Functional Analysis of the Hydrophobic Patch on Nuclear Transport Factor 2 Involved in Interactions with the Nuclear Pore in vivo*

Received for publication, June 1, 2001, and in revised form, July 6, 2001
Published, JBC Papers in Press, August 6, 2001, DOI 10.1074/jbc.M105054200

B. Booth Quimby‡§, Sara W. Leung‡, Richard Bayliss¶, Michelle T. Harreman‡,
Geetha Thirumala‡, Murray Stewart¶, and Anita H. Corbett¶**

From the ‡Department of Biochemistry, Emory University School of Medicine, Atlanta, Georgia 30322 and ¶Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, United Kingdom

Nuclear transport factor 2 (NTF2) is a small homodimeric protein that interacts simultaneously with both RanGDP and FxFG nucleoporins. The interaction between NTF2 and Ran is essential for the import of Ran into the nucleus. Here we use mutational analysis to dissect the in vivo role of the interaction between NTF2 and nucleoporins. We identify a series of surface residues that form a hydrophobic patch on NTF2, which when mutated disrupt the NTF2-nucleoporin interaction. Analysis of these mutants in vivo demonstrates that the strength of this interaction can be significantly reduced without affecting cell viability. However, cells cease to be viable if the interaction between NTF2 and nucleoporins is abolished completely, indicating that this interaction is essential for the function of NTF2 in vivo. In addition, we have isolated a dominant negative mutant of NTF2, N77Y, which has increased affinity for nucleoporins. Overexpression of the N77Y protein blocks nuclear protein import and concentrates Ran at the nuclear rim. These data support a mechanism in which NTF2 interacts transiently with FxFG nucleoporins to translocate through the pore and import RanGDP into the nucleus.

Nucleocytoplasmic transport occurs through nuclear pore complexes (NPCs),† large proteinaceous channels that perforate the nuclear membrane. Protein cargoes to be imported into the nucleus contain internal sequences, termed nuclear localization signals (NLS), that target them to the nucleus (1–3). However, cargo does not interact directly with the NPC but is transported bound to soluble transport receptors of the importin-β family of proteins (4, 5). The recent purification and analysis of the yeast NPC indicates that the majority of the ~40 proteins that make up the NPC, collectively termed nucleoporins, are localized symmetrically throughout the pore complex (6). A subset of these proteins contains a phenylalanine-glycine (FG) repeat motif. These proteins appear to line the nuclear pore channel and can be subdivided further into either GLFG-repeat-containing proteins or FxFG proteins (6, 7). Although the precise mechanism by which the transport receptor-cargo complex translocates through the NPC is largely unknown, importin-β family transport receptors have been shown to interact with both the GLFG repeat and the FxFG repeat nucleoporins (8–16) suggesting that these proteins are involved directly in the translocation process.

The small GTPase Ran is central to nucleocytoplasmic transport. Although Ran is localized throughout the cell, ~80% is concentrated within the nucleus (17). The Ran guanine nucleotide exchange factor is tethered to DNA in the nucleus (18, 19), suggesting that the majority of nuclear Ran is GTP-bound. On the other hand, RanGAP (Ran GTPase-activating protein) is predominantly cytoplasmic (20–22), suggesting that the majority of cytoplasmic Ran is GDP-bound. Thus, the nucleotide bound state of Ran may act as a cellular marker that allows the transport machinery to distinguish between the nuclear and cytoplasmic compartments of the cell. This strict compartmentalization of the Ran effectors also suggests that Ran must shuttle between the nucleus and cytoplasm to undergo a complete round of GTP hydrolysis. It is hypothesized that RanGTP exits the nucleus complexed with importin-β-like transport receptors, and RanGDP is then re-imported into the nucleus by the small homodimeric protein NTF2 (23–25) to replenish the nuclear stores of Ran.

NTF2 was first identified as a factor required for efficient import of proteins into the nucleus (26). Consistent with the role of NTF2 in importing Ran into the nucleus, NTF2 has been shown to interact at non-overlapping sites with both Ran and a subset of nuclear pore proteins containing FxFG repeats (27). NTF2 specifically interacts with the GDP-bound form of Ran, and this interaction has been extensively characterized through mutational analysis of both NTF2 and Ran (27–31) as well as analysis of the NTF2-Ran co-crystal structure (32). These studies indicate that the interaction between NTF2 and Ran is required to concentrate Ran in the nucleus and consequently for protein transport between the nucleus and the cytoplasm (33).

The interaction between NTF2 and nucleoporins is predicted to be more complex than the Ran-NTF2 interaction because NTF2 is capable of interacting with multiple FxFG nucleoporins that line the central channel of the pore (34). Recent in vitro binding studies indicate that the interaction between NTF2 and nucleoporins is relatively weak (35, 36). This suggests a
model in which NTF2 transiently interacts with nucleoporins enabling the NTF2-RanGDP complex to move through the pore by hopping from one repeat to another (37). If this is the case, one would predict that decreasing or increasing the affinity of NTF2 for FxFG nucleoporins could have a detrimental effect on NTF2 function. However, the lack of an NTF2-FxFG co-crystal structure has made it difficult to engineer mutants crucial to testing this hypothesis.

Bayliss et al. (35) utilized the crystal structure of NTF2 bound to RanGDP to predict which residues in NTF2 might be involved in binding to nucleoporins. They identified residue Trp-7 as a potential site for FxFG binding and engineered a mutation in the rat NTF2 protein, W7A NTF2, with a reduced affinity for FxFG nucleoporins. They went on to demonstrate that the W7A NTF2 protein only weakly stimulates nuclear import of RanGDP in vitro. However, a recent study by Ribbeck and Görlich (38) that examined the rate of translocation of both NTF2 and W7R NTF2 showed that W7R NTF2 enters the nucleus quite rapidly compared with a control green fluorescent protein (GFP). This led these authors (38) to suggest that other hydrophobic residues in NTF2 in addition to Trp-7 are critical for NTF2-nucleoporin interactions in vivo.

