covalently coupled to protein A-coated beads (anti-Nup85p-coated beads) were mixed with purified hexameric Nup84p complex (1 μg) or with
enous Nup84p complex, we tested whether antibodies against Nup85p (a subunit of the complex) could co-immunoprecipitate Nup133p and the Nup84p complex present in fraction 3 from the Superdex 200 column (Fig. 4, B and C). Anti-Nup85 antibodies coupled to protein A-coated beads did co-immunoprecipitate Nup133p and the Nup84p complex (Fig. 6, lane 2). Control experiments conducted in the presence of competitor Nup85p monomers confirmed that Nup133p precipitates specifically with the Nup84p complex (Fig. 6, lane 3). Thus, we conclude that Nup133p is a stable member of the endogenous Nup84p complex.

**Complex III: Nup2p-Kap60p-Kap95p: the Importin Kap95p-Kap60p Can Tether Nup2p to the FG Region of Nup100p**

Nup2p was among the proteins captured by several FG Nups in affinity-capture experiments, including Nup42p, Nup49p, Nup57p, Nup100p, Nup116p, and Nup60p (4). Interestingly the interaction of Nup2p with these FG Nups (except Nup60p) decreased in a manner similar to importins when the concentration of Gsp1p-GTP was elevated in the extracts (4). This suggests that Nup2p interacts directly with several FG Nups in a Gsp1p-GTP-sensitive manner or that Nup2p is tethered to FG Nups indirectly via an adapter (such as the Kap95p-Kap60p heterodimer) whose binding to FG Nups is disrupted by Gsp1p-GTP. Our data suggest that the latter case is true. First, Nup2p and the importins Kap95p and Kap60p co-fractionate in the sizing column after salt extraction from Nup100p-coated beads (Fig. 4, B and C, fractions 2–4). Second, Kap95p and Kap60p are the only karyopherins captured by Nup2p in affinity-capture experiments (4).

To test whether Nup2p binds directly to the FG region of Nup100p or whether it needs Kap95p-Kap60p as an adapter, we sought to reconstitute this quaternary complex in a solution binding assay using all purified proteins. Kap95p-Kap60p heterodimers and Nup100p (AA 1–640) were incubated with immobilized GST-Nup2p either alone or in combination (Fig. 7). We found that Kap95p-Kap60p heterodimers bind directly to Nup2p as expected (Fig. 7, lane 3) and that the FG region of Nup100p binds to immobilized Nup2p but only in the presence of Kap95p-Kap60p (compare lanes 2 and 4). These results demonstrate that Nup2p interacts with the FG region of Nup100p via the Kap95p-Kap60p heterodimer. It is likely that the observed interaction of Nup2p with other FG Nups (excluding Nup60p) (4) is also mediated by Kap95p-Kap60p.

**Complexes IV and V and Monomers**

Kap104p and Nab4p were captured by several FG Nups in affinity-capture experiments (Figs. 4 and 8) (4). Kap104p is an importin, and Nab4p is one of its cargoes (36). Kap104p-Nab4p complex (Complex IV) and its interaction with various FG Nups have been documented previously (36). Likewise a complex between Kap95p and Kap60p (Complex V) and its interaction with various FG Nups have been documented.
complexes (grouped by study we resolved which of the captured proteins are part of each complex (marked by small circles). The Nup85p subunit (Fig. 6) hexameric Nup84p complex binds directly to Nup49p and the Nups via Crm1p. Below we discuss the possible functional roles of these novel interactions in the mechanisms of nuclear pore complex function.

Finally, the Rna1p-Crm1p-Gsp1p-GTP complex binds to FG region of Nup100p. The diagram depicts the reconstituted Nup2p-Kap60p-Kap95p-Nup100p complex. GST-Nup2p-coated beads (2 μg in 10 μl of beads) were incubated with soluble Nup100pΔC (1 μg) or Kap95p-Kap60p heterodimers (2 μg) as indicated. Bound and unbound proteins were collected, resolved by SDS-PAGE, and visualized by Coomassie Blue.

