The GLFG Regions of Nup116p and Nup100p Serve as Binding Sites for Both Kap95p and Mex67p at the Nuclear Pore Complex*

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Our previous studies have focused on a family of S. cerevisiae nuclear pore complex (NPC) proteins that contain domains composed of repetitive tetrapeptide glycine-leucine-phenylalanine-glycine (GLFG) motifs. We have previously shown that the GLFG regions of Nup116p and Nup100p directly bind the karyopherin transport factor Kap95p during nuclear protein import. In this report, we have further investigated potential roles for the GLFG region in mRNA export. The subcellular localizations of green fluorescent protein (GFP)-tagged mRNA transport factors were individually examined in yeast cells overexpressing the Nup116-GLFG region. The essential mRNA export factors Mex67p, Mtr2p, and Dbp5p accumulated in the nucleus. In contrast, the localizations of Gle1, Gle2, and Gle3-GFP remained predominately associated with the NPC, as in wild type cells. The localization of Kap95p was also not perturbed with GLFG overexpression. Coimmunoprecipitation experiments from yeast cell lysates resulted in the isolation of a Mex67p-Nup116p complex. Soluble binding assays with bacterially expressed recombinant proteins confirmed a Mex67p-Nup116p complex. In vitro binding of Mex67p to the GLFG region. To map the Nup116-GLFG subregion(s) required for Kap95p and/or Mex67p association, yeast two-hybrid analysis was used. Of the 33 Nup116-GLFG repeats that compose the domain, a central subregion of nine GLFG repeats was sufficient for binding either Kap95p or Mex67p. Interestingly, the first 12 repeats from the full-length region only had a positive interaction with Kap95p, whereas the last 12 were only positive with Mex67p. Thus, the GLFG domain may have the capacity to bind both karyopherins and an mRNA export factor simultaneously. Taken together, our in vivo and in vitro results define an essential role for a direct Mex67p-GLFG interaction during mRNA export.

To move between the nuclear and cytoplasmic compartments of a eukaryotic cell, all molecules must pass through nuclear pore complexes (NPCs)1 embedded in the nuclear envelope. Ions, metabolites, and small proteins may diffuse through an ~9-nm aqueous channel in the NPC. In contrast, the movement of large macromolecules, including proteins and RNA, is energy-dependent and facilitated (reviewed in Refs. 1–3). The NPCs are built by the oligomerization of over 30 different polypeptides collectively referred to as nucleoporins (4, 6, 7). This includes three integral membrane proteins, a subset of proteins with predicted coiled-coil domains, several proteins with novel primary structure, and a family of 13 nucleoporins with phenylalanine-glycine (FG) repeat domains. All FG repeat domains share the common feature of multiple FG dipeptide repeats with variable length spacers (8). However, there are at least two distinct subclasses: 1) glycine-leucine-phenylalanine-glycine (GLFG) repeat domains, which are separated by spacers that lack acidic residues and are enriched in serine, threonine, glutamine, and asparagine residues, and 2) phenylalanine-amy amino acid-phenylalanine-glycine (FXFG) repeat domains, which have charged spacers. In vertebrates and yeast, different FG nucleoporins reside in each of the NPC substructures, and there is significant evidence for a direct involvement of FG nucleoporins in both nuclear import and export (4, 6, 9).

Recently, several reports have documented in vitro biochemical interactions between shuttling transport factors and individual components of the NPC (reviewed in Ref. 9). In particular, there has been considerable focus on the interactions between FG repeat nucleoporins and karyopherins (also known as importins/exportins). Karyopherins are a family of shuttling transport factors that recognize specific nuclear import or export signals in their respective cargo (reviewed in Refs. 2, 3, 10, and 11). All yeast FG nucleoporins have been shown to bind at least one karyopherin in vitro (reviewed in Ref. 9). Current models for the mechanism of docking and translocation through the NPC are based on direct karyopherin-nucleoporin binding. Karyopherins also interact with the small GTPase

Gal4DBD, GAL4 DNA binding domain; GFP, green fluorescent protein; GLFG, glycine-leucine-phenylalanine-glycine; GST, glutathione S-transferase; LexADBD, LexA DNA binding domain, MBP, maltose-binding protein; SC, synthetic complete; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol.

