Molecular Determinants of Binding between Gly-Leu-Phe-Gly Nucleoporins and the Nuclear Pore Complex*

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The vertebrate nucleoporin Nup98 can be expressed in two distinct forms from differentially spliced mRNAs, either as a 98-kDa protein or as the 195-kDa Nup98/Nup96 polyprotein. Both forms undergo autoproteolytic processing to generate the 90-kDa Nup98 and either an 8-kDa tail or the nucleoporin Nup96. An equivalent cleavage event occurs in one yeast ortholog, Nup145, to produce Nup145N and Nup145C. We previously proposed that Nup145N, and possibly the other orthologs Nup116 and Nup100, might bind to Nup145C as demonstrated for Nup98 and Nup96. Here we have further investigated the interaction of both yeast and vertebrate Gly-Leu-Phe-Gly nucleoporins with the nuclear pore. We find that dynamic Nup98 binding can be recapitulated in vitro and that simultaneous translation and folding as a polypeptide are not required to allow subsequent binding between Nup98 and Nup96. We show that Nup145N and Nup145C do indeed bind to each other, and we have determined the dissociation constants for these interactions in vitro. Additionally, we characterize two sites of molecular interaction for each binding pair. Of the yeast orthologs, Nup116 binds far less robustly to Nup145C than does Nup145N, and Nup100 binding is barely detectable. Thus, we conclude that Nup116 and Nup100 likely use means of incorporation into the nuclear pore complex that are distinct from those used by Nup145N.

Transport between the nucleus and cytoplasm of a eukaryotic cell is mediated by nuclear pore complexes (NPCs), highly selective gateways embedded in the nuclear envelope. NPCs are composed of about 30 different protein species, known as nucleoporins or Nups. Each NPC has numerous copies of each nucleoporin and an approximate total mass of 40 or 60 MDa in yeast or metazoans, respectively.

Structural studies, most recently by cryoelectron microscopy, have revealed with considerable detail an NPC with 8-fold symmetry about an axis perpendicular to the plane of the envelope, being enriched on but not exclusive to one face or the other. Most of the mass of the complex consists of rings on each face of the nuclear envelope with spokes in between. Fibrils extend from the rings into either the cytoplasm or nucleoplasm and on the nucleoplasmic face join to form a basket-like structure. Nups of the core region are distributed symmetrically with respect to the plane of the envelope, whereas the cytoplasmic and nuclear fibrils are each composed of a distinct set of nucleoporins. In yeast, a few Nups have been shown to have a biased distribution relative to the nuclear envelope, being enriched on but not exclusive to one face or the other.

Many Nups associate into subcomplexes that act as building blocks in NPC assembly, underscoring the modular nature of this symmetrical structure (2, 5, 6). Additionally there are interspersed throughout the NPC a class of Nups containing repeat domains, each with many copies of the sequence Phe-Gly (FG) or variants of this sequence such as FXFG (were X is any amino acid) or Gly-Leu-Phe-Gly (GLFG). These domains function as natively unstructured regions responsible for interaction with transport receptors (7, 8), for the permeability barrier of the NPC (9, 10), and for the association of some nucleoporins with the pore (11, 12).

The preceding findings present a static, architectural picture of the NPC. However, more recent studies in vertebrate cells indicate that NPC structure is, at least in part, dynamic. Although many Nups reside stably in the pore, others associate more transiently, and, in some cases, shuttle between nuclear and cytoplasmic compartments (13, 14). To fully understand the function of the NPC, it will be necessary to characterize the molecular structures of the individual nucleoporins and the nature of the interactions between them, both stable and dynamic.