The transport receptor importin-β also interacts with FxFG nucleoporins (8-16) presumably to allow the translocation of importin-β-cargo complexes through the NPC. One could envision that the import of RanGDP by NTF2 via the ability of NTF2 to interact with nucleoporins is analogous to the import of protein cargo by importin-β. If so, the recent co-crystal structure of importin-β bound to five FxFG nucleoporin repeats from Nsp1p (37) might give some clues about how NTF2 and FxFG nucleoporins interact. The importin-β-FxFG structure revealed that the phenylalanines of the FxFG repeat cores are buried in a hydrophobic pocket on importin-β, with the phenylalanines of the FxFG repeat core forming stacking interactions with themselves and hydrophobic residues in importin-β. This observation is consistent with the finding that a hydrophobic residue, Trp-7, is involved in the NTF2-FxFG nucleoporin interaction. However, mutations in Trp-7 appear only to weaken and not eliminate the interaction between NTF2 and nucleoporins (35, 38), suggesting that other hydrophobic residues in NTF2 are also involved in this interaction. To fully understand the function of NTF2, it is imperative to define all residues that are critical for NTF2-nucleoporin interactions in vivo.

In this study, we have used mutational analysis to probe the interaction between NTF2 and FxFG nucleoporins in vivo. First, we engineered a series of mutant NTF2 proteins that disrupt the interaction between NTF2 and FxFG nucleoporins to varying extents. Our data show that the strength of NTF2 binding to nucleoporins can be reduced significantly without reducing cell viability. However, if this interaction is abolished completely, cells can no longer survive. Second, we isolated a mutant of NTF2 that has an increased affinity for FxFG nucleoporins. This mutation renders the NTF2 protein non-functional in vivo, and overexpression of this mutant protein blocks NLS-mediated protein import. Together, these results suggest a model in which the interaction between NTF2 and FxFG nucleoporins occurs at a sufficiently low affinity to enable NTF2 to move freely from one FxFG repeat to another as it translocates through the pore complex. Furthermore, our data demonstrate that the fine-tuned interaction between NTF2 and FxFG nucleoporins is critical in vivo.

### MATERIALS AND METHODS

All chemicals were obtained from Sigma or United States Biological unless otherwise noted. All DNA manipulations were performed according to standard methods (39), and all media were prepared by standard procedures (40). All plasmids used in this study are described in Table 1.

### Table 1: Plasmids used

| Plasmid | Description |
|---------|-------------|
| pAC18   | GAL1-10, 2 μ, URA3, amp |
| pAC78   | GSP1, CEN, TRP1, amp |
| pAC82   | NTF2, GAL1-10, 2 μ, URA3, amp |
| pAC117  | NTF2, CEN, LEU2, amp |
| pAC130  | nft2D21A, Bluescript |
| pAC149  | nft2D21A, GAL1-10, 2 μ, URA3, amp |
| pAC150  | nft2D21A, CEN, TRP1, amp |
| pAC163  | nft2N77Y, CEN, LEU2, amp |
| pAC197  | nft2N77Y-GFP, 2 μ, URA3, amp |
| pAC240  | NTF2, Bluescript |
| pAC253  | nft2N77Y, GAL1-10, 2 μ, URA3, amp |
| pAC267  | nft2F5A, Bluescript |
| pAC345  | nft2F5A, CEN, LEU2, amp |
| pAC410  | GSP1-GFP, 2 μ, URA3, amp |
| pAC411  | nft2F5A-GFP, 2 μ, URA3, amp |
| pAC420  | S. cerevisiae Ntf2pF5A bacterial expression vector |
| pAC628  | NTF2, CEN, URA3, amp |
| pAC629  | myc-GSP1, CEN, TRP1, amp |
| pAC629  | S. cerevisiae Ntf2p bacterial expression vector |
| pAC697  | pADH-NLS-GAL4AD-GFP, 2 μ, LEU2 (42) |
| pAC709  | NTF2-GFP, 2 μ, URA3, amp |
| pAC715  | YRB, CEN, TRP, amp |
| pAC738  | nft2Y112A, CEN, LEU2, amp |
| pAC739  | nft2Y112D, CEN, LEU2, amp |
| pAC744  | nft2Y112A, Bluescript |
| pAC745  | nft2Y112D, Bluescript |
| pAC746  | Δnft2, CEN, LEU2, amp |
| pAC750  | S. cerevisiae Ntf2pY112A bacterial expression vector |
| pAC751  | S. cerevisiae Ntf2pY112D bacterial expression vector |
| pAC760  | nft2F5A/Y112A, CEN, LEU2, amp |
| pAC792  | pADH-NLS-GAL4AD-GFP, 2 μ, LEU2 (42) |
| pAC797  | NTF2-GFP, 2 μ, URA3, amp |
| pAC798  | nft2Y112D-GFP, 2 μ, URA3, amp |
| pAC799  | nft2F5A/Y112A-GFP, 2 μ, URA3, amp |
| pAC812  | S. cerevisiae Ntf2pF5A/Y112A bacterial expression vector |
| pAC814  | nft2D21A-GFP, 2 μ, URA3, amp |
| pAC821  | S. cerevisiae Ntf2pF5A bacterial expression vector |
| pAC913  | Δnft2p bacterial expression vector |
| pAC914  | ΔNf2p-GFP, 2 μ, URA3, amp |