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Ran, which acts as a molecular switch regulating the association of karyopherins with their cargo and the NPC (2, 12). The crystal structures of different karyopherins (or karyopherin domains) complexed with either RanGTP or an FGF repeat region has suggested binding of RanGTP to importin-β when complexed with either RanGTP or an FGF repeat region has suggested binding of RanGTP to importin-β may induce a conformational change in importin-β that occludes the FGF repeat binding site. This would release importin-β from the nucleoporin. However, it is unclear if such a mechanism contributes to vectoral movement along the central axis of the NPC or to a terminal release step from the NPC. Several factors with specific roles in mRNA export have also been shown to physically or genetically interact with nucleoporins. For example, in S. cerevisiae, the RNA helicase Dbp5p directly associates with Nup159p (16). The mRNA export factor Gle2p physically associates with Nup116p (17, 18), and gle2 mutants are synthetically lethal with a nup100 null mutant (19). The essential factor Gle1p interacts with Dbp5p (20), and gle1 mutants are synthetically lethal with either rip1/nup42 or nup100 null mutants (19, 21). The essential mRNA export factor Mex67p forms a heterodimeric complex with Mtr2p, and Mtr2p mediates association with Nup85p (22). The vertebrate Mex67p homologue, TAP, directly associates with vertebrate Nup214p, Nup85p, and hCG1 (23, 24). Interestingly, all of these nucleoporins, except Nup85p, are GF family members. Taken together, it is intriguing that both karyopherins and mRNA export factors interact with GF nucleoporins.

Although much progress has been made in defining interactions between soluble transport factors and nucleoporins, the exact molecular mechanisms for translocation through the NPC are still unknown. To reveal critical events that mediate NPC translocation, we have analyzed the interface between dynamic transport factors and two homologous components of NPC translocation, we have analyzed the interface between soluble transport factors and nucleoporins, the GLFG nucleoporins Nup116p and Nup100p. Nup116p and Nup100p are localized on both sides of the NPC (6, 25), and their C-terminal regions interact with Nup100p. Nup116p and Nup100p are localized on both sides of the NPC. Our previous studies have documented an in vivo requirement for Nup116p and Nup100p in nuclear transport (19, 26–29). The unique N-terminal region of Nup116p binds the mRNA export factor Gle2p (17, 18), whereas the GLFG regions of Nup116p and Nup100p directly bind Kap95p (27, 28) and other karyopherins (20, 30–35). Interestingly, overexpression of the GLFG region inhibits mRNA export and cell growth (27). This suggests an essential role for the GLFG region in mRNA export. However, a direct role for karyopherins in mRNA export has not been documented. We have found that the Nup116p and Nup100p GLFG regions interact in vivo and in vitro with both the karyopherin Kap95p (27, 28) and the mRNA export factor Mex67p (this work). Furthermore, Kap95p and Mex67p bind to both common and distinct aspects of the Nup116p-GLFG region.

**MATERIALS AND METHODS**

**Yeast Strains and Plasmas**—All yeast strains used in this study are listed in Table I. The sequence encoding the green fluorescent protein (GFP) was fused in frame before the stop codons for the chromosomal MEX67, DBP5, GLE1, and MTR2 genes. This was achieved using the gene integration method of Baudin et al. (36). Respective polymerase chain reaction products were generated with oligonucleotides and a template containing genes for GFP and the Schizosaccharomyces pombe HIS3 (gfp-HIS3; gift of J. Atchison). The resulting DNA fragments were introduced into SWY518 by standard transformation methods and colonies selected on media lacking histidine. Correct integration was confirmed by immunoblot and direct fluorescence microscopy. The resulting strains were back-crossed to a wild type yeast strain, and the GFP-tagged prey gene was used in this study. For GFP tagging the genomic copy of GLE2, a similar strategy was used except the S. cerevisiae HIS3 gene served as the selectable marker, and the polymerase chain reaction fragment was transformed into the diploid SWY1920. The resulting diploid was sporulated and dissected to obtain SWY1920.

The plasmids used in this study are listed in Table II and were maintained in either BL21 (pSW156, pSW304, and pSW329) or DH5α (all others).