One case in which interaction between two nucleoporins has been described at the molecular level is the pore-targeting interaction of the Nup98 C-terminal domain with Nup96. Nup98 is the sole GLFG repeat nucleoporin in metazoans, although there is a family of orthologs in Saccharomyces cerevisiae, Nup145, Nup116, and Nup100. Nup98 is found on both cytoplasmic and nucleoplasmic faces of the NPC and is a relatively dynamic Nup (13, 14). Nup98 can be translated from two major alternatively spliced mRNA transcripts. The shorter transcript encodes a 920-amino acid, 98-kDa protein. The larger transcript encodes a 195-kDa Nup98/Nup96 polyprotein (Fig. 1). Post-translationally, both proteins are cleaved by cis-autoproteolysis to yield an N-terminal cleavage product of 90 kDa (generally referred to as Nup98) and a C-terminal cleavage product of either a 57 amino acid peptide (referred to here as the Nup98 "tail") or the nucleoporin Nup96, which is identical.
in its first 51 amino acid residues to the Nup98 tail (15). An equivalent cleavage event takes place in the yeast ortholog, Nup145, yielding the nucleoporins Nup145N and Nup145C (16, 17). Two other yeast orthologs, Nup100 and Nup116, are homologous only to the N-terminal cleavage product, Nup98, and do not carry out autoproteolysis.

The targeting of Nup98 to the NPC is, in part, through binding between its C-terminal domain and Nup96 (18–20), and in keeping with this, the products of Nup98 autoproteolysis can bind each other noncovalently in vitro. Because of the sequence identity between the Nup98 tail and Nup96, both bind identically to Nup98 and can compete with each other for binding (18). Therefore in vivo, if Nup98 is expressed from the smaller transcript, the tail peptide must be released to allow Nup98 to associate with Nup96 at the NPC (18, 19). Furthermore, although Nup96 incorporates into a large and highly stable subcomplex of the NPC, Nup96 expressed in vivo independently of Nup98 did not enter the nucleus and did not incorporate into NPCs with normal efficiency (15). Thus, proper assembly of Nup98 and Nup96 into the NPC requires a complex series of interactions and processing. The crystal structure of the Nup98 C-terminal domain revealed molecular details of the binding interaction between Nup98 and Nup96. Binding is mediated in large part by interactions between the first seven residues of the tail/Nup96 and a binding groove in Nup98 (19). In addition, a run of acidic residues in the tail/Nup96, although not directly visible in the structure, were constrained to lie in proximity to a basic patch in Nup98; electrostatic interactions in this region would further contribute to binding between Nup98 and Nup96.

Amino acids involved in both autocatalysis and binding are generally conserved between yeast Nup145 and human Nup98. We, therefore, postulated that Nup145N and Nup145C were likely to interact analogously at the yeast NPC (19). Although Nup116 and Nup100 lack residues required for autocatalytic cleavage, molecular modeling predicts some structural similarity to both Nup98 and Nup145N, including conservation of the patch of basic amino acids. Thus, Nup145C might act also as a binding site for Nup116 and Nup100 in addition to Nup145N.

Here we have further investigated the nature of the interactions between these GLFG nucleoporins and the NPC. We find that the dynamics of the Nup98/Nup96 interaction can be recapitulated in vitro using purified protein domains, indicating that additional factors are not required to facilitate such exchange. Binding can occur between the independently expressed Nup98 pore-targeting domain and the Nup98 tail/Nup96 fragment, suggesting that simultaneous translation and folding are not required to produce the appropriate structural folds. Using fluorescence polarization, we have determined the binding constant for this interaction. Furthermore, we extended our analysis to the yeast GLFG orthologs and find that, as expected, Nup145N utilizes the same mechanism for interaction with Nup145C. However, despite sharing considerable sequence homology, Nup116 binds far less robustly to Nup145C, and significant binding between Nup100 and Nup145C was not detected, suggesting that these nucleoporins use, at least in part, distinct means of incorporation into the NPC.

**EXPERIMENTAL PROCEDURES**

**Production of Recombinant Proteins**—For expression of GST fusions, fragments of Nup98, Nup145, Nup100, or Nup116 genes were cloned into pGEX-4T vectors and expressed in BL21(DE3) cells induced at either 37 °C or 17 °C overnight. Frozen cell pellets from 1 liter of culture were resuspended in binding buffer 1 (BB1; 10 mM Tris, pH 8.0, 100 mM NaCl, 1 mM β-mercaptoethanol, 1 mM EDTA) with 0.1% Triton X-100 and COMPLETE protease inhibitors (Roche Applied Science) to a final volume of 15 ml/liter of culture and lysed in a French press. Insoluble material was removed by centrifugation, and clarified lysates were either frozen for later use or incubated with glutathione-Sepharose (GE Healthcare) at 700 μl of beads/liter of culture for 1 h at 4 °C. The beads were washed extensively, first in BB1 with 0.1% Triton-X and then in BB1 alone. Bound fusion proteins were eluted with 100 mM reduced glutathione in 100 mM Tris, pH 8.0, 120 mM NaCl, and dialyzed against BB1. Products were analyzed by SDS-PAGE and Coomassie Blue staining and judged to have purity in excess of 90%. Purified proteins or clarified lysates were stored in aliquots at −80 °C after addition of glycerol to 7% final concentration.