1. The wild-type (PSY560) and NTF2 deletion strains (ACY114) used in this study have been described (41).

**Mutagenesis—**Site-directed mutagenesis was performed on pBSNTF2 (pAC240) using the QuickChange PCR-based mutagenesis kit from Stratagene (La Jolla, CA). Mutated sequences were confirmed by DNA sequencing. ΔNFT2F was made using a PCR-based strategy. The NTF2 promoter was amplified from pAC117 with 5’-GCC ACC GGT CAT TAT AAA GAT AAT AGT ATT AAA ACC-3’ and 5’-GGG ACC GCT CTT ATA TGG TTC TGG GGC GTA GGC TA-3’ as primers. The resulting PCR product was digested with AgeI and SacI and subcloned into pAC715 (pRS314YRB1) digested with AgeI and SacI. The Ntf2p coding region from Gln-10 to the stop codon including the 3’-untranslated region was amplified from pAC117 with 5’-GCC ACC GGT CAT TAT AAA GAT AAT AGT ATT AAA ACC-3’ and 5’-GGG ACC GCT CTT ATA TGG TTC TGG GGC GTA GGC TA-3’ as primers. The resulting PCR product was digested with AgeI and Xhol and subcloned into pAC175 containing the NTF2 promoter digested with AgeI and Xhol. The six-amino acid deletion was confirmed by DNA sequencing then subcloned into the pSac1 and Xhol sites of pPS155 (CEN, LEU plasmid).

Localization of Ntf2p Proteins and secRan in Vivo—Wild-type and mutant Ntf2p-GFP fusion proteins were transformed into the NTF2 deletion strain, ACY114, maintained by a wild-type copy of GSP1 (scRan) (pAC78). secRan-GFP was transformed into ACY114 expressing each of the mutant alleles of NTF2 as the only copy of NTF2. The GFP fusion proteins were localized by viewing the GFP signal directly in living cells through a GFP-optimized filter (Chroma Technology) using an Olympus BX60 epifluorescence microscope equipped with a Photometrics QuantaX digital camera.

**NLS-GFP Import Assay—**The NLS-GFP import assay was performed as described previously (42). Briefly, cells were grown to early mid-log phase in synthetic media containing 2% glucose (w/v) at 25 °C, pelleted, resuspended in 1 ml of 10 mM sodium azide, 10 mM 2-deoxy-d-glucose in glucose-free synthetic media, and incubated at 25 °C for 45 min. The cells were then pelleted, washed with 1 ml of ice-cold dH2O, repelleted, resuspended in 100 μl of glucose-containing synthetic media pre-
warmed to 37 °C, and incubated at 37 °C. For scoring, 2–μl samples were removed every 2.5 min, and cells were observed and counted through a GFP-optimized filter (Chroma Technology) using an Olympus BX60 epifluorescence microscope. Cells were scored as “nuclear” if the nucleus was both brighter than the surrounding cytoplasm and a nuclear membrane boundary was visible. At least 100 cells were counted at each time point.

**Functional Analysis of NTF2 Mutant Alleles** — The *in vivo* function of each of the Ntf2 mutant proteins was tested by using a plasmid shuffle technique. The NTF2 deletion strain, ACY114, was transformed with plasmids encoding each of the Ntf2 mutant proteins. Single transformants were grown in liquid culture to saturation. The transformed cultures were serially diluted (1:10) and spotted on fluoroorotic acid (5-FOA) plates to eliminate the URA3 plasmid-encoded wild-type Ntf2p (43). This results in cells that express each of the mutant Ntf2 proteins on a low copy centromeric plasmid as the sole copy of Ntf2p.

**Ntf2p Purification and Immobilization** — All Ntf2p proteins were purified from *Escherichia coli* essentially as described (25) with the following changes. After lysis and clarification, *E. coli* lysates were applied to DEAE ion exchange column pre-equilibrated with 10 mM Tris-HCl, pH 8.0. Ntf2p was eluted from the column with a gradient of 0 to 1 M NaCl. Fractions containing Ntf2p were pooled, concentrated using a Centriprep-10 (Amicon) concentrator, and applied to a column of Sephacryl HR100 pre-equilibrated in 20 mM Tris-HCl, pH 7.5. Fractions containing purified Ntf2p werecollected, pooled, and dialyzed against 50 mM Tris-HCl, 0.5 M glycerol, 10% glycerol. Purified Ntf2 proteins were cross-linked to cyanogen bromide-Sepharose beads as described previously (25).

**Bead-binding Assays** — Yeast cell extracts were prepared as described (25). Two mg of BQY65 (MATa ura3–52 trpl63 leu2-31 his3A200 GSP1::His3 GSP2::His3 myc-GSP1) cell extract was incubated with 50 μl of Ntf2p-Sepharose beads. For the competition experiment, either 300 μg of wild-type Ntf2p or 300 μg of bovine serum albumin was added in addition to the yeast cell extract. Binding was carried out in PBSM (PBS, 2.5 mM MgCl2) in a total volume of 500 μl at 4 °C for 1 h. Following the binding, the beads were pelleted, and unbound lysate was removed from the beads. This fraction was designated the unbound sample, and 20 μg of total protein, corresponding to 1% of the total unbound volume, was analyzed. After the unbound sample was removed, beads were washed once for 10 min in 1 ml of PBSM and twice for 10 min in PBSMT (PBS, 2.5 mM MgCl2, 0.5% Triton X-100). The beads that contained the bound fraction were then boiled in sample loading buffer (100 μl total volume), 10% of the total bound proteins (10 μl) was loaded onto an SDS-polyacrylamide gel. Because the bound proteins were eluted directly into sample buffer, it was impossible to quantify the total amount of bound sample. However, within each experiment the percentage of total bound protein loaded was equal, so direct comparisons could be made between each of the bound lanes within each experiment. This is also the case for the unbound samples. Samples (bound and unbound) were resolved by polyacrylamide gel electrophoresis, and transferred to nitrocellulose for immunoblotting.