**Microscopic Localization of Yeast Strains Overexpressing Nup116p-GLFG**—pSW163 (Nup116p-GLFG under a galactose-inducible promoter), pSW384 (Nup100p-GLFG under a galactose-inducible promoter), or pNLS-E1 (vector) were transformed into the respective GFP-tagged strains by standard methods. Stationary phase cultures grown in synthetic complete (SC) media lacking uracil (ura) with 2% glucose were pelleted, washed once in an equal volume of SC–ura, 2% raffinose and used to inoculate SC–ura, 2% raffinose at an initial A600 of 0.015. When the A600 reached 0.1 to 0.2, galactose (J. T. Baker Inc.) was added to one-half of the culture at a final concentration of 2%. The raffinose and galactose cultures were incubated 5 h at 30 °C. A small portion of the live cells was removed for viewing direct fluorescence, and the remaining cells were prepared for indirect immunofluorescence microscopy as described previously (27, 37). To test for nucleolar fragmentation in the GLFG-overexpressing strains, fixed cells were incubated overnight with tissue culture supernatant monoclonal antibody D77 (38) (1:10) for detection of Nop1p. Bound antibody was detected with a rhodamine donkey anti-rabbit antibody (Chemicon; 1:400). For detection of Kap95p, a rabbit polyclonal antibody (Chemicon; 1:400). For detection of Kap95p, a rabbit polyclonal antibody (Jackson ImmunoResearch; 1:200). Images were collected with a × 100 objective on an Olympus BX50 microscope using a Dage-MTI CCD-300-RC camera (Dage MTI, Michigan City, IN).

**Whole Cell Lysates and Immunoprecipitations**—Whole cell lysates of SWY518 (MEX67) and SWY2131 (MEX67-GFP) were prepared in 20 mM Tris-HCl, pH 6.5, 150 mM NaCl, 5 mM MgCl2, 2% Triton X-100 by glass bead lysis. Immunoprecipitations were conducted as described previously (25). For immunoprecipitations, 1 μl of affinity-purified rabbit polyclonal anti-GFP antibody (gift from M. Linder) was used per 100 μl of cell extract. Blots were probed with an anti-GFP antibody at a 1:1000 dilution (16 h, 4 °C), affinity-purified rabbit polyclonal anti-Nup116p-GLFG antibody WU956 (39) at a 1:2000 dilution (16 h, 4 °C), or affinity-purified rabbit polyclonal antibody WU600 (27) raised against the Nup116p C-terminal region at a 1:1000 dilution (16 h, 4 °C). Bound

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**TABLE I**

**Yeast strains**

| Strain          | Genotype                                   | Source |
|-----------------|--------------------------------------------|--------|
| SWY518          | MATα ade2–1::ADE2 ura3–1 leu2–3,112 trp1–1 his3–11,15 can1–100 | 51     |
| SWY505          | MATα/a ade2–1::ADE2/ade2–1::ADE2 ura3–1/ura3–1 leu2–3,112/leu2–3,112 trp1–1/1 | 51     |
| SWY1920         | MATα GLE2::GFP:HIS3 ade2–1::ADE2 ura3–1 leu2–3,112 trp1–1 his3–11,15 can1–100 | This study |
| SWY2131         | MATα MEX67-GFP:HIS5 ade2–1::ADE2 ura3–1 leu2–3,112 trp1–1 his3–11,15 can1–100 | This study |
| SWY2154         | MATα MEX67-GFP:HIS5 ade2–1::ADE2 ura3–1 leu2–3,112 trp1–1 his3–11,15 can1–100 | This study |
| SWY2353         | MATα GLE1-GFP:HIS3 ade2–1::ADE2 ura3–1 leu2–3,112 trp1–1 his3–11,15 can1–100 | This study |
| SWY2257         | MATα MTR2-GFP:HIS3 ade2–1::ADE2 ura3–1 leu2–3,112 trp1–1 his3–11,15 can1–100 | This study |
| SWY2261         | MATα DBP5-GFP:HIS3 ade2–1::ADE2 ura3–1 leu2–3,112 trp1–1 his3–11,15 can1–100 | This study |
| L40             | MATα his33200 trp1–901 leu2–3,112 ade2 LYS2::(LexAop)5 HIS3 URA3::(LexAop)8-lacZ | S. Hollenberg |
| PJ66–4A         | MATα trp1–901 leu2–3,112 ura3–52 his3–200 gal4A gal80A LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7- LacZ | 42     |
antibodies were detected with alkaline phosphatase-conjugated anti-
rabbit IgG (Promega) diluted 1:7500 (1 h, 23 °C). After incubation with 
nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-1-phosphate
were developed for color visualization.

**Purification of Proteins from Bacteria and Soluble Binding Assay—**
For purification of the glutathione-S-transferase (GST) fusion proteins
GST-Nup116-GLFG or GST-Nup100-GLFG, *Escherichia coli* cells containing
pSW304 or pSW433, respectively, were grown in 1 liter of
42°C; pSW301 (27).