To produce C-terminal GFP fusions, fragments of Nup98, Nup98/96, Nup145, or Nup145C were cloned upstream of a modified GFP2 gene (21) in pET-28a and expressed in BL21(DE3) cells induced at 37° for 3 h. For purification of GFP-hexahistidine-tagged proteins, frozen cell pellets were resuspended in nickel binding buffer consisting of 50 mM sodium phosphate, pH 7.4, 250 mM NaCl, 0.1% Triton X, 1 mM phenylmethylsulfonyl fluoride and lysed in a French press. After the pelleting of insoluble material, the protein was bound to a HiTrap chelating column (GE Healthcare) and eluted with a gradient of 0–500 mM imidazole in nickel binding buffer. Fractions containing the protein were pooled and dialyzed against BB1 containing 10% glycerol, separated into aliquots, and stored at −80 °C. Protein concentrations were determined using the Bio-Rad Protein Assay with bovine serum albumin as a protein standard.

**Mutagenesis**—Site-directed mutagenesis was performed using QuikChange mutagenesis (Stratagene; Austin, TX), and mutations were verified by sequencing. Mutant proteins were expressed and purified as described above.

**Bead Binding Assays**—For trans binding assays, 75 μg of purified GST-fusion protein and 37.5 μl of washed glutathione-Sepharose beads in a total volume of 250 μl of BB1 were incubated for 75 min at 4 °C. After incubation, beads were pelleted and washed 3 times in cold BB1 with 0.1% Triton X and once with BB1 alone. 1.5 mg of purified tail-GFP. His-tagged protein in 500 μl of BB1 was then added to the washed beads and incubated for 3 h at 4 °C to allow binding. After incubation, beads were pelleted and washed once in BB1 with 0.1% Triton-X and 3 times with BB1 alone. Bound proteins were then eluted by boiling in Laemmli gel sample buffer (22) and analyzed on 14% acrylamide gels followed by Coomassie Blue R250 staining. Final loads were adjusted to give approximately equal amounts of GST head protein.
Binding between GLFG Nucleoporins and the NPC

For exchange binding assays, 37.5 μl of washed beads were added to 500 μl of clarified lysate containing GST fusion protein, corresponding to 80 ml of expression culture. These samples were incubated to allow binding and washed in the same manner as the trans binding assays. 1.5 mg of purified GFP-His-tagged protein in 1 ml of BB1 was then added to the washed beads, and the samples were incubated, washed, and processed in the same manner as trans binding assays.

For immunoblots of bead binding assays, proteins were transferred from polyacrylamide gels to Immobilon membranes (Millipore; Billerica, MA), and tail fragments were detected using a mouse antibody against the hexahistidine tag (H-15; Santa Cruz Biotechnology; Santa Cruz, CA) and goat anti-mouse horseradish peroxidase conjugate (Zymed Laboratories Inc.; South San Francisco, CA).

Fluorescence Polarization Assays—For polarization assays, three fluorescently labeled peptides (sequences given in Table 1) were prepared by solid phase peptide synthesis at the Keck Biotechnology Resource Center at Yale University, New Haven, CT. After the actual Nup96 or Nup145C sequence, each peptide terminates with a diglycine linker followed by an ε-(5/6-carboxyfluorescein)-l-lysine residue, referred to as K(fam), and a final glycine required C-terminal to K(fam) in the synthetic process. High performance liquid chromatography analysis revealed no significant peaks other than those corresponding to the intended products.