For many of the experiments some low level of nonspecific or low affinity binding of Ntf2p to the beads (whether they were charged with Ntf2 protein or not) was observed. This Nup2p was readily washed from the beads by the PBSM/PBSMT washes used in our protocol and could be recovered in a wash fraction by trichloroacetic acid precipitation (data not shown). This means that in some experiments, the amount of total protein (summing the unbound and bound lanes) is not equal despite the fact that the same lysates with identical amounts of Nup2p were used for each sample within the experiment. For all experiments the amount of unbound (1% of total unbound volume) and bound (10% of total bound volume) sample loaded in each lane was identical, and therefore direct comparisons can be made between the bound lanes shown for all samples and between the unbound lanes shown for all samples.

**Immunoblot Analysis** — Immunoblot analysis was performed essentially as described (44). Ntf2p-GFP was detected by incubation with 1:10,000 dilution of rabbit anti-GFP polyclonal antibody. Nup2p was detected by incubation with 1:3000 dilution of mAb414 (Babco). Myc-Gsp1p was detected with 1:500 dilution of mouse anti-Myc monoclonal antibody (Santa Cruz Biotechnology).

**Dominant Negative Screen** — Dominant negative alleles of NTF2 were generated using random PCR mutagenesis. A 4.0-kilobase pair product that included the entire NTF2 open reading frame was generated using *Taq* polymerase under mutagenic conditions (7 mM MgCl2) and purified on a Qiaquick PCR column from Qiagen (Chatsworth, CA). The plasmid pAC82 was linearized with BanHI and Sall to remove a 475-base pair insert containing the coding region of NTF2. Gap repair ligation (56) was achieved by co-transforming the wild-type strain PSY580 with the PCR product and linearized the GAL1-NTF2 plasmid (pAC82). Colonies were selected on synthetic complete media plus 2% (w/v) glucose lacking leucine (SD–Leu). The plates were then replica plated to synthetic complete media plus 2% galactose (w/v) lacking leucine (Sgal–Leu) supplemented with 7.5 mg of the vital dye erythrosin B/agar and incubated at 30 °C. Addition of galactose to the media initiates the overexpression of the plasmid encoded Ntf2 protein. Taking of the erythrosin B dye by dead cells, which results in a pink colony, was used to identify colonies that died upon overexpression of the Ntf2 protein. Plasmids were rescued from dominant negative colonies and retransformed into wild-type cells (PSYS580) to confirm plasmid linkage and the dominant negative phenotype. Plasmids that retested were sequenced to identify the mutations.

**Growth and Viability** — Cells were grown in SD–Leu overnight at 30 °C, diluted 1:200 into synthetic complete media supplemented with 2% raffinose (w/v) lacking leucine, grown at 30 °C to ~107 cells/ml, and induced with 2% galactose (w/v). Cells were counted every 2 h. Two hundred cells were plated at each time point on SD–Leu media and incubated for 2 days at 30 °C. Viability was determined by counting the number of colonies that arose from the 200 cells plated.

**RESULTS**

**Modeling of Saccharomyces cerevisiae Ntf2p Reveals a Surface Patch of Hydrophobic Residues** — As the two phenylalanines in the FxFG repeat core have a very hydrophobic nature, one would anticipate that their binding site on NTF2 would also be hydrophobic, analogous to the hydrophobic FxFG binding site identified on importin-β (37). Previous work consistent with this hypothesis has shown that residue Trp-7 of rNTF2 contributes to the interaction between rNTF2 and FxFG nucleoporins (35) and that mutation of Trp-7 of rNTF2 to alanine reduces binding to FxFG nucleoporins (35). Moreover, in crystal structures of both wild-type and mutant rNTF2 (35, 45), the hydrophobic patch surrounding Trp-7 is often involved in hydrophobic crystal contacts between adjacent molecules in the crystal lattice (29, 45). In particular, close interactions between the aromatic ring of Phe-126 and both Trp-7 and Trp-112 are found in the crystal structures of rat NTF2 (35, 45). As shown in Fig. 1B, both chains in the NTF2 dimer have a substantial hydrophobic patch on their surface (indicated by the black shading) that includes residues Phe-5 and Tyr-112 in yNTF2, which are analogous to Trp-7 and Trp-112 in rNTF2. This hydrophobic patch therefore appeared to be an attractive candidate for the FxFG nucleoporin binding site on NTF2. To test this hypothesis, we mutated both Phe-5 and Tyr-112 to alanine and also generated the F5A/Y112A double mutant. As a more drastic change, we mutated the Tyr-112 to an aspartic acid, thus changing a hydrophobic aromatic residue to a charged nonaromatic residue. As a more severe change to the hydrophobic patch, we constructed a deleted form of yNTF2 (Δ6Ntf2), which deletes residues 2–8 including Phe-5 and the surrounding residues (See Fig. 1A).