**Mex67** and full-length Nup116p (MBP-Nup116) were purified as above
and the overexpressed Nup116-GLFG region localizes in both
yeast cells results in nucleolar fragmentation, accumulation of
polyadenylated RNA within the nucleus, and cell lethality (27). The
GLFG overexpression phenotype does not result in any
detectable NPC or nuclear envelope structural perturbations, and the
overexpressed Nup116-GLFG region localizes in both the
cytoplasm and nucleus (27). We previously suggested this
phenotype may be due to titration of an essential GLFG-interact-
acting factor away from the NPC. Recently, several *S. cerevi-
siae* factors have been identified that are specifically required
for mRNA export and dispensable for the transport of proteins
and other classes of RNA (44). These factors include Gle1p,
Dbp5p, Mex67p, and Mtr2p. All of these factors except
Gle2p, Dbp5p, Mex67p, and Mtr2p. All of these factors except
Gle2p are essential, and in wild type cells all localize predom-
nantly at the NPC with some also showing low levels of cyto-
plasmic or nucleoplasmic staining (19, 22, 29, 45–47). Therefore,
each was a candidate for perturbation by the
overexpression of the Nup116-GLFG region.

To test directly whether the subcellular localizations of any of
these mRNA export factors were perturbed upon overexpression
of Nup116-GLFG, a panel of yeast strains harboring a
respective GFP-tagged transport factor was examined. Yeast
strains were generated with the sequence encoding GFP chro-

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**TABLE II**

| Plasmid | Construction | Source |
|---------|--------------|--------|
| pNLS-E1 backbone | GLFG region of NUP116 (bp 532–2145) in BamHI/SacI | 27 |
| pSW163 | GLFG region of NUP100 (bp 4–1782) in BamHI/SacI | 27 |
| pSW384 | | |
| pGEX-3X backbone | GLFG region of NUP116 (bp 481–2190) in BamHI | 28 |
| pSW304 | GLFG region of NUP100 (bp 4–1830) in BamHI | This study |
| pSW433 | | |
| pQE-32 backbone | KAP95 in BamHI | This study |
| pSW1261 | | |
| pMAL-cRI | NUP116 in BamHI | 2 |
| pSW156 | MEX67 in EcoRI/SalI | This study |
| pSW1237 | | |
| pCH432 backbone | KAP95 in BamHI | 27 |
| pSW319/332 | | |
| pGBD-C1 backbone | MEX67 in EcoRI/SalI | This study |
| pSW1254 | | |
| pSW291 backbone | Full-length NUP116 GLFG region (bp 478–2196) in NcoI/Xhol | This study |
| pSW291 | | |
| pSW1232 | Repeats 1–25 of NUP116 GLFG region (bp 478–1809) in NcoI/Xhol | This study |
| pSW1233 | | |
| pSW1234 | Repeats 9–33 of NUP116 GLFG region (bp 925–2196) in NcoI/Xhol | This study |
| pSW1235 | | |
| pSW1236 | Repeats 9–25 of NUP116 GLFG region (bp 925–1809) in NcoI/Xhol | This study |
| pSW1246 | | |
| pSW1247 | Repeats 9–21 of NUP116 GLFG region (bp 925–1605) in NcoI/Xhol | This study |
| pSW1248 | | |
| pSW1249 | Repeats 13–21 of NUP116 GLFG region (bp 1084–1605) in NcoI/Xhol | This study |
| pSW1290 | | |
| pSW1291 | Repeats 12–12 of NUP116 GLFG region (bp 478–1086) in NcoI/Xhol | This study |
| pSW1291 | | |
| pSW1291 | Repeats 22–33 of NUP116 GLFG region (bp 1606–2196) in NcoI/Xhol | This study |

