The \textit{Saccharomyces cerevisiae} Nucleoporin Nup2p Is a Natively Unfolded Protein*

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Little is known about the structure of the individual nucleoporins that form eukaryotic nuclear pore complexes (NPCs). We report here in vitro physical and structural characterizations of a full-length nucleoporin, the \textit{Saccharomyces cerevisiae} protein Nup2p. Analyses of the Nup2p structure by far-UV circular dichroism (CD) spectroscopy, Fourier transform infrared (FTIR) spectroscopy, protease sensitivity, gel filtration, and sedimentation velocity experiments indicate that Nup2p is a “natively unfolded protein,” belonging to a class of proteins that exhibit little secondary structure, high flexibility, and low compactness. Nup2p possesses a very large Stokes radius (79 Å) in gel filtration columns, sediments slowly in sucrose gradients as a 2.9 S particle, and is highly sensitive to proteolytic digestion by proteinase K; these characteristics suggest a structure of low compactness and high flexibility. Spectral analyses (CD and FTIR spectroscopy) provide additional evidence that Nup2p contains extensive regions of structural disorder with comparatively small contributions of ordered secondary structure. We address the possible significance of natively unfolded nucleoporins in the mechanics of nucleocytoplasmic trafficking across NPCs.~

In eukaryotic cells, the translocation of biomolecules between the nucleus and cytosol occurs through nuclear pore complexes (NPCs),¹ supramolecular protein structures embedded in the double lipid membrane of the nuclear envelope (1–3). The \textit{Saccharomyces cerevisiae} NPC is a 60-MDa structure (4) formed by 30 different Nups present in multiple copies per NPC (5); 13 of these Nups contain regions with multiple phenylalanine-glycine repeats (FG Nups) that are believed to be involved directly in the transport mechanism (6). The yeast NPC contains a core ring structure with 8-fold symmetry measuring 95 nm in diameter and 35 nm in depth (4). Most of the Nups are distributed symmetrically on the nucleoplasmic and cytoplasmic faces of the ring, forming a central transporter that is the conduit for macromolecular transport. However, a subset of Nups form filaments that extend into the cytoplasm, and another subset form a basket structure that extends into the nucleoplasm (5). The Nups of the cytoplasmic fibrils and nuclear basket are proposed to function in the initiation and termination of karyopherin-mediated transport reactions (7–11).

In \textit{S. cerevisiae}, proteins, mRNA, tRNA, and other biomolecules (collectively referred to as “cargo”) larger than ~30 kDa are actively transported across the NPC via association with karyopherins (Kaps: importins, exportins, and transportins) (12–14). Kaps bind the import or export signals of cargo destined for transport across the NPC and interact with the FG Nups, which may serve as stepping stones for Kaps as they traverse the NPC (6, 15–17). The GTPase Gsp1p (the yeast homolog of Ran) governs the interaction of Kaps with transport cargo and Nups by binding directly to Kaps (6, 15, 18). In export reactions, Gsp1p-GTP stabilizes the cargo-exportin interaction and enhances the binding of this export complex to FG Nups (19); in import reactions, Gsp1p-GTP induces the release of cargo from importins and prevents the association of importins and FG Nups (6, 7, 15). A high concentration of Gsp1p-GTP is maintained in the nucleus by Prp20p, the Ran guanine nucleotide exchange factor (RanGEF) (20, 21); the Ran GTpase activating protein (RanGAP), Rna1p, maintains low cytoplasmic concentrations of Gsp1p-GTP (22–24).

The \textit{S. cerevisiae} protein Nup2p is a 78-kDa nucleoporin that contains 720 amino acid residues, including 16 FxFG peptide repeats (25). Although Nup2p is not essential under normal physiological conditions, yeast lacking Nup2p (nup2Δ) exhibit defects in nuclear import of Kap60p-Kap95p (importin αβ or karyopherin αβ)-dependent cargoes and defects in the Cse1p-mediated recycling of Kap60p (Srp1p/importin α/Kap α) from the nucleus to the cytoplasm (26–28). Nup2p interacts with Kap95p and Los1p via its FxFG repeats (15, 27, 29). In addition, the N terminus of Nup2p binds directly to Kap60p (27, 28) and functions as a KaRf (karyopherin release factor) to induce the dissociation of cargoes from Kap60p (7). Nup2p also contains a low affinity binding site for Gsp1p-GTP at its C terminus (30, 31). Electron microscopic studies have localized Nup2p to the nuclear basket structure of the NPC where it binds directly to Nup60p in a Gsp1p-GTP-sensitive manner (27, 28, 31, 32). At this location, Nup2p is proposed to function in the termination of import reactions involving the Kap95p-Kap60p heterodimer and the initiation of Kap60p export (7, 26). Interestingly, Nup2p associates with Kap60p in a dynamic manner dependent on Gsp1p-GTP, allowing Nup2p to shuttle between the nucleoplasm and cytoplasm under conditions of low nuclear Gsp1p-GTP concentrations (31, 32).