**Mutations in Hydrophobic Residues Alter the Localization of Ntf2p in Vivo** — To analyze the effect the engineered mutants have on binding to nucleoporins in vivo, each mutant Ntf2 protein was fused at the C terminus to the GFP and expressed in living cells. Ntf2p is a homodimeric protein that is required for viability in *S. cerevisiae* (41); however, overexpression of yeast Ran, GSP1, can compensate for the deletion of NTF2 (46). Therefore, to eliminate the possibility that any of the engineered mutants might dimerize with endogenous wild-type Ntf2p, plasmids expressing each of the GFP fusion Ntf2 proteins were trans-
formed into the NTF2 deletion strain maintained by a plasmid-encoded Gsp1p. As shown in Fig. 2A, although Ntf2p-GFP was concentrated at the nuclear rim, the engineered mutants of Ntf2p showed a range of localization patterns from Ntf2p structure showing positions of residues mutated in this study. Both chains of the scNTF2 dimer are shown (in pink and blue), and thus two copies of all described features are visible. Residues Phe-5 and Tyr-112 form part of a hydrophobic patch (F5, Y112, black) exposed on the surface of both chains. Residue Aan-77 (N77, yellow) is located in close proximity to the dimerization interface and between the two hydrophobic patches. Residue Asp-21 (D21) is also identified.

To determine whether the engineered mutations specifically affect the ability of Ntf2p to bind FxFG nucleoporins without disrupting the binding to Gsp1p, we performed in vitro binding assays. Each of the mutant proteins (with the exception of Y112D, which we found encoded an unstable protein (see Fig. 3B)) was expressed and purified from bacteria and attached to Sepharose beads as described under “Materials and Methods.” The beads were then incubated with yeast lysates expressing Myc-tagged Gsp1p (BQY65). After several washes, proteins that bound to the Ntf2p-beads (10% of total bound sample, Fig. 2B, upper panel, or 30% of total bound sample, Fig. 2B, lower panel) as well as 1% of the total unbound sample (Fig. 2B, upper panel) were analyzed by immunoblot analysis using anti-Myc antibodies to detect Gsp1p and mAb414 to detect nucleoporins. mAb414 was originally isolated from antisera raised against Triton X-100-treated rat liver nuclei (47) and has been shown to cross-react with two yeast nuclear envelope proteins, p110, which corresponds to the FxFG nucleoporin Nup2p,2 and p95 (48). Consistent with the in vivo localization data, the mutants varied significantly in their extent of binding to Nup2p (Fig. 2B). Although the interaction between Ntf2pF5A and Nup2p was comparable with the interaction observed for wild-type Ntf2p, Ntf2pY112A, Ntf2pF5A/Y112A, and ΔN6Ntf2p, all exhibited reduced Nup2p binding. As shown in Fig. 2C, all of the mutant proteins retained the ability to interact with Gsp1p, indicating that their reduced affinity for FxFG nucleoporins was not because they had failed to fold correctly. In summary, the engineered series of mutant Ntf2 proteins showed varying degrees of reduction in binding to FxFG nucleoporins without affecting binding to Gsp1p. Therefore, these mutants can be used to examine the interaction between Ntf2p and nucleoporins in vivo.

The Interaction between Ntf2p and FxFG Nucleoporins Is Essential in Vivo—To address whether the binding of Ntf2p to nucleoporins is crucial for the function of Ntf2p in vivo, a plasmid shuffle approach was used to determine whether each of the mutant proteins could function as the sole copy of the essential Ntf2 protein in cells. An NTF2 deletion strain, ACY114 (41), maintained by a wild-type copy of NTF2 on a URA3 plasmid was transformed with genomic clones of each of the ntf2 mutants. Cells that contain both the wild-type and the mutant plasmid are viable regardless of which mutant is analyzed, as demonstrated by growth of serially diluted (1/10) cells on the URA- plate (Fig. 3A, left panel). Cells were also serially diluted on to 5-FOA plates to select against the URA3 plasmid (43) that encodes wild-type Ntf2p. Thus, the right panel in Fig. 3A (5-FOA) shows the growth of cells that contain only the mutant NTF2 plasmids (or control plasmids) indicated in the absence of any other copy of NTF2. Results from this experiment indicated that growth of the ntf2F5A and ntf2Y112A mutants was comparable with the wild-type control and growth of the ntf2F5A/Y112A double mutant was only slowed slightly compared with wild-type control cells. In contrast, the ntf2Y112D mutant grew slowly, and the ΔN6ntf2 mutant was not viable in the absence of wild-type Ntf2 protein. Similar results were observed at 16, 25, and 37 °C. The phenotypes observed were confirmed through the analysis of growth in liquid media (data not shown). We performed immunoblots using lysates from cells expressing each of the mutant GFP proteins to ensure that each of the mutant proteins was expressed at a comparable level. Although all of the mutant proteins were expressed (Fig. 3B), Ntf2pY112D was expressed at a significantly lower level than wild-type Ntf2p, suggesting that the Y112D mutation affects the stability of the Ntf2 protein in vivo; therefore, we excluded the Y112D mutant protein from subsequent analyses. However, the ΔN6ntf2 protein was

\[^2\] B. B. Quimby and A. H. Corbett, unpublished data.

\[^2\] S. cerevisiae.
expressed at comparable levels to wild-type Ntf2p, indicating that its loss of in vivo function was not the result of instability or low levels of expression.

The Strength of the Interaction between Ntf2p and FxFG Nucleoporins Influences Protein Import into the Nucleus—The generation of a series of Ntf2 mutant proteins that varied in the strength of their binding to nuclear pores but remained viable (with the exception of Δ6Ntf2p, which cannot be included in the following analysis because cells expressing this mutant protein as the sole copy of Ntf2p are not viable (See Fig. 3A)) allowed us to assess how the strength of the interaction with FxFG nucleoporins affects the in vivo nuclear transport function of Ntf2p. To examine nuclear import, a kinetic assay that monitors nuclear import of an NLS-GFP reporter protein was employed (42). The F5A/Y112A mutation, which had a severe effect on the binding of Ntf2p to Nup2p, significantly reduced the import of labeled NLS protein into the nucleus (Fig. 4A, filled squares). In contrast, Ntf2pY112A, which reduced the interaction with nucleoporins less dramatically, appeared to have no detectable effect on protein import (Fig. 4A, open circles). These results suggest that the interaction between Ntf2p and nucleoporins can be reduced substantially without producing a significant decrease in the rate of NLS-mediated protein import.