*All nucleotide positions given begin with the initiation codon (bp = base pairs). Vector backbone references: pNLS-E1 (52); pGEX-3X (Amer sham Pharmacia Biotech); pQE-32 (Qiagen); pMAL-cRI (New England Biolabs); pCH432 for LexA binding domain fusions (41); pGBD-C1 (42); pSW291 (27).
mosomally integrated in-frame at the sequence for the C terminus of the particular transport factor (see “Materials and Methods”). A multicopy plasmid containing the Nup116-GLFG region under control of the GAL10 promoter was introduced into each strain. In all of the strains, overexpression of the Nup116-GLFG region was confirmed by immunoblot, and the overexpressing strains failed to grow on media containing galactose (data not shown). Nucleolar fragmentation was documented for all strains by indirect immunofluorescence microscopy with antibodies to Nop1p (data not shown). After induction of GLFG overexpression, the localization of the GFP-tagged factor was determined by direct fluorescence microscopy. The results are summarized in Table III. In wild type control cells, Mex67-GFP, Mtr2-GFP, and Dbp5-GFP were predominantly localized in a punctate pattern at the nuclear periphery (Fig. 1A and Table III), identical to the published localizations (22, 45–47). Strikingly, in Nup116-GLFG-overexpressing cells, Mex67-GFP, Mtr2-GFP, and Dbp5-GFP accumulated within the nucleus, and the punctate pattern at the nuclear periphery was absent (Fig. 1G and Table III). In contrast, the localizations of Gle1-GFP and Gle2-GFP were not perturbed by overexpression of the GLFG region (Table III). Gle1-GFP and Gle2-GFP were localized predominantly at the nuclear rim in both control and Nup116-GLFG-overexpressing cells. Because Nup116-GLFG has been previously shown to interact directly with Kap95p (27, 28), we also examined the localization of Kap95p by indirect immunofluorescence microscopy. The distribution of Kap95p was not perturbed, and it was localized in the nucleus, the cytoplasm, and at the NPC of both control and GLFG-overexpressing cells (Table III). Thus, overexpression of Nup116-GLFG specifically altered the distribution of Mex67-GFP, Mtr2-GFP, and Dbp5-GFP.

Since Nup116p and Nup100p have a high degree of structural and functional similarity, we investigated the localization of Mex67-GFP in cells overexpressing the Nup100-GLFG region. The level of Mex67-GFP in the nuclei of cells overexpressing Nup100-GLFG was greater than that in the control cells (Fig. 1, compare I and K). However, some peripheral punctate localization was detectable in a fraction of the Nup100-GLFG-overexpressing cells (Fig. 1K). This contrasts with the Nup116-GLFG-overexpressing cells where Mex67-GFP was no longer localized in a punctate pattern at the nuclear rim (Fig. 1G). These results suggest that Nup116-GLFG was more effective than Nup100-GLFG at perturbing the localization of Mex67-GFP. These results also correlate with our previous observations (27) that only Nup116-GLFG overexpression severely inhibits cell growth.

Mex67p and Nup116p Form a Complex in Vivo—The mislocalization of Mex67-GFP, Mtr2-GFP, and Dbp5-GFP in cells overexpressing the Nup116-GLFG region suggested that these factors may directly or indirectly associate in vivo with Nup116p. Izaurralde and co-workers (23) recently reported that TAP, the vertebrate homologue of Mex67p, associates with the vertebrate GLFG nucleoporin Nup98p. Therefore, Mex67p was a good candidate for Nup116p and Nup116p interacting. To investigate whether Mex67p and Nup116p physically interact, we performed coimmunoprecipitation experiments from whole cell yeast lysates. Cell lysates from wild type and Mex67-GFP-expressing cells were incubated with an affinity-purified rabbit polyclonal antibody raised against GFP. Antibody-bound Mex67-GFP and coprecipitating proteins were isolated with protein A-Sepharose, and the unbound and bound fractions were separated by SDS-PAGE and analyzed by immunoblotting. Immunoblotting with anti-GFP antibodies showed that the antibodies recognized an ~105-kDa protein representing Mex67-GFP. This band was present in the lysate from Mex67-GFP cells but not in the lysate from the untagged cells (Fig. 2, lanes 1 and 2). The anti-GFP antibody also recognized several endogenous yeast proteins on immunoblots; however, the immunoprecipitation was specific for Mex67-GFP as the 105-kDa band was the only one observed in the bound fractions (Fig. 2, lanes 5 and 6). The immunoisolation depleted a significant fraction of the Mex67-GFP, with very little remaining in the unbound fraction (Fig. 2, lane 4).

To determine whether a GLFG protein(s) coprecipitated with Mex67-GFP, the samples were probed with polyclonal antibodies raised against the Nup116-GLFG region. The anti-GLFG antibody specifically recognized an ~120-kDa band in the bound fraction of the Mex67-GFP stain (Fig. 2, lanes 9 and 10). Because the anti-Nup116-GLFG antibody has the potential to react with other GLFG proteins in yeast, we confirmed that the ~120-kDa band was Nup116p by probing the samples with an antibody monospecific for the Nup116p C-terminal region. The anti-Nup116p antibody recognized the ~120-kDa band (Fig. 2, lane 14). Monospecific antibodies recognizing Nup100p are not available, and we could not directly test for the presence of Nup100p in the Mex67-GFP immunoprecipitate. Under the cell lysis conditions used, Nup116p is solubilized into distinct subcompartments, and the immunoprecipitation does not represent mostly intact NPCs (25). Thus, Mex67-GFP and Nup116p specifically associate in a complex in yeast cells. However, not all of the Nup116p was coprecipitated with Mex67-GFP (Fig. 2, compare lanes 10 and 14 with 8 and 12, respectively). This is consistent with Nup116p being a binding site for multiple dynamic transport factors.
MBP-Mex67 binding to GST-Nup116-GLFG was specific as it was competed by the addition of MBP-Nup116 (full-length) but not MBP alone (Fig. 3E, right panel). These results demonstrate a direct association between Mex67p and the Nup116-GLFG region.