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¹ The abbreviations used are: NPC, nuclear pore complex; Nup, nucleoporin; FG Nup, Nup with multiple phenylalanine-glycine repeats; FTIR, Fourier transform infrared spectroscopy; AA, amino acids; BSA, bovine serum albumin; Rs, Stokes radius; GST, glutathione S-transferase; KaRF, karyopherin release factor.

This paper is available on line at http://www.jbc.org
In this study, we employ a variety of biophysical methods to examine the physical and structural characteristics of the nucleoporin Nup2p as a model for nucleoporin structure. From these studies, we have concluded that Nup2p is a natively unfolded protein. We speculate that the intrinsic disorder of Nup2p is important for its functions, as it may facilitate rapid association and dissociation reactions with Kaps while allowing simultaneous interactions with binding partners such as Nup60p, Gsp1p, and Prp20p.

**MATERIALS AND METHODS**

**Preparation of *S. cerevisiae* Extract—**GPY60 yeast were grown in 1 liter of YPD medium at 30 °C to an A600 = 2.0. Yeast were harvested by sedimentation at 5,000 × g for 10 min and resuspended in 20 mM Hapes, pH 6.5, 150 mM KOAc, 250 mM sorbitol, 2 mM Mg(OAc)2, and protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM aprotinin, and 2 mM phenylmethylsulfonyl fluoride) to a final volume of 40 ml. Yeast were lysed in a French Press cell and cell debris was 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 mM Hapes, pH 6.8, 150 mM KOAc, and 2 mM MgOAc2. Pooled fractions were supplemented with 0.1% Tween, 2 mM dithiothreitol, and protease inhibitors.

**Construction and Purification of Recombinant Proteins—**Recombinant Nups and Kaps were expressed as glutathione S-transferase (GST) fusions using vector pGEX-2TK (Amersham Biosciences) that incorporates a thrombin cleavage site at the fusion junction. The NUP2, KAP95, KAP60 genes and portions of NUP2 were amplified from yeast genomic DNA (Promega) by PCR. The PCR products were ligated into vector pGEX-2TK and transformed into BL21 codon plus (Promega) by PCR. The PCR products were further purified by fast protein liquid chromatography in gel fractionation mixer (Hoefer) and recombinant Nup2p (5 μg), with or without recombinant Kap95p and Kap60p were digested in solution with 100 μg/ml proteinase K (Sigma) at 37 °C. At various time points, aliquots were removed and mixed with Laemmli sample buffer containing 1 mM phenylmethylsulfonyl fluoride (Sigma) to stop proteolysis. Samples were then resolved by SDS-PAGE and stained with Coomassie Blue to detect the products of proteinase K digestion. For protease digestion of native Nup2p in the context of intact NPCs, yeast nuclei were purified as described (37) and resuspended to 3.6 mg/ml in 20 mM Hapes, pH 6.8, 150 mM KOAc, 250 mM sorbitol, 2 mM MgOAc2, 50% glycerol. Proteinase K (Sigma) was added to the nuclei at 300 ng/ml. The reaction was incubated at 37 °C, and aliquots were removed at the indicated time points. Digestions were stopped with 2× Laemmli sample buffer plus 1 mM phenylmethylsulfonyl fluoride followed by heating at 95 °C for 10 min. The fraction of full-length Nups remaining was quantified by SDS-PAGE followed by Western blotting with anti-Nup antibodies and 125I-Protein A (Amersham Biosciences). Radioactive blots were exposed to phosphoimager screens and then digitized and quantified using ImageQuant software (Amersham Biosciences). Anti-Nup2p antibodies were generated as described previously (31). Anti-Kap95p, -Kap60p, -Nup85p, and -Pom152p rabbit polyclonal antibodies were raised against Kap95p (full-length), Kap60p (full-length), Nup85p (full-length), and Pom152p (AA 210–533 and AA 1182–1337).

**RESULTS**

The *S. cerevisiae* NPC is formed by 30 different Nups that provide a detailed physical and structural characterization of the yeast nucleoporin Nup2p.