Ntf2p is required for the import of Ran (Gsp1p) into the nucleus (23–25). To determine the importance of the Ntf2p-nucleoporin interaction on RanGDP nuclear import, we examined Ran-GFP localization in each mutant NTF2 strain. In wild-type cells, Ran-GFP localized throughout the cell, with a clear concentration in the nucleus as observed previously (25).

Similar to the effects of the mutations on nuclear protein import, ntf2F5A and ntf2Y112A had little effect on Ran localization, although ntf2F5A showed marginally higher levels of cytoplasmic Ran than wild type. However, in cells expressing Ntf2pF5A/Y112A, a greater amount of Ran-GFP was mislocalized to the cytoplasm (Fig. 4B) indicating that Ran import into the nucleus is less efficient in these cells than in wild-type cells.

It should be noted that although the Ntf2pF5A/Y112A cells are larger than wild-type cells, they consistently showed a more cytoplasmic distribution of Ran-GFP than any of the other cells analyzed.

In summary, we have identified several residues within a hydrophobic patch on the surface of the yNTF2 protein that are involved in Ntf2p binding to FxFG nucleoporins and have shown that when two of these hydrophobic residues are mutated, a more significant effect on both the strength of Ntf2p binding to FxFG nucleoporins in vitro and the function of Ntf2p in RanGDP nuclear import in vivo is observed. The strength of the Ntf2-nucleoporin interaction can be reduced substantially with little effect on cell viability; although cells do not survive if the interaction is eliminated completely. Although these data indicate that the Ntf2p-FxFG interaction is essential in vivo, they also suggest that this interaction can be substantially weakened without producing a serious effect on cell viability consistent with a model in which Ntf2p only interacts with nucleoporins transiently (with a low binding affinity) so that it may pass from one nucleoporin to the next to traverse the NPC (37).

Isolation of a Dominant Negative Mutant of NTF2 with Increased Binding to FxFG Nucleoporins—Our experiments
make two predictions about Ntf2p-mediated nuclear import of Ran. First, Ntf2p must interact with nucleoporins to mediate Ran import in vivo. Second, a functional interaction between Ntf2p and nucleoporins can occur with low affinity. This raises the question of whether the low affinity interaction between Ntf2p and nucleoporins is critical for the in vivo function of Ntf2p. To test whether increasing the affinity of Ntf2p for nucleoporins interferes with Ntf2p function, we devised a genetic screen for NTF2 mutations that alter the Ntf2p-nucleoporin interaction. We hypothesized that overexpression of a mutant Ntf2 protein with increased affinity for nucleoporins would block nucleocytoplasmic transport in vivo and inhibit cell growth. Therefore we conducted a screen to identify dominant negative mutants of Ntf2p that inhibited cell growth when expressed at high levels from a galactose-inducible promoter. Mutants identified in this screen were then tested to identify those with altered binding to nucleoporins. We identified one mutation that met the criteria of the screen. This mutation results in a change from asparagine to tyrosine at residue 77 (N77Y).

Overexpression of NtF2N77Y inhibits cell growth in vivo (Fig. 5A). It should be noted that the overexpression of wild-type NTF2 is slightly dominant negative (compare vector and NTF2 in Fig. 5A). To further characterize the growth inhibitory phenotype of the N77Y mutant, growth in liquid medium was analyzed. As shown in Fig. 5B, overexpression of the N77Y mutant protein results in a strong growth-inhibitory effect (~30% of wild-type growth after 10 h).

During the course of our work, it was reported that a mutation in mammalian NTF2, D23A NTF2, exhibited a dominant negative phenotype (49). These studies indicated that the D23A NTF2 protein maintained the ability to interact with Ran while blocking FxFG nucleoporins more efficiently. In addition, when the D23A NTF2 protein was added in excess in in vitro nuclear protein import assays, importin-β-mediated protein import was blocked (49). To analyze the in vivo effect of this mutation on cellular function, we made the complementary mutation in yeast NTF2, ntF2/2D21A (See Fig. 1). As shown in Fig. 5, A and B, this mutation indeed caused a dominant negative effect on growth in vivo, although slightly less of an effect was observed (~24% of wild-type growth after 10 h) than with the N77Y mutant protein (~30% of wild-type).

To ensure that the dominant negative mutant proteins were expressed to comparable levels and to determine the effect, if any, these mutations have on the stability of the Ntf2p protein, each of the mutant proteins was fused at the C terminus to GFP. These fusion proteins were introduced into the NTF2 deletion strain, ACY114, maintained by a plasmid containing GSP1 (to prevent the possible dimerization with endogenous wild-type Ntf2p). Lysates from individual transformants were analyzed by immunoblotting with anti-GFP. As shown in Fig. 5C, the mutant proteins were expressed to comparable levels that were slightly higher than those observed with wild-type protein. Because overexpression of wild-type Ntf2p is slightly toxic, it was possible that the higher expression levels of the mutant proteins could contribute to their toxicity. However, this was thought to be unlikely because although each mutant protein was expressed at approximately the same level, we observed a difference in the level of toxicity between the two mutants.

To determine why overexpression of the N77Y mutant protein inhibits cell growth, we first examined the effect that overexpression had on protein import. Overexpression of wild-type Ntf2p had a slight effect on the localization of an NLS-GFP reporter. Cells that overexpress wild-type Ntf2p showed a
small increase in cytoplasmic GFP fluorescence (Fig. 6A) consistent with the observation that high levels of wild-type Ntf2p are slightly toxic to cells (See Fig. 5A). Overexpression of the N77Y protein caused significant mislocalization of the NLS reporter protein to the cytoplasm.