To obtain binding curves, purified GST head fragments in concentrations ranging from 1 nM to greater than 50 μM were incubated at room temperature with 1.0 × 10⁻⁸ M fluorescein-labeled peptide in BB1 for at least 2.75 h to reach full equilibrium. Fluorescence polarization was measured at 25 °C over 15 read cycles on a Beacon 2000 Fluorescence Polarization System (Invitrogen). For each combination of head fragment and tail peptide, measurements were made at 20 or more different concentrations of head protein resulting from serial dilutions of 1:1.6. Fluorescent peptide was kept at 1.0 M and then incubated with a second form of this same Nup98 tail-GFP fragment was assessed by gel electrophoresis as an additional factor might be required to release the tail peptide. To test this we developed an exchange binding assay in which a GST-tagged Nup98 NPC-targeting domain including the cleavage site and tail peptide (amino acids 712–920; “GST-head-tail”) was bound to glutathione beads and then incubated with a second form of this same Nup98 domain in which the tail peptide was fused to GFP (Fig. 2A; “head-tail-GFP”). After incubation, the beads with bound GST-Nup98 head were retrieved, and the presence of the tail-GFP fragment was assessed by gel electrophoresis as an indication of dynamic exchange between the head and tail fragments. When the GST was fused to a wild type Nup98 head-tail construct, which could undergo autocatalytic cleavage to separate head and tail fragments, there was clearly dynamic exchange in which some of the original tail peptide was replaced by tail-GFP (Fig. 2A, lanes 3 and 4). When GST was fused to a mutant form of Nup98 (S864A), which we had previously shown to be inactive for autocatalytic cleavage and release of the tail (19), there was no exchange, and no tail-GFP was recovered on the beads (Fig. 2A, lanes 5 and 6). We concluded, therefore, that dynamic interaction between Nup98 and the tail/Nup96 residues down-app.
stream of the cleavage site did not require additional factors from the NPC; the interaction remained dynamic in vitro.

The autocatalytic activity of Nup98 identifies it as a member of a family of self-processing proteases that includes both intein and NTN proteins (for review, see Ref. 23). It has been suggested for some members of this family that expression as a polyprotein followed by autocatalytic cleavage to separate the components is necessary to obtain protein conformations that can only be achieved by simultaneous folding as a single polypeptide (24). Such conformations would not be obtainable from components expressed independently and then combined, as was shown in the case of glycosyl asparaginase (25). Similarly, simultaneous folding in the form of Nup98 with its tail peptide or the Nup98/Nup96 polyprotein might be essential for Nup98 and Nup96 to achieve conformations that allow correct recognition of each other as binding partners. To test this possibility, we used a trans binding assay (Fig. 2B) in which GST-Nup98 head truncated at the site of cleavage (amino acids 712–863) was bound to glutathione beads and incubated with independently expressed tail-GFP by PAGE and Coomassie staining. Expression in bacteria leads to removal of the initiator methionine, thus leaving the authentic serine as the first residue of the tail-GFP construct. When these separately produced partners were mixed, we found that the two components had each independently folded into a conformation that was compatible with binding; tail-GFP was retrieved on glutathione beads along with GST-head (Fig. 2B, lanes 2 and 3). As before, the non-cleavable mutant head, which cannot release the tail peptide, did not bind the tail-GFP protein (Fig. 2B, lanes 4 and 5).