Because Mex67-GFP was also perturbed in cells overexpressing Nup100-GLFG, we investigated whether Mex67p and Nup100-GLFG directly interact in vitro. Nup100-GLFG was purified as a GST fusion from bacteria and immobilized on Glutathione-Sepharose (Fig. 3A). GST-Nup100-GLFG did not bind MBP (Fig. 3B, right panel). Binding of His8-Kap95 (Fig. 3C, right panel) and full-length MBP-Mex67 (Fig. 3D, right panel) was detected with GST-Nup100-GLFG. Therefore, Mex67p directly binds the GLFG regions of Nup116p and Nup100p in vitro.

Others have shown (22) Mex67p and Mtr2p form a complex in yeast cells, and Mtr2p mediates the association of Mex67p with the nucleoporin Nup85p. We have not detected direct binding between bacterially expressed, recombinant Mtr2p and either GST-Nup116-GLFG or GST-Nup100-GLFG (data not shown). Therefore, Mex67p may bind some nucleoporins directly and other nucleoporins through heterodimerization with Mtr2p (see “Discussion”).

Mex67p and Kap95p Bind Distinct Regions of the Nup116-GLFG Domain—The Nup116-GLFG domain consists of 33 GLFG tetrapeptide repeats separated by polar spacer regions (37). It is unknown whether all of the repeats and spacers are functionally or structurally equivalent. Because the GLFG region binds both Mex67p and Kap95p in vitro, we wanted to test whether each factor had the capacity to interact with overlapping or distinct subregions of the Nup116-GLFG domain. Yeast two-hybrid analysis was used to map the Mex67p- and Kap95p-binding sites on the Nup116-GLFG domain. A series of sequential deletions of four or eight GLFG repeats from each end of

![Fig. 1. Mex67-GFP accumulates in the nucleus of yeast cells overexpressing the GLFG region. Yeast cells expressing chromosomal Mex67-GFP were transformed with an empty vector (pNLS-E1) (A–D), a vector with Nup116-GLFG under control of a galactose-inducible promoter (pSW163) (E–H), or a vector with Nup100-GLFG under control of a galactose-inducible promoter (pSW384) (I–L). The cells were grown in raffinose (A, B, E, F, I, and J) or shifted to growth in galactose (C, D, G, H, K, and L) for 5 h before visualization of direct fluorescence (A, C, E, G, I, and K). The corresponding Nomarski panels are shown to the right of the respective fluorescence panel (B, D, F, H, J, and L).](http://www.jbc.org/)

![Fig. 2. Mex67-GFP coimmunoprecipitates Nup116p from yeast cell lysates. Whole cell lysates from wild-type or Mex67-GFP-expressing cells were immunoprecipitated with anti-GFP antibodies. Input, unbound, and bound fractions were separated by SDS-PAGE and immunoblotted with either anti-GFP (A), anti-GLFG (B), or anti-Nup116C (C) antibodies. The anti-GFP antibody recognizes several endogenous yeast proteins on immunoblots. Mex67-GFP is indicated by two asterisks. The large band at ~55 kDa in the bound fractions represents IgG.](http://www.jbc.org/)

(Fig. 3D, middle panel). MBP-Mex67 binding to GST-Nup116-GLFG was specific as it was competed by the addition of MBP-Nup116 (full-length) but not MBP alone (Fig. 3E, right panel). These results demonstrate a direct association between Mex67p and the Nup116-GLFG region.