**Two-step Purification of Nup2p from Yeast Extract—**As a part of our ongoing biochemical investigations into the mechanism of Kap95p (importin β/karyophilin β) movement across the NPC, we incorporated the yeast extract with a GST-Kap95p affinity resin to isolate specific Kap95p-interacting proteins. Among the most abundant proteins that eluted with 250 mM MgCl2 was the nucleoporin Nup2p (Fig. 1A, lane 2). Nup2p is a member of the FxFG family of nucleoporins and is tethered primarily to Nup60p in the nuclear basket structure of...
Physical and Structural Characteristics of Nup2p

Fig. 1. Purification and gel filtration sizing of Nup2p. A, two-step purification of Nup2p from yeast extract. A yeast extract containing 10 mg of soluble protein (lane 1) was incubated with GST-Kap95p (100 μg) immobilized on glutathione-Sepharose beads (100 μl). Proteins bound to the GST-Kap95p resin were eluted with buffer containing 250 mM MgCl2 (lane 2), concentrated, and separated further by gel filtration in a Superose-6 column equilibrated with elution buffer. Nup2p containing fractions were pooled, resolved via SDS-PAGE, and stained with Coomassie Blue (lane 3). The identity of Nup2p was confirmed by Western blot (not shown). B, gel filtration sizing of recombinant Nup2p and Nup2p-Kap complexes. Recombinant Nup2p with and without recombinant Kap95p and/or Kap60p (100 μg each) were resolved via SDS-PAGE, and stained with Coomassie Blue (top panel, Nup2p alone; second panel, Nup2p-Kap60p; third panel, Nup2p-Kap95p; and bottom panel, Nup2p-Kap95p·Kap60p).

Table I

| Protein/complex | Predicted Mc | Stokes radius, Re (Å) ± 5% | Sedimentation coefficient, s20,w (x10^-13 s ± 1% | Calculated Mc |
|-----------------|--------------|-----------------------------|-----------------------------------------------|--------------|
| Nup2p           | 78           | 79                          | 2.9                                           | 89           |
| Nup2p·Kap60p'   | 138          | 79                          | 4.3                                           | 149          |
| Nup2p·Kap95p'   | 173          | 76                          | 6.2                                           | 183          |
| Nup2p·Kap95p·Kap60p' | 233     | 76                          | 7.1                                           | 221          |
| Nup2p''         | 78           | 101                         |                                               |              |
| Nup2p·Kap60p''  | 138          | 97                          |                                               |              |

a Predicted molecular weight if all proteins present in a single copy.

b Estimated error.

c Molecular weight calculated from Re and s20,w values as described under "Materials and Methods."

d Purified recombinant protein.

e Purified from yeast extract.

The NPC (31, 32); Nup2p also shuttles between the nucleoplasm and cytoplasm (32). A second chromatography step was sufficient to obtain purified Nup2p in complex with its binding partner Kap60p (data not shown). Briefly, the MgCl2 eluate from the GST-Kap95p affinity resin was separated by gel filtration in a Superose-6 column equilibrated in physiological buffer. Nup2p co-purified with Kap60p, and no additional proteins were visible in the same fractions as detected by Coomassie Blue stain (data not shown). To purify Nup2p to homogeneity the Superose 6 column equilibrated in buffer with 250 mM MgCl2. Under such conditions, Nup2p that eluted from the Superose-6 column was pure as verified by Coomassie Blue (Fig. 1B, bottom panel; Table I). The addition of equimolar concentrations of Nup2p binding partners (Kap60p and Kap95p) yielded no significant changes in the elution volumes of Nup2p in Nup2p-Kap complexes versus Nup2p alone (Fig. 1B, bottom panels; Table I).

It is unclear why the recombinant Nup2p produced in E. coli exhibits a smaller Stokes radius than the Nup2p purified from yeast. Post-translational modifications of Nup2p in S. cerevisiae have not been reported, but sulfation or phosphorylation may account for the difference. However, recombinant and endogenous Nup2p migrate identically in SDS-acrylamide gels (data not shown). It is also possible that structural differences may result from the different purification procedures or the presence of a 9-AA tag at the N terminus of the recombinant Nup2p. Regardless, recombinant and native Nup2p behave identically in their ability to bind Kap60p and Kap95p (Figs. 1 and 2; data not shown), suggesting that the two versions of Nup2p were functionally identical.
The large hydrodynamic dimension of Nup2p is consistent with either (i) a homo-oligomeric assembly of Nup2p or (ii) a nonglobular structure of low compactness (38, 39). Sedimentation velocity experiments also provide information regarding the mass, size, and degree of compactness of proteins or protein complexes. To calculate the sedimentation coefficient of Nup2p, recombinant Nup2p with and without its Kap binding partners was layered on linear sucrose gradients (5–20% or 10–40%) and subjected to centrifugation at 259,000 × g for 12 h at 4 °C. Eighteen × 83-μl aliquots were collected from the top and mixed with Laemmli sample buffer. Proteins were resolved via SDS-PAGE and stained with Coomassie Blue.