FIGURE 2. Interaction between Nup98 and Nup96 is dynamic in vitro and does not require simultaneous folding in the form of the polyprotein precursor. A: top, schematic of the exchange assay. N-terminal GST-tagged Nup98 C terminus was immobilized on beads and incubated with a soluble version of the same region of Nup98 carrying a C-terminal GFP tag. The arrowhead indicates the position of autocatalytic cleavage. Bottom, retrieval of the GFP-tagged tail on GST-head beads indicates dynamic exchange between the two post-cleavage complexes. The GST-Nup98 C terminus (712–920; lanes 3 and 4) or an uncleavable mutant form of the same fusion protein (S864A; lanes 5 and 6) were immobilized on glutathione-Sepharose beads and incubated with buffer (lanes 3 and 5) or soluble Nup98 C terminus (712–920)-GFP (lanes 4 and 6). Bound proteins were analyzed by SDS-PAGE and Coomassie Blue stain. Lane 1, input Nup98 C terminus-GFP showing both autodigestion products. Lane 2, binding assay performed with empty beads. A small amount of breakdown product is always present in the uncleavable mutant preparation but does not correspond to cleavage at the autocatalytic site. The 8-kDa untagged tail protein is too small to appear in this 12% PAGE. B: top, schematic of trans binding assay. Immobilized GST-Nup98 C terminus head was incubated with soluble tail-GFP. Bottom, GST-Nup98 head (712–863; lanes 2 and 3) or the uncleavable mutant form as above (712–920, S864A; lanes 4 and 5) were immobilized on glutathione-Sepharose beads and incubated with buffer (lanes 2 and 4) or soluble Nup98 tail (864–920)-GFP. Bound proteins were analyzed by SDS-PAGE and Coomassie Blue stain. Lane 1, input tail protein. WT, wild type.
Nup98/Nup96 Binding Constant Determined by Fluorescence Polarization—Because we appeared to have recapitulated authentic dynamic binding in vitro, we next determined the binding constant for this interaction. For these measurements, we used fluorescence polarization assays in which GST-Nup98 head (amino acids 712–863) was incubated with a synthetic fluorescent peptide corresponding to the first 14 amino acids of the tail/Nup96 followed by a diglycine linker and a fluorescently labeled lysine residue (see the sequence in Table 1). This sequence includes the tail/Nup96 residues previously observed by crystallography to directly bind to Nup98 as well as the stretch of acidic residues we predicted to be positioned for interaction with a cluster of basic residues in Nup98 (Ref. 19 and Table 1). The binding curve obtained from the polarization assays indicated a dissociation constant of 0.11 μM (Fig. 3 and Table 1). When the tail peptide was shortened by removal of the acidic residues, the binding constant increased ~10-fold to 1.3 μM, indicating a significant contribution of the acidic residues to binding.

S. cerevisiae Nup145N and Nup145C Bind Each Other in a Manner Equivalent to Nup98/Nup96—In the budding yeast S. cerevisiae, Nup98 has three orthologs Nup145, Nup116, and Nup100 (26). Of these three proteins, only Nup145 undergoes autocatalytic cleavage to yield Nup145N and Nup145C. Nup145N resembles Nup98 in both the C-terminal and GLFG repeat domains, although it lacks a binding site for the mRNA export factor Rae1/Gle2 (Fig. 1). It is not established whether Nup145N interacts stably or dynamically with the yeast NPC; however, based upon our results with Nup98, we predicted that Nup145N would bind to the post-cleavage N terminus of Nup145C. To test this possibility, we expressed GST-Nup145N and Nup145C analogous to the GST-Nup98 head and Nup98 tail-GFP proteins and utilized these in both exchange (Fig. 4A, left panel) and trans (Fig. 4A, right panel) binding assays. Like Nup98, Nup145 fragments undergo dynamic interactions in vitro and are capable of binding each other efficiently when expressed in trans. As expected, the homologous uncleavable mutation in Nup145 (S606A) was completely unable to bind to the Nup145 tail-GFP in an exchange assay (Fig. 4A, lanes 4 and 5 and lanes 8 and 9).

To further test our prediction that the post-cleavage interaction between Nup145N and Nup145C is equivalent to the interaction seen for Nup98 and Nup96, we synthesized a fluorescent Nup145C peptide analogous to the Nup98 tail peptide for fluorescence polarization binding analysis (27). This peptide corresponded to the first 17 amino acids of Nup145C including the acidic residues (Table 1). Again, there was a clear contribution of the acidic residues to both these heterologous interactions since, in their absence, the affinity of binding between the Nup98 head and Nup98 tail was reduced 10-fold exactly as had been observed for binding between the Nup98 head and Nup98 tail (Fig. 3 and Table 1). This value is remarkably similar to that obtained from the Nup98 assays, suggesting an equivalent and conserved binding interaction. Indeed, we found that the Nup98 head could bind to the Nup145 tail with only slightly more than a 2-fold decrease in affinity compared with binding between the Nup98 head and Nup98 tail (Fig. 3 and Table 1). Conversely, the Nup145N head construct bound the Nup98 tail with virtually the same affinity as it bound its natural partner (Fig. 4B and Table 1). Again, there was a clear contribution of the acidic residues to both these heterologous interactions since, in their absence, the affinity of binding between the Nup145N head and the Nup98 tail was reduced 10-fold exactly as had been observed for binding between the Nup98 head and Nup98 tail.