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the Nup116-GLFG domain were generated and fused in-frame behind the transcriptional activation domain of Gal4p (Gal4AD). These plasmids were cotransformed into reporter strains with plasmids expressing either Kap95p fused to the DNA binding domain of LexA (LexABD) or Mex67p fused to the DNA binding domain of Gal4p (Gal4BD). The two-hybrid interaction was assayed by growth on SC media lacking histidine (for Kap95p) or media lacking histidine and adenine (for Mex67p). Both Kap95p and Mex67p resulted in positive two-hybrid interactions with full-length Gal4AD-Nup116-GLFG (Fig. 4, GLFG repeats 1–33). Kap95p (27) and Mex67p (this work) also yielded a positive interaction with Gal4 AD-Nup100-GLFG in the two-hybrid assay (data not shown). Deletion of eight Nup116-GLFG repeats from each end of the full-length Nup116-GLFG region did not abolish the positive signal (Fig. 4, GLFG repeats 1–25, 9–33, and 9–25). Subsequent deletions of four more repeats from each end also did not abolish the positive interaction (Fig. 4, GLFG repeats 9–21, 13–25, and 13–21). Therefore, the central region composed of Nup116-GLFG repeats 13–21 was sufficient for a two-hybrid interaction with either Kap95p or Mex67p.

To test if the nine GLFG repeats from 13–21 were necessary for binding Kap95p and Mex67p, Gal4AD fusions were generated with the N- and C-terminal GLFG segments that flank GLFG repeats 1–25, 9–33, and 9–25. Subsequent deletions of four more repeats from each end also did not abolish the positive interaction (Fig. 4, GLFG repeats 9–21, 13–25, and 13–21). Therefore, the central region composed of Nup116-GLFG repeats 13–21 was sufficient for a two-hybrid interaction with either Kap95p or Mex67p.

To test if the nine GLFG repeats from 13–21 were necessary for binding Kap95p and Mex67p, Gal4AD fusions were generated with the N- and C-terminal GLFG segments that flank GLFG repeats 13–21 (repeats 1–12 and 22–33, respectively) (Fig. 4). Strikingly, GLFG repeats 1–12 were sufficient for a two-hybrid interaction with Mex67p, and GLFG repeats 22–33 were sufficient for interaction with Kap95p but not vice versa.
Therefore, although both transport factors bind the central portion of the GLFG region, Mex67p alone associates with the N-terminal portion of the GLFG region, and Kap95p alone associates with the C-terminal portion.

**DISCUSSION**

To fully understand how the GLFG nucleoporins Nup116p and Nup100p function in nuclear transport, it is critical to characterize their protein interacting partners. Direct in vitro interactions between these GLFG regions and members of the karyopherin transport factor family have been definitively established (28, 30, 32, 33). Genetic and in vivo evidence further supports a role for a Kap95p-GLFG interaction during transport (29). Here we report in vivo and in vitro documentation that the mRNA export factor Mex67p directly interacts with the GLFG regions of Nup116p and Nup100p. This conclusion is based on multiple pieces of evidence. Mex67p-GFP was mislocalized in yeast cells overexpressing the Nup116p and Nup100p GLFG regions; Nup116p was coisolated from yeast cell lysates in a complex with Mex67p-GFP, purified recombinant MBP-Mex67 and GST-Nup116-GLFG or GST-Nup100-GLFG directly bound in vitro, and Mex67p yielded a positive two-hybrid interaction with both GLFG regions. Interestingly, the localization of Kap95p was not altered by overexpression of Nup116p-GLFG. Moreover, Mex67p and Kap95p exhibited differential two-hybrid interactions with subregions of the Nup116p-GLFG domain. Direct in vitro interactions between the vertebrate Mex67p homologue, TAP, and the vertebrate FG nucleoporins Nup120p and Nup88p have been recently reported (23, 24). In addition, while our study on the homologous yeast proteins was in progress, Hurt and coworkers (48) reported in vitro interactions between the heterodimeric Mex67p-Mtr2p complex and the FG domains from several different yeast nucleoporins. This included in vitro binding of a recombinant Mex67p-Mtr2p complex to the Nup116p-GLFG region. Our studies independently corroborate these findings and extend our knowledge in several important ways. There are key implications for our results in terms of both the mechanism of Mex67p action in mRNA export and the global role of the Nup116p and Nup100p GLFG regions in nuclear transport.

In all other reported *S. cerevisiae* mutant phenotypes, wild type Mex67p has only been observed to accumulate in the cytoplasmic side of the NPC (6, 49); however, interactions with Mex67p have not been documented. The other FG nucleoporins that to date have been reported as interacting with the Mex67p-Mtr2p complex in vitro are either localized exclusively on the cytoplasmic side of the NPC (Rip1/Nup42p and Nup159p) or localized symmetrically on both sides (Nsp1p) (4, 6, 48). Overall, our results provide novel evidence for an essential in vivo Mex67p-GLFG interaction during mRNA export.