The number of proteins shown in vitro to contain a natively unfolded domain(s) under physiological conditions has increased exponentially in the last 10 years. This growing data base has permitted the identification of amino acid compositions peculiar to natively unfolded proteins. Natively unfolded proteins contain a low percentage of hydrophobic amino acids and often have a large net charge at neutral pH as the result of non-neutral pI values (38). Compared with globular proteins in the Protein Data Bank, natively unfolded proteins were significantly depleted of amino acids Ile, Leu, Val, Trp, Phe, Tyr, Cys, and Asn, and were enriched in amino acids Glu, Lys, Arg, Gly, Gln, Ser, Pro, and Ala (39). Hypothetically, the hydrophobic amino acids in the former group contribute “order” to a protein, whereas the charged or polar amino acids of the latter group facilitate unfolding and “disorder.”

Nup2p contains only six tyrosines (Tyr), one cysteine (Cys), and no tryptophans (Trp); as a result, 25.8% of the Nup2p amino acid sequence was composed of order conferring amino acids, compared with an average of 38.1% for all proteins in the S. cerevisiae proteome (Fig. 3A). Conversely, 57.6% of the Nup2p sequence was derived from the group of AA that confer disorder, compared with 46.0% for all S. cerevisiae proteins (Fig. 3A). Although Nup2p has a high frequency of charged amino acids (Asp, Glu, Lys, and Arg), its 104 positive charges and 104 negative charges compensate at neutral pH, yielding an isoelectric focusing point (pI) of 7 that was uncharacteristic of natively unfolded proteins (Fig. 3A).

A recent study compared the mean hydrophobicity (<H>) and mean net charge (<R>) of natively folded and natively unfolded amino acid sequences, establishing that natively unfolded proteins occupy a clearly demarcated region of the
charge-hydrophobicity space (42). Fig. 3C shows the location of natively folded proteins (black squares) and unfolded proteins (gray circles) from the original study, as well as Nup2p (large open circle) in the charge hydrophobicity plot; the line represents the boundary between folded and unfolded polypeptides and was defined by the equation, 
\[ <H> = <R> + \frac{1.151}{2.785} \]
Although Nup2p has a mean net charge of 0, its low mean hydrophobicity (Fig. 3B) places it near the boundary line, but clearly in the natively unfolded region of the charge hydrophobicity plot (Fig. 3C). Thus, the amino acid composition of Nup2p and its low mean hydrophobicity predict that its structure was intrinsically disordered.

CD and FTIR Analyses Show That Nup2p Contains a Large Contribution of Unordered Secondary Structure—Natively unfolded proteins typically have a low content of ordered secondary structure. To characterize the secondary structure of Nup2p and fragments thereof (see diagram in Fig. 4A), we used CD and FTIR spectroscopic techniques. Far-UV CD and FTIR allow estimations of the α-helical, β-sheet, and random coil content of a protein. Typically, the far-UV CD spectra of polypeptides with extensive α-helical structure have two characteristic minima near 208 and 222 nm; β-sheet structure yields a minimum at 215 nm; and random coil is characterized by a negative peak in the vicinity of 200 nm and low ellipticity at 222 nm. The CD spectrum of full-length Nup2p shows an intense minimum at 202 nm that indicates a large unordered contribution (Fig. 4B). However, Nup2p was not composed entirely of random coil as the minimum was shifted from 200 to 202 nm; additionally, the slight negative ellipticity at 222 nm points to a small but detectable contribution of α-helical structure.

The frequency and distribution of the apparent unordered and ordered secondary structure (α-helical and β-sheet structures) cannot be determined from the CD spectrum of full-length Nup2p. For example, the ordered secondary structure contribution could be manifested in at least two possible ways: (i) there may be a propensity for the entire length of Nup2p to form short, unstable segments of secondary structure; or (ii) small, specific domains of Nup2p may form relatively stable ordered secondary structure (α-helices and β-strands) with intervening domains that were primarily unordered. To distinguish between the two possible Nup2p conformations, fragments of Nup2p were also analyzed by CD (Fig. 4, A–E). The N terminus of Nup2p (AA 1–185), which binds directly to Kap60p and targets Nup2p to the NPC (26, 27), contains a higher fraction of ordered secondary structure than full-length Nup2p as indicated by the emergence of a second minimum at 222 nm (Fig. 4C). The C-terminal fragment of Nup2p (AA 562–720), which includes the Gsp1p-binding domain (30), contains less ordered secondary structure than the N terminus, albeit more than the full-length protein as evidenced by a weak second minimum at 222 nm (Fig. 4E). Despite the content of secondary structure in the N- and C-terminal fragments of Nup2p compared with full-length Nup2p, both termini contained significant contributions of disorder as indicated by their minima at 207 and 204 nm, respectively. In contrast, the large FXFG region of Nup2p (AA 186–561) that binds Kap95p/Kap60p heterodimers (Fig. 4A) displays an intense peak of negative ellipticity at 200 nm characteristic of random coil (Fig. 4E). Thus, full-length Nup2p appears to be composed of N- and C-terminal domains with small contributions of ordered secondary structure, and an extensive FXFG region that was highly unordered. This indicates that the majority of Nup2p was similar to the coil-like class of natively unfolded proteins, which contain mostly random coils (38).