Assessment of Binding between Nup116, Nup100, and Nup145—Based upon sequence homology between the C-terminal domains of Nup98 and the three GLFG nucleoporin orthologs in yeast, we had previously proposed that Nup145

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**TABLE 1**

| N-terminal fragment | C-terminal peptide | C-terminal peptide sequence | $K_d$ μM |
|---------------------|-------------------|-----------------------------|----------|
| Nup98               | Nup98/96 β-strand plus acidic residues | SKYGLQEDDEEDEEEGK*G          | 0.11 ± 0.01 |
| Nup98               | Nup98/96 β-strand only | SKYGLQEDDEEDEEEGK*G          | 2.6 ± 0.1   |
| Nup98               | Nup145 β-strand plus acidic residues | SIWGLVNEEDAEIDDDDKG*G        | 0.25 ± 0.01 |
| Nup145              | Nup145 β-strand plus acidic residues | SIWGLVNEEDAEIDDDDKG*G        | 0.12 ± 0.01 |
| Nup145              | Nup98/96 β-strand plus acidic residues | SKYGLQEDDEEDEEEGK*G          | 0.16 ± 0.01 |
| Nup145              | Nup98/96 β-strand only | SKYGLQEDDEEDEEEGK*G          | 1.6 ± 0.1   |
| Nup116              | Nup145 β-strand plus acidic residues | SIWGLVNEEDAEIDDDDKG*G        | 1.3 ± 0.1   |
| Nup100              | Nup145 β-strand plus acidic residues | SIWGLVNEEDAEIDDDDKG*G        | 36 ± 1      |
might provide a binding site for Nup100 and Nup116 in the yeast NPC. In particular, conservation among these proteins is striking in the cluster of basic residues that appear positioned to interact with the acidic residues of Nup145 near the cleavage site, with “overhangs” of three and four amino acids, respectively (Fig. 5A). To test our hypothesis that Nup100 and Nup116 bind to Nup145C, we expressed GST fusions to the C-terminal domains of Nup100 (amino acids 802–959) and Nup116 (amino acids 956–1113) and used these in exchange binding assays with the Nup145 head-tail-GFP protein as above. Surprisingly, neither Nup100 nor Nup116 displayed any detectable binding to the Nup145C tail (data not shown). On considering these results, we reasoned that the exchange binding reaction could in this case represent a form of competition assay with the Nup145N head present as an alternative and higher affinity interactor. We, therefore, carried out trans binding assays in which the Nup116 or Nup100 head was incubated with only the Nup145 tail-GFP. When the bound fractions were analyzed as above by Coomassie staining, a band at the position of the tail-GFP could now be detected binding to Nup116, albeit at significantly lower levels than were bound to Nup145 (Fig. 5B, upper panel). However, even using the greater sensitivity of immunoblotting, we could not detect an association between Nup100 and the Nup145 tail (Fig. 5B, lower panel lanes 4 and 5).

In the Nup98 crystal structure, the majority of binding between the Nup98 head and tail is mediated by the first five residues after the cleavage site (SKYGL); within this sequence YGL make extensive contacts with residues in the head domain (19). In Nup145C, this same position corresponds to the conserved sequence WGL and is expected to be similarly involved in binding between Nup145N and Nup145C. We, therefore, considered the possibility that the overhangs of Nup100 and Nup116 posed varying degrees of steric interference to binding the Nup145C tail. To test this possibility, we truncated each of the constructs at the equivalent of the Nup145 cleavage site, removing the final three or four amino acids, respectively. In additional constructs we also mutated the final amino acid to Phe to generate versions of Nup100 and Nup116 that paralleled Nup145N by terminating in the sequence His-Phe. For Nup116, these modifications had little to no effect on binding to Nup145N (Fig. 5C, lanes 7–9). In the case of Nup100, the modifications were unable to promote any interaction with Nup145C even when probed at the higher sensitivity of immunoblots (Fig. 5C, lanes 4–6, upper and lower panels).