The molecular basis for the in vivo GLFG overexpression phenotype (toxicity and mRNA export defects) may be partially due to sequestering Mex67p in the nucleus. However, we also observed accumulation of Mtr2-GFP and Dbp5-GFP in the nucleus of cells overexpressing the Nup116p-GLFG region (Table III). Therefore, the phenotype could be a combined effect from coincident sequestering of three essential mRNA export factors in the nucleus. Since Mex67p and Mtr2p physically interact (22), it is not unexpected that Mtr2p-GFP would accumulate in the nucleus through its association with Mex67p-GFP. We have not detected a direct binding between recombinant, bacterially expressed Mtr2p and Nup116p-GLFG.

2 Genetic or biochemical associations between Dbp5p and GLFG nucleoporins or between Mex67p/Mtr2p and Dbp5p have not been reported. Further studies will be needed to examine Dbp5p interactions and reveal the mechanism of the Dbp5p mislocalization in GLFG-overexpressing cells.

The recent study by Hurt and coworkers (48) concludes that in vitro Mex67p binding to a FG nucleoporin requires Mtr2p and formation of the heterodimeric Mex67p-Mtr2p complex. They reported Mex67p or Mtr2p alone does not bind to Nup116p-GLFG. In contrast, we have found that Mex67p does independently interact in vitro with Nup116p-GLFG or Nup100p-GLFG. Therefore, Mtr2p is not strictly required for Mex67p binding to the GLFG regions. However, it is possible that Mtr2p increases the affinity of a Mex67p-GLFG interaction. Our results showing a direct GLFG-Mex67p interaction more closely parallel the results of vertebrate TAP directly binding vertebrate FG nucleoporins (23). We believe that yeast Mex67p and vertebrate TAP share a similar mechanism for interacting with GLFG nucleoporins.

Finally, our analysis of Mex67p overexpression in the Nup116p-GLFG region suggests that the GLFG region has the capacity to bind Mex67p and karyopherins simultaneously.

These results indicate that all of the GLFG repeats and spacer

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2 L. A. Strawn and S. R. Wente, unpublished data.
regions are not necessarily functionally equivalent. This is surprising and should change the way we think about the function of FG repeat domains. Most prior investigations have treated subregions of FG repeat domains as functionally representative of the full-length domains. The results in this study now suggest that distinct subregions of the GLFG domain may serve as unique binding sites for different transport factors.

How do such binding interactions between nucleoporins and transport factors result in movement through the NPC portal?

FG nucleoporins are located on both the cytoplasmic and nuclear NPC faces as well as along the central axis of the NPC (summarized in Refs. 4 and 6). Recent models have suggested that karyopherins and mRNA export factors could move from one FG repeat domain to another along the filament and central channel. Besides the potential distinct binding of transport factors to subregions of an individual FG repeat domain, there is a growing body of evidence that the FG repeat domains among the family members are also not equivalent. In yeast cells, the GLFG region of Nup116p cannot be functionally replaced with the FXXFG region of Nsp1p (27). Mutants of transport factors show contrasting perturbations on their respective binding capacity for different FG nucleoporins (13, 23, 28). For example, a yeast Kap95p mutant with diminished GLFG interaction is not altered for FXXFG interaction (28). Deletions of vertebrate TAP that abolish binding to Nup214p are not defective in vitro for binding Nup89p (23). Thus, the interactions of a given transport factor with GLFG versus FXXFG repeats may be mechanistically distinct in terms of facilitating, directing, or even propelling entry and exit through the NPC portal.

Another important emerging theme is that multiple different mRNA export factors have binding sites on a single nucleoporin. Mex67p and Gle2p have distinct binding sites on Nup116p in the GLFG and N-terminal regions, respectively (this work and Refs. 17, 18, and 48). Mex67p-mtr2p and Dhp5p can both interact with Nup159p (16, 20, 48). The fact that a single nucleoporin can bind more than one mRNA export factor strongly suggests the particular factors may be required for a common step in mRNA export. There is evidence that vertebrate TAP and hGle2 may interact (23); however, we have not observed any qualitative effects of yeast Gle2p on Mex67p binding to full-length Nup116p. Finally, the characterization of a higher order, cytoplasmically localized Nup116p-Nup82p-Nup159p complex suggests there is a juxtaposition of binding sites for different mRNA export factors at a discrete NPC substructure (25, 50). Taken together, these interactions may reflect sequential steps in the mRNA export mechanism. Revealing how Nup116p/Nup100p and GLFG binding influences the movement of distinct transport factors through the NPC is a future goal.

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