High Levels of the GTPase Ran/TC4 Relieve the Requirement for Nuclear Protein Transport Factor 2*

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The GTPase Ran/TC4 and the 14-kDa protein nuclear transport factor 2 (NTF2) are two of the cytosolic factors that mediate nuclear protein import in vertebrates. Previous biochemical studies have shown that NTF2 binds directly to the GDP-bound form of Ran/TC4 and to proteins of the nuclear pore complex that contain phenylalanine-glycine repeats. In the present study we have used molecular genetic approaches to study the Saccharomyces cerevisiae homologue of NTF2. The scNTF2 gene encodes a protein that is 44% identical to the human protein. We found that deletion of the scNTF2 gene is lethal and that repression of scNTF2p expression by a regulatable promoter results in gross structural distortions of the nuclear envelope. In a screen for high copy number suppressors of a scNTF2 deletion, the only gene we isolated other than scNTF2 itself was GPS1, the S. cerevisiae homologue of Ran/TC4. Furthermore, we found that high levels of Ran/TC4 can relieve the requirement for NTF2 in a mammalian-permeabilized cell assay for nuclear protein import. These data suggest that certain of the nuclear protein import functions of NTF2 and Ran/TC4 are closely linked and that NTF2 may serve to modulate a transport step involving Ran/TC4.

Exchange of macromolecules between the cytoplasm and nucleus is specified by signals encoded within transported proteins. The signals that specify nuclear protein import, which are called nuclear localization signals (NLSs), usually consist of short stretches of amino acids enriched in basic amino acid residues (1). A group of cytosolic factors that mediate nuclear import of proteins containing these basic-type NLSs has been identified. These include the α subunit of the NLS receptor (α importin/α karyopherin; Refs. 2–4) and its β subunit (p97/β importin/β karyopherin; Refs. 5–7), the GTPase Ran/TC4 (8, 9), and the homodimeric protein nuclear transport factor 2 (NTF2/p10; Refs. 10 and 11). According to current working models (1, 12), the pathway for import of proteins with basic-type NLSs begins in the cytoplasm, where the α subunit of the receptor binds to the NLS of a protein destined for import. This complex then binds to the cytoplasmic surface of the nuclear pore complex (NPC) via the β subunit, an event that is referred to as the initial binding or docking step of transport. Subsequently, the substrate-receptor complex is delivered to the central channel of the NPC and then translocated into the nucleoplasm (events collectively referred to as the translocation step; Ref. 13). The latter processes very likely involves Ran/TC4 and NTF2.

A number of binding interactions of Ran/TC4 and NTF2 have been described by in vitro biochemical studies, and these associations can provide a framework for studying the precise functions of these factors in nuclear import. The GTP-bound form of Ran/TC4 can bind to three distinct proteins implicated in nuclear import: the β subunit of the NLS receptor (14, 15), RanBP1 (16), and the NPC protein RanBP2/Nup358 (17, 18). The GDP form of Ran/TC4 can bind to NTF2 (14, 19) as well as to a complex containing RanBP1 and the β subunit of the NLS receptor (20). NTF2 itself can bind directly to multiple proteins of the NPC containing FACTG repeat motifs, including p62 (10, 19, 21). It has become clear that elucidating the mechanism of nuclear protein import will require understanding how the cytosolic transport factors interact with each other and with the NPC at specific transport steps and how these interactions are coupled to the GTPase cycle of Ran/TC4 (1, 12).

The mechanisms of nuclear protein import are predicted to be conserved between vertebrates and Saccharomyces cerevisiae. Basic-type NLSs within proteins such as the glucocorticoid receptor and the SV40 large T antigen are fully functional in yeast, and the primary structures of the cytosolic transport factors show 40–80% identity when human and yeast proteins are compared. The basic architecture of the NPC appears to be conserved as well (22). We have, therefore, used a molecular genetic approach to characterize NTF2 in the budding yeast S. cerevisiae. We have found that the scNTF2 gene is required for viability and that deletion of scNTF2p leads to nuclear structure defects similar to those observed with mutants of certain nuclear pore complex proteins. We carried out a genetic selection for multicopy suppressors that would compensate for loss of scNTF2p. This analysis revealed that the S. cerevisiae homologue of Ran/TC4 is a multicopy suppressor of a scNTF2 deletion strain, thus providing the first in vitro evidence that certain functions of NTF2 and Ran/TC4 are linked. Furthermore, we have used recombinant proteins to show that Ran/TC4 can relieve the requirement for NTF2 in a permeabilized cell import assay in mammalian cells. Taken together, these data support the hypothesis that NTF2 serves to modulate a transport step(s) involving Ran/TC4.

EXPERIMENTAL PROCEDURES

Plasmids—To generate the plasmid used for methionine-regulated expression of scNTF2p, the scNTF2 ORF was amplified by PCR and cloned into the unique BamHI site immediately downstream of the MET3 promoter in the plasmid pRS405-MET3 (generously provided by
The screen for multicopy suppressors was carried out in the strain BY4. Log phase yeast cells were transformed by using polyethylene glycol and lithium acetate (25) with a *S. cerevisiae* genomic library constructed in YEp24 (26) and plated onto synthetic medium containing 5 mM methionine and lacking uracil. We plated 1% of the transformation onto media lacking methionine and determined that this procedure yielded a total of ~10^6 Ura− transformants. The 480 Ura− colonies that grew under the condition of scNTF2p repression were replated onto methionine-containing medium. Whole cell PCR was used to screen the 480 colonies for the presence of the library plasmids that contained the scNTF2 gene.