A recent analysis of native coil proteins and native pre-molten globule proteins established a clear relationship between the molecular weight of proteins from each conforma-
All far-UV CD spectra were measured at 25 °C. Nup2p (AA 186–720) of the spectra. Vettes with path lengths of 1 mm for Nup2p (0.022 mg/ml) and Nup2p (AA 186–561) (0.50 mg/ml). Note the different scales of the spectra.

**Fig. 4.** Far-UV CD spectroscopy of Nup2p and Nup2p fragments shows extensive regions of structural disorder. A, schematic drawing of Nup2p, its domains, and some of its known and predicted binding partners. B–E, CD spectra. B, full-length Nup2p. C, the N terminus of Nup2p (AA 1–185). D, the middle FxFG region of Nup2p (AA 186–561); and E, the C terminus of Nup2p (AA 562–720). All far-UV CD spectra were recorded at 25 °C at neutral pH in cuvettes with path lengths of 1 mm for Nup2p (0.022 mg/ml) and Nup2p (AA 562–720) (0.11 mg/ml) or 0.1 mm for Nup2p (AA 1–185) (0.69 mg/ml) and Nup2p (AA 186–561) (0.50 mg/ml). Note the different scales of the spectra.

FTIR spectroscopy also provides a powerful method for the quantification of protein secondary structure. The primary advantage of FTIR in comparison with CD spectroscopy was that it could identify and quantify protein secondary structure. The primary advantage of FTIR in comparison with CD spectroscopy was that it could identify and quantify protein secondary structure.

**TABLE II**

| Conformation state | Stokes radius (Å) |
|--------------------|-------------------|
| Natively folded    | 35 ± 1            |
| Molten globule     | 38 ± 1            |
| Pre-molten globule (PMG) | 51 ± 1          |
| Natively unfolded (coil-like) | 73 ± 1         |
| Natively unfolded (PMG-like) | 54 ± 1          |

Because disordered proteins frequently acquire folded structure when in complex with other proteins (39, 44), it is possible that Nup2p folds upon binding Kap95p and Kap60p, its likely partners in the cell (31, 45). However, the binding of Kap95p-Kap60p heterodimers failed to improve the resistance of Nup2p to the protease treatment (Fig. 6B), suggesting that bound Kaps do not alter the flexibility of Nup2p or cause it to fold. As expected, the presence of Kap60p or Kap95p monomers also failed to change the sensitivity of Nup2p to the protease treatment (data not shown). Both Kap95p and Kap60p are natively folded proteins with high α-helical content (40, 41) and were resistant to proteinase K digestion in these experiments (Fig. 6B and data not shown).

It is also possible that Nup2p folds when bound to Nup60p in the NPC. Previously, it was shown that Nup2p is bound to NPCs in purified yeast nuclei (32). To test the protease sensitivity of Nup2p in its native context within intact NPCs, we exposed purified yeast nuclei to proteinase K and probed Western blots with antibodies against full-length Nup2p and other Nup2p proteins. Endogenous Nup2p was highly sensitive to proteinase digestion (Fig. 6, C and D), indicating that Nup2p retains its flexible and disordered characteristics even when incorporated into the yeast NPC. By comparison, endogenous Kap95p, Kap60p, Nup85p, and Pom152p (all of which co-purify with the nuclei) were highly resistant to proteinase K digestion (Fig. 6, C and D). Note that Nup85p was partially clipped by proteinase K (Fig. 6D, third panel) but not fully digested (Fig. 6, C and D). This indicates that proteinase K can access Nup85p within the nuclei, but that its structure or interactions with other proteins prevents digestion. Although indirect, these results demonstrate that the structure of Nup2p was highly flexible in purified form and its native context within the yeast NPC, supporting the conclusion that Nup2p was natively unfolded.

**Heat Treatment of Nup2p Does Not Affect Its Binding Properties**—Unlike most natively folded proteins, intrinsically disordered proteins retain their activity and are not denatured following high temperature incubations (39). To test its thermotolerance, Nup2p was heated to 90 °C for 1 h. Pre-heated Nup2p was then incubated with GST, GST-Kap95p, or GST-Nup60p conjugated to glutathione-Sepharose beads to test its binding activity in solution (Fig. 7). As expected, the heat-treated Nup2p bound equally well to GST-Kap95p and GST-Nup60p as compared with untreated Nup2p. Neither Nup2p nor heat-treated Nup2p bound to GST (data not shown). The ability of Nup2p to retain its binding activities following extreme heat treatment was consistent with its classification as a natively unfolded protein.
**DISCUSSION**