To determine whether the block in NLS-mediated protein import was secondary to the inability of the N77Y protein to import RanGDP into the nucleus, we analyzed the localization of Ran-GFP in cells overexpressing the N77Y protein. Overexpression of wild-type Ntf2p results in a slight increase in cytoplasmic Ran-GFP (Fig. 6B). Overexpression of the N77Y protein resulted in a brighter punctate nuclear rim signal (see arrows in Fig. 6B) together with a decrease in nuclear staining. The effect observed is subtle but is reproducible in numerous independent experiments. These results suggest that Ran may accumulate at NPCs, possibly because of an increased affinity of the N77Y mutant proteins for nucleoporins, thus resulting in a reduced rate of transport of RanGDP through the pore. Alternatively, the N77Y protein may have an increased affinity for RanGDP resulting in less efficient release of RanGDP into the nucleus at the nucleocytoplasmic face of the pore.

Fig. 7A shows that, like wild-type Ntf2p, the N77Y protein localizes to the nuclear rim. This observation suggests that N77Y retains its interaction with the nuclear pore but cannot distinguish whether it might have enhanced affinity for the pore. To examine the interaction of the N77Y protein with the nucleoporins, N77Y was expressed and purified from bacteria and attached to beads. The beads were incubated with yeast lysate and washed, and proteins that bound were eluted and analyzed by immunoblotting for Nup2p and Gsp1p binding as described under “Materials and Methods.” In this bead
The N77Y mutation increases the affinity of Ntf2p for nucleoporins. A, localization of Ntf2p-GFP. A 2µ plasmid encoding either wild-type or mutant Ntf2p fused at the C terminus to GFP was transformed into the NTF2 deletion strain ACY114 maintained with a CEN plasmid encoding Gsp1p. Single transformants were inoculated into Ura − liquid and grown to log phase at 30 °C. Ntf2p-GFP was viewed directly in living cells. Interactions with liquid Nup2p (B and C) were examined using bead-binding assays as described under “Materials and Methods.” B, Ntf2 proteins were bound to beads and incubated with wild-type yeast lysate. Bound and unbound samples were resolved by gel electrophoresis and transferred by standard methods (see “Materials and Methods”). Blots were probed with mAb414 to detect Nup2p. U indicates the unbound fraction (1% of total unbound), and B indicates the bound fraction (10% of total bound). C, Nup2p competition assay. Bead-binding assays were performed (see “Materials and Methods”) in the presence of 300 µg of either bovine serum albumin or competitor wild-type Ntf2p. Unbound (U) and bound (B) fractions were probed for Nup2p. D, binding to Gsp1p. Bound samples in panel B were probed for Myc-tagged Gsp1p using an anti-Myc antibody. Both N77Y and D21A bound Gsp1p at levels comparable with wild-type Ntf2p. E, functional analysis of N77Y Ntf2p. The NTF2 deletion strain, ACY114, maintained by a plasmid encoding wild-type Ntf2p was transformed with a CEN plasmid encoding either wild-type or N77Y Ntf2p. Cultures were grown to saturation, serially diluted (1:10), and spotted on plates. For each plasmid, the Ura− plate illustrates that each of the strains can grow in the presence of a wild-type copy of NTF2. The 5-FOA plate demonstrates that the ntf2N77Y is unable to grow in the absence of a wild-type copy of NTF2. Vector alone is shown as a control to demonstrate that the deletion strain requires a functional copy of NTF2 for viability. Plates were grown at 30 °C.

Increasing the Affinity of Ntf2p for FxFG Nucleoporins Is Detrimental to Cellular Function—To test the effect of increasing the affinity of Ntf2p for FxFG nucleoporin on the in vivo function of Ntf2p, we transformed a plasmid-borne copy of ntf2N77Y into the yeast NTF2 deletion strain and asked whether these transformants could grow in the absence of wild-type NTF2. As shown in Fig. 7E, cells expressing the N77Y protein as the sole form of Ntf2p were not viable. Thus, increasing the affinity of Ntf2p for FxFG nucleoporins is detrimental to cells. These data support the hypothesis that the interaction between Ntf2p and nucleoporins must be weak enough so that the interaction is sufficiently transient to allow Ntf2p to traverse the NPC (36).

**DISCUSSION**

The recent analysis of the co-crystal structure of residues 1–442 of importin-β with a peptide containing five tandem FxFG repeats from the nucleoporin Nsp1p showed a primary and a secondary binding site for the FxFG repeats (37). Both binding sites are hydrophobic pockets that permit hydrophobic contacts between importin-β and the phenylalanines of the FxFG repeat cores as well as stacking of the core phenylalanines themselves. Our modeling of the S. cerevisiae NTF2 protein based on the crystal structure of rat NTF2 showed an analogous hydrophobic patch on the surface of yeast NTF2. This hydrophobic patch is separate and distinct from the RanGDP binding site on NTF2. Two amino acids residues in this patch were thought to be likely candidates to form hydrophobic contacts with the phenylalanines of FxFG nucleoporins: Phe-5, which corresponds to Trp-7 in the rat NTF2 protein, and Tyr-112 that, by analogy with Trp-112 in rNTF2, probably protrudes into this hydrophobic pocket in yNTF2 (Fig. 1B). Ribbeck and Görlich (38) recently reported that mutating Trp-7 to arginine in rat NTF2 significantly reduces the rate of transport passage of NTF2 through the NPC (38). However, this
This model and proposes that translocation proceeds through a binding site at or within the NPC. This hypothesis would also predict that overexpression of wild-type NTF2 would compete for binding to nucleoporins and result in less efficient NLS-mediated protein import. Indeed, our results demonstrate that overexpression of wild-type Ntf2p is slightly toxic to cells over time (≥5 h, Fig. 5C) although we did not observe any effect on NLS-mediated protein import 3 h after induction (Fig. 6B).