Previously (3), Kap60p is often considered to be a cargo for Kap95p because it contains a nuclear localization signal-like sequence (the importin-β binding domain), which is recognized by Kap95p. Finally, Nup100p captured Msn5p, Kap121p, Crm1p, and Sxm1p from yeast extracts, possibly as monomers or perhaps in complexes with very small proteins.

**DISCUSSION**

**Deciphering Networks of Protein Interactions**—A summary of all protein interactions identified thus far using our affinity-capture assay is shown in Fig. 8. Proteins captured by each of the immobilized Nups and Kaps are listed vertically. In this study we resolved which of the captured proteins are part of complexes (grouped by boxes). We also identified the subunit of each complex (marked by small circles) that physically contacts the immobilized Nup or Kap. We demonstrate that the Nup84p complex includes Nup133p as a subunit and binds to FG repeat regions of Nups via the Nup85p subunit (Fig. 6). The Nup2p-Kap60p-Kap95p complex also binds the same regions of FG Nups, most likely via the Kap95p subunit. Finally, the Rna1p-Crm1p-Gsp1p-GTP complex binds to FG Nups via Crm1p. Below we discuss the possible functional roles of these novel interactions in the mechanisms of nuclear pore complex function.

**Binding of the Nup84p Complex to FG Repeat Regions of Nups**—Here we showed using all purified proteins that the hexameric Nup84p complex binds directly to Nup49p and the FG region of Nup116p and that the interaction is mediated by the Nup85p subunit (Fig. 6B). The binding of the Nup84p complex to these and additional FG Nups is reminiscent of karyopherins and implies that the Nup84p complex also shuttles across the NPC via interaction with FG Nups as karyopherins do. However, the interaction of the Nup84p complex with FG Nups does not change in response to Gsp1p-GTP, whereas Gsp1p-GTP affects binding of Kaps to Nups (4).

Furthermore, it was recently shown that the vertebrate homologue of the yeast Nup84p complex (the Nup107 complex) does not shuttle between the cytoplasm and nucleus of cells but is instead a stationary component of the NPC located in the central symmetric scaffold (29). In accordance, we suggest that the yeast Nup84p complex may play a structural role in the NPC, possibly as a component of the rings of the central scaffold (Fig. 9A) (1, 6).

Why does the Nup84p complex interact with so many FG Nups? The FG repeat regions of Nups (typically between 200–700 amino acids long) are mostly composed of random coils that lack secondary structures (37). Thus, FG Nups are thought to form a loosely packed meshwork of filaments at the NPC (38). In a hypothetical model whereby the density of filaments within the center of the NPC determines the exclusion size of the permeability barrier (37), a weak interaction between FG regions of Nups and the Nup84p complex (in particular Nup85p) may help to maintain a “seal” between the filamentous meshwork and the NPC scaffold (see Fig. 9A).

The weak affinity between Nup85p and the FG region of Nup116p (Kc = 1.5 μM) is physiologically significant considering that the concentration of Nup85p and Nup116p within the NPC is estimated to be 200 μM. Indeed a low affinity interaction between Nup85p and FG repeat regions may guarantee that such interactions only occur within the NPC and not in the cytoplasm during Nup biosynthesis. Because NUP85 is not an essential gene, we suggest that additional nucleoporins in the scaffold interact weakly with FG repeats to cooperatively seal the borders around the filamentous meshwork. Not all FG repeat regions in Nups seem to participate in this interaction. Notably, the FG region of Nup1p, Nup2p, Nup60p, Nsp1p (the FXFG subset of FG Nups), and Nup159p do not bind stably to Nup85p and/or the Nup84p complex. Interestingly these Nups (except Nsp1p) are components of peripheral structures of the NPC such as the nuclear basket and cytoplasmic fibers (1).

The physiological relevance of the interaction between Nup85p and FG regions of Nups needs to be addressed in vivo. We plan to perform mutational analysis of NUP85 to generate variants that cannot interact with FG Nups. These mutant yeast will be examined for phenotypes related to the integrity of the permeability barrier (Fig. 9B), the efficiency of mRNA export (20), and defects in NPC biogenesis (39).