To more quantitatively evaluate the relative interactions among the yeast nucleoporins, we tested for binding between the Nup100 and Nup116 heads and the Nup145C tail by fluorescence polarization and compared these results to those obtained with Nup145N head (Fig. 6). In keeping with the results observed in gel analysis of the trans binding assays, Nup116 bound to the Nup145 tail peptide with a binding constant that indicated 10-fold lower affinity than the homologous Nup145N-Nup145 tail peptide interaction ($K_D = 1.3 \mu M$ versus $0.16 \mu M$; Fig. 6 and Table 1). This is in very good agreement with a previous measurement using isothermal calorimetry; however, in that case, binding between Nup145N and Nup145C was not measured for comparison (28). Binding between Nup100 and the Nup145 tail peptide was substantially weaker. At the concentrations obtainable in our assay system we were unable to achieve saturation of binding; however, we estimated a dissociation constant of $\sim 36 \mu M$ for the interaction between Nup100 and Nup145. For comparison, this corresponds to 100–200-fold lower affinity than was observed for binding between the heterologous human and yeast partners and a more than 20-fold decrease in affinity relative to Nup116 binding to Nup145C (Table 1). Thus, although Nup145C may anchor some fraction of the Nup116 protein to the yeast NPC.
it seems unlikely that Nup145C is a significant binding site for Nup100.

**DISCUSSION**

Previously we showed that Nup98 binds to Nup96 at the NPC. This was unexpected since Nup98/Nup6 is synthesized as a polyprotein; it is, therefore, unclear why the components should be cleaved apart only to reassociate with each other. One clue to this is our finding that Nup98 is a dynamic component of the NPC. Nup98 can shuttle between nucleus and cytoplasm, and a fraction of the protein is found within in the nucleus. When at the pore Nup98 binds to Nup96, which itself is assembled into an extremely stable subcomplex found on both faces of the NPC. Therefore, to achieve dynamic interaction with the pore, Nup98 must be separable from Nup96.

Here we have further investigated the nature of the Nup98/Nup96 binding interface. We find that an N-terminal fragment of Nup96 exchanges readily between soluble and tethered forms of Nup98; thus, the binding between Nup98 and Nup96 is dynamic in vitro in the absence of other nucleoporins, transport factors, or cargo. The dissociation constant for the Nup98/Nup96 binding was determined by fluorescence polarization to be 0.11 μM. This moderate binding affinity is in keeping with the intermediate rate of exchange observed for Nup98 at the pore in vivo (13, 14). From the crystal structure of the complex we had previously proposed two points of interaction between these regions of Nup98 and Nup96. The electron densities of the first seven amino acids of the tail peptide/Nup96 were aligned as a third β-strand on one face of the Nup98-fold. Major interactions are formed between residues 3–5 (Tyr-Gly-Leu) and the side chains and peptide backbone of the adjacent β-strand and α-helix of Nup98. A second site of interaction was hypothesized to be formed by a series of acidic residues of Nup98 that, although not visible in the structure, were constrained to be centered over a patch of basic residues in Nup98. We found in the current study that these paired charge clusters do indeed contribute significantly to binding between Nup98 and Nup96. In the absence of the acidic residues of Nup96 the dissociation constant increased 10-fold. It is interesting to speculate that binding between these charge clusters might possibly have significance to the regulation of binding between Nup98 and the NPC. Nup98 is
known to be phosphorylated during mitosis when the NPC is disassembled (29). Indeed Nup98 is one of the relatively few nucleoporins dissociated from the NPC in *Aspergillus*, where the NPC is only partly disassembled (30). A serine is positioned within the basic region of Nup98 such that, if phosphorylated, it might disrupt the charge interaction and destabilize binding between Nup98 and Nup96. This possibility remains to be tested.