Previous structural and physical analyses of nucleoporin and nucleoporin-containing complexes have been limited to electron microscopic and atomic force microscopic visualization of intact NPCs and purified Nups and Nup complexes (4, 46–52). Although these images illustrate the overall shape of the NPC and Nups, they are of insufficient resolution to reveal the structural nature of nucleoporins. We therefore subjected Nup2p to biophysical, structural, and conformational analyses to gain insight into the structure of nucleoporins. We specifically chose Nup2p as a model nucleoporin because: (i) it contains a large, characteristic domain of FG repeats (and thus belongs to the family of FG Nups); (ii) much is known about its biochemical and genetic interactions; and (iii) Nup2p exhibits a dynamic interaction with the NPC, existing in both NPC-bound and freely diffusible/mobile forms (25, 31, 32). Our *in vitro* studies of purified Nup2p are particularly relevant to the structure of Nup2p when it is in its mobile form as it is possible (although not certain) that Nup2p undergoes structural changes upon incorporation into the NPC. The findings described here constitute the first detailed structural and physical analyses of a full-length nucleoporin.

**Nup2p Is a Natively Unfolded Protein**—Intrinsically unordered or natively unfolded proteins are identified by low hydrophobicity, high net charge, low compactness, absence of globularity, lack of secondary structure, and high flexibility (38, 39, 42–44). Consistent with these characteristics, Nup2p contains a very low frequency of hydrophobic amino acids (such as Trp, Tyr, and Cys), which contributes to its low overall hydrophobicity (Fig. 3). The under-representation of hydrophobic amino acids in a protein may inhibit formation of a compact hydrophobic core (38, 39, 42, 43), resulting in unexpectedly large hydrodynamic dimensions. Indeed, gel filtration and sucrose gradient experiments show that Nup2p possesses a large Stokes radius (78–100 Å) and a small coefficient of sedimentation (2.9 S), characteristics that are indicative of low compactness (Figs. 1 and 2). CD and FTIR measurements demonstrate that Nup2p and particularly its middle FxFG region (AA 186–561) are highly disordered, lacking extensive contributions of ordered secondary structure (Figs. 4 and 5). Finally, Nup2p is hypersensitive to proteinase K digestion, consistent with a highly flexible tertiary structure (Fig. 6A). It remains possible that Nup2p acquires structure in complex with binding partners in its cellular context(s) (44). However, the high protease sensitivity of native Nup2p within purified nuclei suggests that Nup2p retains considerable disorder and flexibility even when bound to NPCs (Fig. 6, C and D).

The conclusion that Nup2p behaves as a natively unfolded protein was unexpected and provocative. As discussed below, the structural disorder of Nup2p has interesting implications regarding its function and the mechanism of trafficking across NPCs. It also raises the possibility that other Nups may be natively unfolded.

**Why Is Nup2p Natively Unfolded?**—Computer analyses of the yeast proteome predict that 30% of *S. cerevisiae* proteins contain disordered domains of at least 50 amino acids in length (39). Among the hundreds of proteins and protein fragments that have been characterized as intrinsically disordered, there is in an eclectic range of cellular functions including transcription factors, cytoskeletal components, heat shock proteins, chromosome-binding proteins, hormone receptors, and more (39, 43, 44). The evolutionary impetus for the emergence of natively unfolded proteins is not clear; yet, natively unfolded proteins share functional commonalities that suggest at least two significant advantages of structural disorder.

First, natively unfolded proteins often contain multiple binding domains and are thus capable of simultaneous interactions with multiple protein partners (39, 43, 44, 53). Nup2p binds directly to Kap60p, Kap95p, Gsp1p-GTP, Nup60p, and Prp20p (26–28, 30, 31). Furthermore, Nup2p binds simultaneously to Nup60p, Gsp1p-GTP, and Kap60p to form a tetrameric complex that facilitates Cse1p-mediated export of Kap60p (31). The flexible, unfolded structure of Nup2p may allow it to serve as a scaffold for the assembly of multisubunit complexes. Additionally, the structural disorder of Nup2p may be related to its ability to assemble onto the NPC during the biogenesis of NPCs.

Second, disorder can dramatically affect association and/or dissociation rates of the protein with binding partners. For example, natively unfolded proteins often associate rapidly with binding partners. Although their large hydrodynamic dimensions slow down diffusion, their size provides a large target for initial molecular collisions that facilitate association with binding partners (39, 54). More importantly, the lack of rigid binding pockets permits multiple approach orientations for a binding partner that may increase the probability of productive interactions (39). Rapid association and dissociation rates may be an important feature for interactions of Nup2p with its binding partners. For example, Nup2p displays KaRF activity, as it accelerates the dissociation rates of import cargoes containing the classic nuclear localization signal from Kap60p (7). As disassembly of import complexes is rate-limiting in the Kap95p-Kap60p-dependent import pathways, fast binding of Nup2p to Kap60p cargo
complexes is likely necessary to ensure rapid release of cargo to the nucleus and rapid recycling of Kap60p to the cytoplasm. Thus, large hydrodynamic size and high flexibility of Nup2p may allow it to perform its KaRF activity efficiently at the nuclear basket structure.