**The Importin Kap95p-Kap60p Can Tether Nup2p to FG Nups**—Using purified proteins, we showed that the importin Kap95p-Kap60p can tether Nup2p to the FG region of Nup100p (Fig. 7). Based on that finding, we suggest that Kap95p-Kap60p heterodimers are also responsible for tethering Nup2p to addi-
A likely explanation for these interactions is that Kap95p-Kap60p heterodimers facilitate translocation of Nup2p across the NPC during its biogenesis. Nup2p resides in the nuclear basket structure of the NPC and in the nucleoplasm, so it needs to be transported across the NPC from its site of synthesis in the cytoplasm. However, Nup2p is also unique among yeast nucleoporins in that it can shuttle between the nucleoplasm and cytoplasm of cells (40). Thus, Kap95p-Kap60p may also carry Nup2p across the NPC when it shuttles. In both scenarios, Nup2p may simply represent a cargo molecule for Kap95p-Kap60p.

Nup2p is tethered to the nuclear basket structure of the NPC via direct interaction with Nup60p (5, 40). However, when the nucleoplasmic concentration of Gsp1p-GTP drops, Nup2p detaches from Nup60p and relocates to the cytoplasmic face of the NPC (5, 40). We suggest that Kap95p-Kap60p heterodimers are responsible for dissociating Nup2p from Nup60p (4, 5) and for tethering Nup2p to various FG Nups throughout the NPC. Based on 1) the filamentous random coil structure of Nup2 and FG Nups in general (37) and 2) the hypothesis that the density of FG Nup filaments within the center of the NPC determines the exclusion size of the permeability barrier (21, 37), we suggest that relocation of Nup2p serves to regulate the permeability of the NPC (Fig. 9B). Indeed newly deposited Nup2p filaments at the cytoplasmic face of the NPC would increase the exclusion limit of the permeability barrier by adding filaments to the FG Nup meshwork thereby increasing its density (Fig. 9B). Alternatively, positioning Nup2p throughout the NPC may serve to thwart nuclear import reactions as Nup2p would trigger premature dissociation of cargoes from Kap60p using its karyopherin release factor activity (10).

What could cause a drop in the nucleoplasmic concentration of Gsp1p-GTP or at least locally at a particular NPC? It is possible that a breach in the permeability barrier of the NPC (i.e. gaps or holes) may be caused by accidental or regulated loss of FG Nups, which occurs when cells are blocked in secretion (41). Alternatively a breach may result from proteolysis of the FG Nup meshwork (“wear and tear”) as FG Nups are natively unfolded proteins that are hypersensitive to proteases (21, 37). Because Gsp1p-GTP is close in size to the diffusion limit of the permeability barrier of the NPC, it could easily diffuse toward the cytoplasm across damaged NPCs. When normal Gsp1p-GTP concentrations are restored, Gsp1p-GTP would dissociate Nup2p-Kap60p-Kap95p-FG Nup complexes throughout the NPC (via binding to Kap95p).
and would promote binding of Nup2p to Nup60p at the nuclear basket structure where it is predominantly localized (5).

**Tethering of Rna1p to the NPC via Crm1p**—We showed using all purified proteins that the exportin Crm1p directly tethers Rna1p to Nup159p in a Gsp1p-GTP-dependent manner (Fig. 3). This could seem impossible as Rna1p functions to stimulate hydrolysis of Gsp1p-bound GTP; however, Gsp1p-GTP bound to Crm1p is protected from Rna1p action while Crm1p is bound to Nup42p and Nup159p (42) and possibly other FG Nups, thus making this interaction possible. The interaction between Rna1p, Crm1p, and FG Nups may reflect partial reactions in the transport of Rna1p across the NPC as Rna1p shuttles between the cytoplasm and nucleoplasm of yeast and Crm1p functions as its exportin (17). Consistently, we found that Nup49p-, Nup57p-, Nup100p-, and Nup116p-coated beads also capture Rna1p from yeast extracts (data not shown). In all cases, capture only occurred when the cell extracts (which contain wild-type Rna1p) were supplemented with Gsp1p-GTP (Q71L), a mutant Gsp1p that cannot hydrolyze GTP but associates stably with Rna1p (data not shown). This predicts that Rna1p may be free to stimulate GTP hydrolysis of Gsp1p at the cytoplasmic fibrils of the NPC and possibly throughout the NPC when shuttling (2).