A central question outstanding in nuclear transport is how molecules actually traverse through nuclear pores. Addressing this complex question will probably require a higher resolution structure of the nuclear pore than currently exists, but mapping the protein-protein interactions that occur between soluble transport factors and the pore, and that are likely to mediate this process, is also an important prerequisite to understanding the molecular mechanism of nuclear trafficking. In the present study we have identified a hydrophobic patch on NTF2 that is crucial for the NTF2-NPC interaction both in vivo and in vitro and have used this information to engineer mutants to dissect the functional significance of this interaction. This is an important step toward understanding the pathway that NTF2 takes in the course of transporting RanGDP through the nuclear pore to maintain the RanGTP gradient. Furthermore, our work suggests that both NTF2 and importin-β are probably translocated through NPCs by analogous hydrophobic interactions involving the cores of FxFG nucleoporins.

Acknowledgments—We are grateful to members of the Corbett and Stewart laboratories for helpful discussions and comments on the manuscript. We thank Drs. K. Marfatia and P. Fanara for extremely helpful comments on the manuscript.

REFERENCES

1. Gorlich, D., and Kutay, Ü. (1999) Annu. Rev. Cell Dev. Biol. 15, 607–660
2. Adam, S. A. (1999) Curr. Opin. Cell Biol. 11, 402–406
3. Bayliss, R., Corbett, A. H., and Stewart, M. (2000) Traffic 1, 448–456
4. Gorlich, D. (1998) EMBO J. 17, 2721–2727
5. Melchior, F., and Gerace, L. (1998) Trends Cell Biol. 8, 175–179
6. Rout, M. P., Aitchison, J. D., Suprapto, A., K. Hjertaas, Zhao, Y., and Chait, B. T. (2000) J. Cell Biol. 148, 635–652
7. Rout, M. P., and Wente, S. R. (1994) Trends Cell Biol. 4, 357–365
8. Racu, A., Bibol, G., and Moore, M. S. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1769–1773
9. Bezach, M., and Blobel, G. (1995) Cell 83, 683–692
10. Chi, N. C., Adam, J. H. E., and Adam, S. A. (1999) J. Cell Biol. 139, 265–274
11. Hu, T., Guan, T., and Gerace, L. (1996) J. Cell Biol. 134, 589–601
12. Shah, S., and Forbes, D. J. (1999) Curr. Biol. 9, 1378–1388
13. Kishimoto, T., and Yoneda, T. (1999) J. Cell Biol. 143, 327–330
14. Seedorf, M., Danelim, M., Kahana, J., Taura, T., and Silver, P. A. (1999) Mol. Biol. Cell. 12, 1547–1555
15. Kehlenbach, R. H., Dickmanns, A., Kehlenbach, A., Guan, T., and Gerace, L. (1999) J. Biol. Chem. 274, 28575–28580
16. Grote, M., Kubitscheck, U., Reichelt, R., and Peters, R. (1995) J. Cell Sci. 108, 2963–2972
17. Bayliss, R., Ribbeck, K., Akin, D., Kent, H. M., Feldherr, C. M., Gorlich, D., and Stewart, M. (1999) J. Mol. Biol. 288, 379–393
36. Chaillan-Huntington, C., Braslavsky, C. V., Kuhlmann, J., and Stewart, M. (2000) J. Biol. Chem. 275, 5874–5879
37. Bayliss, R., Littlewood, T., and Stewart, M. (2000) Cell 102, 99–108
38. Ribeck, K., and Görlich, D. (2001) EMBO J. 20, 1320–1330
39. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
40. Rose, M. D., Winston, F., and Hieter, P. (1990) Methods in Yeast Genetics: A Laboratory Course Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
41. Corbett, A. H., and Silver, P. A. (1996) J. Biol. Chem. 271, 18477–18484
42. Shulga, N., Roberts, P., Gu, Z., Spitz, L., Tabb, M. M., Nomura, M., and Goldfarb, D. S. (1996) J. Cell Biol. 135, 329–339
43. Boeke, J. D., Truehart, J., Natsoulis, G., and Fink, G. (1987) Methods Enzymol. 154, 164–175
44. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
45. Bullock, T. L., Clarkson, W. D., Kent, H. M., and Stewart, M. (1996) J. Mol. Biol. 260, 422–431
46. Paschal, B. M., Delphin, C., and Gerace, L. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7679–7683
47. Davis, L. I., and Blobel, G. (1986) Cell 45, 699–709
48. Aris, J. P., and Blobel, G. (1989) J. Cell Biol. 108, 2059–2067
49. Lane, C. M., Cushman, I., and Moore, M. S. (2000) J. Cell Biol. 151, 321–332
50. Stöffler, D., Fahrenkrug, B., and Aebi, U. (1999) Curr. Opin. Cell Biol. 11, 391–401
51. Talcott, B., and Moore, M. S. (1999) Curr. Biol. 9, 312–318
52. Ben-Efraim, I., and Gerace, L. (2001) J. Cell Biol. 152, 411–417
53. Görlich, D., and Mattaj, I. W. (1996) Science 271, 1513–1518
54. Davis, L. I., and Fink, G. R. (1990) Cell 61, 965–978
55. Wong, D. H., Corbett, A. H., Kent, H. M., Stewart, M., and Silver, P. A. (1997) Mol. Cell Biol. 17, 3755–3767
56. Orr-Weaver, T. L., and Szostak, J. W. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 4417–4421