**Is Nup2p Structure a Model for Other Nups?**—A similar biophysical analysis of other yeast nucleoporins to determine whether intrinsic disorder is a common characteristic of Nups is currently underway. Because the middle FxFG region of Nup2p (AA 196–561) shows extensive disorder (Fig. 4), we hypothesize that other Nups containing FG repeat regions will also prove to be unfolded under physiological conditions. Interestingly, the composite cryo-EM images of Xenopus NPCs suggest that disorder is a general feature of Nups that comprise the central transporter region of the NPC. In these images, an unresolved amorphous protein “plug” occupies the central conduit, reflecting either a heterogeneity of cargoes within the transporter or a structural disorder of the Nups in that region (47). As discussed below, Nup disorder could explain some issues regarding the mechanism of facilitated trafficking across the NPC.

Our “natively unfolded Nups” hypothesis supports the current “entropic exclusion” and “selective phase partitioning” models of nuclear transport (3, 5, 55). In these models, nucleoporins are hypothesized to form a barrier meshwork that excludes most macromolecules larger than a threshold size from entering the NPC (3, 5). Only molecules (such as karyopherins) exhibiting physical characteristics compatible with the meshwork hydrophobicity can penetrate the barrier and diffuse to the opposite side of the NPC (55). Natively unfolded Nups would be ideal building blocks for this barrier meshwork because their flexible and unfoldable structures could re-arrange, contort, and re-pack to accommodate a wide range of sizes for karyopherin-cargo complexes. A meshwork barrier composed of unfolded Nups could be maintained behind and in front of the Kap-cargo complexes as they displace Nups during facilitated transport, thereby preserving the diffusional barrier.

Finally, the intrinsic disorder of Nups may reconcile two seemingly contradictory observations: (i) the fast rate of karyopherin movement across the NPC (1–10^3 molecules/NPC/s); and (ii) the high binding affinity between certain Kaps and Nups (7, 55–58). The former observation implies that Nup-Kap interactions must be transient (and therefore of low affinity) to support rapid rates of transport. However, in vitro studies with purified proteins have shown that Kap-Nup interactions can be of very high affinity with dissociation constants (K_D values) in the nanomolar and even picomolar range (7, 56). Assuming typical association rates for protein-protein interactions, the high affinity measured between Kaps and Nups predict slow

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dissociation rates that are incompatible with the rapid rates of nuclear transport. To explain the rapid flux of cargo through NPCs, it has been hypothesized that Gsp1p-GTP destabilizes Nup-Kap complexes (6). We suggest that structural disorder within Nups also facilitates fast transport rates. Natively unfolded proteins and their binding partners often have unexpectedly fast association and dissociation rates (39, 53). Consistent with these observations, Kap95p-Kap60p heterodimers dissociate from the yeast FG nucleoporin Nup1p, a functional homolog of Nup2p (25), much faster than predicted for their high affinity of interaction ($K_D \approx 10^{-11}$ M) (7). Kap60p heterodimers would be expected to dissociate from Nup1p with a half-life of minutes to hours assuming typical association rates ($10^7$ to $10^8$ M$^{-1}$ s$^{-1}$) (54); yet, the measured half-life of Kap95p-Kap60p-Nup1p complexes was $\approx 21$ s and their association rate approached the limits set by diffusion (7). Thus, despite the high affinity between Nups and Kaps, their interactions may be transient as a result of fast association and dissociation reactions.

The mechanism of how Kaps move cargoes rapidly across the NPC remains a central question in the study of nucleocytoplasmic trafficking. As the physical and structural analyses of yeast Nups continue, it will be interesting to determine whether the disordered structure of nucleoporins is indeed intimately related to the mechanism of nuclear trafficking.