**The Affinity-Capture Assay as a Proteomic Tool**—The affinity-capture assay uses GST fusions as bait in cell extracts to obtain the widest possible variety of interacting proteins. This strategy features the advantage of being able to probe various baits against the same extract, thus allowing a comparative analysis between baits and their interaction with the proteome under identical conditions. The technique can be widely applied to study cellular networks of protein interactions in any organism, assuming that the gene of interest can be amplified by PCR, allowing it to be cloned into the pGEX-2TK expression vector (or equivalent pGEX vector). Also it is necessary to make a cell extract from that organism in sufficient quantity for the assay (5–10 mg of total protein per experiment). Cloning
from cDNA would be necessary in cases where the gene of interest contains introns.

GST fusions expressed in E. coli (rather than insect cells or yeast) are particularly useful as baits in the affinity-capture experiments because 1) sufficient amounts of the GST fusion protein can be easily purified from E. coli and 2) the GST fusions are purified free of interacting proteins due to the fact that prokaryotes lack proteins that function in nucleocytoplasmic transport. There are a few disadvantages when working with bacterially expressed GST fusions. First, the protein of interest may require post-translational modifications not present in E. coli and may therefore not be functional in one or more of its binding activities. Second, the protein of interest may not express well or may be easily degraded in E. coli. Third, GST is a dimer so the expressed fusion protein will also be dimeric; in some cases, it may interfere with protein interactions by steric hindrance.

Manipulation of Cells and Extracts to Mimic Distinct Cellular Microenvironments—The affinity-capture assay is versatile in that extracts can be prepared from cells grown under a variety of conditions. For example, extracts can be prepared from cells grown at different cell densities, from cells blocked at different stages of the cell cycle, from cells carrying a mutation, or from cells subjected to stresses such as heat shock and starvation (Fig. 1B). Each growth condition will likely result in different patterns of proteins bound to the immobi-

The affinity-capture assay is also versatile in that cell extracts can be manipulated “postlysis” to mimic a particular intracellular location or condition; the corresponding change in proteins captured may reflect a cell biological response. This was tested and validated using the protein Gsp1p-GTP, which serves as a nucleoplasmic marker. We found that the number and intensity of proteins captured changed in the presence of Gsp1p-GTP (Fig. 2) (4). The effects of additional proteins such as kinases or phosphatases in this or any network of protein interactions can also be analyzed. The affinity-capture assay can also be used to map the domains of proteins responsible for particular interactions (5) because full-length proteins are not needed to generate GST fusions (e.g. Nup159p1NΔC). Finally, affinity-capture assays provide a platform with which to purify proteins or protein complexes in their native form (Fig. 4B) (21).

A direct comparison of results obtained with the affinity-capture assay versus results obtained using additional proteomic approaches such as large-scale two-hybrid screens (45, 46) or large-scale tandem affinity purification tagging screens (47, 48) reveals little overlap in protein “baits” used. This highlights the need for “theme-oriented” proteomic surveys (1, 4, 49, 50). In a few cases, however, the tandem affinity purification tagging technique resulted in results similar to those obtained using the affinity-capture assay; this was the case for Kap95p (51) and Nup2p (40). We anticipate that many protein interactions are missed by the affinity-capture assay, especially those with affinities weaker than 2 μM. This highlights the need to develop more sensitive affinity-capture techniques that retain high specificity as well as automated high throughput mass spectrometers to continue surveying all protein networks in cell biology.

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