Expression of the Nup98/Nup96 polyprotein followed by autocatalytic cleavage is a very ancient mechanism. As early as *Dictyostelium*, organisms encode a single GLFG nucleoporin with homology to the Nup98/96 precursor including conserved catalytic residues. Only *S. cerevisiae* seems to have diverged to the extent of encoding a family of GLFG Nups, dividing among them various functions of Nup98. What is the advantage to producing Nup98 and Nup96 as a polyprotein only to separate these by subsequent cleavage? One possibility would be that simultaneous folding is required to achieve the appropriate structural conformation of Nup98 and/or Nup96. However, we found that, even when the two partners were separately expressed and purified, they retained the ability to bind to each other. Thus, proper folding, at least to the extent required for binding, can be achieved independently. An intriguing alternative is that co-expression could be used to regulate the relative stoichiometry of these two nucleoporins. The polyprotein ensures equal levels of the two proteins. Autocatalytic cleavage ensures that separation is highly efficient and not dependent upon the action of a *trans*-acting independent protease, in keeping with a critical requirement for separation. The level of Nup96 in turn must be coordinated in some way with the levels of other components of its highly stable subcomplex of the NPC (Nup160, Nup133, Nup107, sec13, seh1, Nup43, Nup37). RNA-mediated interference depletion experiments have shown that loss of one member of this complex can result in destabilization and degradation of at least some other members of the complex (31, 32). Human Nup98 was also found to be expressed as an independent protein derived from a differently spliced mRNA; this form of Nup98 must still undergo cleavage to release a C-terminal peptide and permit binding to Nup96. It is intriguing to speculate that the relative levels of Nup98 and Nup96 could, thus, be regulated through control of differential splicing, but levels of the different messages in various tissues have not yet been quantitated or compared.

Having determined the structure of the Nup98 C terminus, we predicted based upon sequence homology that Nup145 would adopt a similar fold and that Nup145C would provide a likely binding partner for 145N at the NPC. We further hypothesized that Nup116 and Nup100 possess sufficient homology to Nup145 and Nup98, particularly near the site of cleavage and in the cluster of basic residues, that they might serve as alternative binding partners for Nup145C. Here we have evaluated this binding through both *in vitro* pulldown and fluorescence polarization binding assays. As predicted, Nup145N and Nup145C bind each other with an affinity almost identical to that determined for Nup98 and Nup96. Indeed, the heterologous proteins (Nup145 “head” with human tail peptide or Nup98 head with Nup145C peptide) bind each other with only minimal reduction in affinity relative to their natural partner, underscoring the conclusion that these interactions are highly similar. In yeast, as in human, we found that the clusters of oppositely charged residues make a significant contribution to binding.

The possibility that Nup116 and Nup100 utilize Nup145C as a binding site at the pore was appealing. Their complementary distributions on the two faces of the NPC (Nup145N biased toward the nuclear face, Nup116 and Nup100 enriched on the cytoplasmic face) would result in a balanced occupancy of Nup145C sites. Several findings, however, suggest that the explanation may not be this simple. Both Nup116 and Nup100 were shown by two-hybrid analysis to bind Nup82, which is present only on the cytoplasmic face of the pore. Intriguingly, Nup145N did not bind to Nup82 in this study (33). We found that, relative to Nup145N, the binding affinities of Nup116 and Nup100 for Nup145C are 10- and 100-fold lower, respectively. Thus, although some Nup116 may bind to Nup145C, it seems unlikely that Nup100 would do so to any significant extent. Additionally, as demonstrated by our attempted exchange assay, Nup145N effectively out-competes Nup116 and Nup100 for binding to Nup145C *in vitro*. We propose that, like other functions of vertebrate Nup98, recognition of different binding partners at the NPC has been largely divided among the different yeast orthologs.

One potential explanation for the observed distribution of GLFG nucleoporins in yeast is that there are additional interactions with other Nups that stabilize the association of Nup116 and Nup100 at the nuclear face of the NPC. In keeping with this, a ts mutation in yeast Nup57 resulted in degradation of the centrally localized Nup57/Nup49/Nsp1 subcomplex and led to loss of Nup116, but not Nup145 or Nup82, from the NPC (34). Alternatively, given that little is known about possible dynamics of the yeast NPC, it could be that the observed distributions of Nup145N, Nup116, and Nup100 correspond only to a steady state snapshot of a changing, dynamic distribution of this GLFG nucleoporin family.
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Note Added in Proof—Similarly, Lutzmann et al. found an interaction between Nup145N and Nup157 (Lutzmann, M., Kunze, R., Stangl, K., Stelter, P., Töth, K. F., Böttcher, B., and Hurt, E. (2005) J. Biol. Chem. 280, 18442–18451). This heterodimer associated weakly with the highly stable Nup84 complex, most likely through the complex members Nup120 and Nup145C. However, they observed no interaction between Nup116 or Nup100 and either Nup157 or the Nup84 complex.

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