REFERENCES

1. Nakielny, S., and Dreyfuss, G. (1999) Cell 99, 677–690
2. Pemberton, L. F., Blobel, G., and Rosenblum, J. S. (1998) Curr. Opin. Cell Biol. 10, 392–399
3. Rout, M. P., and Aitchison, J. D. (2000) Curr. Opin. Cell Biol. 12, 223–234
4. Yang, Q., Rout, M. P., and Akey, C. W. (1998) Mol. Cell 2, 317–326
5. Rout, M. P., Aitchison, J. D., Suprapto, A., Hjertaas, K., Zhao, Y., and Chait, B. T. (2000) J. Biol. Chem. 275, 635–641
6. Rexach, M., and Blobel, G. (1995) Cell 83, 683–692
7. Gilchrist, D., Mykytka, B., and Rexach, M. (2002) J. Biol. Chem. 277, 18161–18172
8. Shah, S., and Forbes, D. J. (1998) Curr. Biol. 8, 1376–1386
9. Kehlenbach, R. H., Dickmanns, A., Kehlenbach, A., Guan, T., and Gerace, L. (1999) J. Cell Biol. 145, 645–657
10. Ullman, K. S., Shah, S., Powers, M. A., and Forbes, D. J. (1999) Mol. Biol. Cell 10, 649–664
11. Yaseen, N. R., and Blobel, G. (1999) J. Biol. Chem. 274, 26493–26502
12. Gerlich, D., and Kuriyan, J. (2000) Annu. Rev. Cell Dev. Biol. 15, 607–660
13. Maiti, I. W., and Englemier, L. (1998) Annu. Rev. Biochem. 67, 265–306
14. Wozniak, R. W., Rout, M. P., and Aitchison, J. D. (1998) Trends Cell Biol. 8, 184–188
15. Allen, N., Huang, L., Burlingame, A., and Rexach, M. (2001) J. Biol. Chem. 276, 28268–28274
16. Rud, A., Moore, M. S., and Blobel, G. (1995) Cell 81, 215–222
17. Bayliss, R., Littlewood, T., and Stewart, M. (2000) Cell 102, 99–108
18. Gerlich, D., Pante, N., Kuriy, U., Aebi, U., and Blobel, G. (1996) EMBO J. 15, 5584–5594
19. Fornerod, M., Ohno, M., Yoshida, M., and Mattaj, I. W. (1997) Cell 90, 1051–1065
20. Bischoff, F. R., and Ponstingl, H. (1991) Nature 354, 80–82
21. Akhtar, N., Hagen, H., Lopilato, J. E., and Corbett, A. H. (2001) Mol. Genet. Genomes. 265, 861–864
22. Gerlich, D., Kuriyan, J., and Blobel, G. (1997) EMBO J. 16, 6535–6547
23. Corbett, A. H., Koppel, D. M., Schlenstedt, G., Lee, M. S., Hopper, A. K., and Silver, P. A. (1995) J. Cell Biol. 130, 1017–1026
24. Becker, J., Melchior, F., Gerke, V., Bischoff, F. R., Ponstingl, H., and Wittighofer, A. (1995) J. Biol. Chem. 270, 11860–11865
25. Leb, J. D., Davis, L. I., and Fink, G. R. (1993) Mol. Biol. Cell 4, 209–222
26. Booth, J. W., Belanger, K. D., Sanella, M. I., and Davis, L. I. (1999) J. Biol. Chem. 274, 32360–32367
27. Solsbacher, J., Maurer, P., Vogel, F., and Schlenstedt, G. (2000) Mol. Biol. Cell. 11, 4868–4879
28. Hood, J. K., Casolari, J. M., and Silver, P. A. (2000) J. Cell Sci. 113, 1471–1480
29. Hellmuth, K., Lauer, D. M., Bischoff, F. R., Kunzler, M., Hury, E., and Simos, G. (1998) Mol. Cell. Biol. 18, 6374–6386
30. Dingwall, C., Kandeles-Lewis, S., and Seraphin, B. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7525–7529
31. Denning, D., Mykytka, B., Allen, N., Huang, L., Burlingame, A., and Rexach, M. (2001) J. Cell Biol. 154, 937–950
32. Dilworth, D. J., Suprapto, A., Pavol, J. C., Chait, B. T., Wozniak, R. W., M. P. R., and Aitchison, J. D. (2001) J. Cell Biol. 153, 1465–1478M. R.
33. Siegel, L. M., and Monty, K. J. (1966) Biochim. Biophys. Acta 112, 346–362
34. McEwen, C. R. (1967) Anal. Biochem. 20, 114–149
35. Oberg, B., Chapman, B. W., and Fink, A. L. (1994) Biochemistry 33, 2628–2634
36. Oberg, B. A., and Fink, A. L. (1998) Anal. Biochem. 256, 92–106
37. Aris, J. P., and Blobel, G. (1991) Methods Enzymol. 191, 735–749
38. Uversky, V. N. (2002) Eur. J. Biochem. 269, 1–10
39. Dunker, A. K., Lawson, J. D., Brown, C. J., Williams, R. M., Romero, P., Oh, J. S., Oldfield, C. J., Campen, A. M., Ratliff, C. M., Hips, K. W., Ausio, J., Nissen, M. S., Reeves, R., Kang, C., Kissinger, C. R., Bailey, R. W., Griswold, M. D., Chiu, W., Garner, E. C., and Obradovic, Z. (2001) J. Mol. Graph. Model. 19, 26–59
40. Conti, E., Uy, M., Leighton, L., Blobel, G., and Kuriyan, J. (1998) Cell 94, 193–204