**Electron Microscopy**—Log phase yeast cells were harvested up to 7 h after the addition of methionine and processed for electron microscopy essentially as described by Byers and Goetsch (27). We used both Spurr and Epon resins, the latter of which yielded slightly better preservation of membranes in our experiments.

**Other Methods**—The permeabilized cell assay used to quantitate nuclear protein import in HeLa cells was described previously (10). The reporter molecule for nuclear protein import was fluorescein isothiocyanate-labeled bovine serum albumin-conjugated with the SV40 large T antigen NLS (10). Pretreatment of HeLa cell cytosol with p62-Sepharose and preparation of recombinant NTF2 and Ran/TC4 have also been described previously (8, 10). The antibody to recombinant human NTF2 was generated in rabbits, affinity purified on NTF2-Sepharose, and used at a final concentration of 2 μg/ml. Anti-serum to the GSP1 protein (28) was used at a dilution of 1:3000 and was kindly provided by Dr. Pierre Belhumeur (University of Montreal). Immunoblots were developed with peroxidase-labeled secondary antibodies and enhanced chemiluminescence.

**RESULTS**

Sequence from the *S. cerevisiae* genome project revealed an open reading frame on chromosome V (cosmid 9537) that is highly related to the sequence of NTF2, a cytosolic factor that facilitates protein transport into the nucleus in mammalian cells (10). The predicted ORF encodes a 125-amino acid protein that is 44.4% identical and 61.5% similar to the 127-amino acid human NTF2 protein (Fig. 1A). The central region of the protein (Phe-12–Pro-76) is 58% identical, whereas the primary structures of the amino and carboxyl termini are unrelated.
except for residues Asn-116–Leu-121. The sequence relationship together with the similarities in size and isoelectric point suggest the yeast ORF encodes the S. cerevisiae homologue of NTF2 and is hereafter referred to as scNTF2p. Evidence that these proteins are functional homologues was obtained in recent work showing that the human cDNA encoding NTF2 can substitute for the yeast gene (29).

*The scNTF2p Is Required for Viability—* We assessed the requirement for the scNTF2p in cell viability by standard gene replacement methods in the yeast strain 15D. To generate an scNTF2 deletion allele, we first used PCR primers that included sequences complementary to the 5' and 3' regions of the scNTF2 gene to amplify the URA3 gene (Fig. 1B). The diploid strain 15D was then transformed with the linear PCR product. Stable Ura+ transformants were screened for integration of the URA3 gene by PCR using oligonucleotides that flank the scNTF2 locus (primers 1 and 2; Fig. 1C). The DNA template from one Ura+ transformant yielded PCR products with sizes of ~560 and ~1300 base pairs. The smaller product is the size expected from amplification of the scNTF2 locus, whereas the larger product is consistent with the replacement of the scNTF2 ORF with the URA3 gene. This was confirmed by showing that a PCR reaction with a primer that flanks scNTF2 (primer 1) and a primer within the URA3 gene (primer 3) produces a ~700-base pair fragment.

The heterozygote deletion strain for scNTF2 (scNTF2/ΔscNTF2::URA3, denoted BPY1) displayed no obvious defects in growth, morphology, or thermosensitivity (data not shown). Sporulation of the BPY1 strain and subsequent analysis of >40 tetrads revealed a 2:0 segregation (live:dead), demonstrating that the scNTF2 gene is required for viability (Fig. 1D). We confirmed that viable segregants were Ura+ by their failure to grow in the absence of uracil. These data are in agreement with those obtained using other strains of *S. cerevisiae* (PSY853, Ref. 29; W303, data not shown, Ref. 19).

**Effects of scNTF2 Depletion *In Vivo*—** As a first step toward analyzing the function of scNTF2 in vivo, we examined the effects of scNTF2p depletion in haploid deletion strains containing plasmids with the scNTF2 gene under the control of different regulatable promoters. Initial experiments were carried out with scNTF2p expression regulated by the GAL10 promoter. We observed that colony growth in haploid deletion strains carrying the GAL10 plasmid was slowed substantially but not completely inhibited (data not shown), suggesting that expression from this plasmid is leaky. We chose the MET3 promoter as an alternative for achieving repressible scNTF2p expression. Transcription driven by this promoter can be strongly repressed by growing cells in the presence of millimolar concentrations of methionine (30). In a haploid deletion strain (BPY4) containing scNTF2 under the control of the MET3 promoter, transcription from the scNTF2 plasmid in the absence of methionine restored growth of this strain to the same level as the WT strain (Fig. 2, –Methionine). In contrast, substantial growth inhibition resulted from including 5 mM methionine in the media, an effect that was manifest as an approximately 100-fold difference in viability as compared with the WT strain (Fig. 2, +Methionine). The BPY4 colonies that did survive selection in the presence of methionine were also visibly smaller than colonies composed of WT cells.

Methionine-repression of the scNTF2 gene in the BPY4 strain resulted in distortion and apparent fragmentation of the nucleus such that the nuclear DNA appeared as small 4.6-diamidino-2-phenylindole dihydrochloride staining domains within these cells (data not shown). Surprisingly, the distorted nuclei present in the mutant cells accumulated a NLS-contain-

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2 D. Goldfarb, personal communication.
nearly 500 colonies that grew in the presence of methionine in both the initial and secondary plating steps. Since we expected that the scNTF2 gene would represent a significant proportion of the putative suppressors, we employed a PCR screen with primers that flanked the scNTF2 gene to rapidly identify library clones containing scNTF2. Since the genomic scNTF2 ORF in the BPY4 strain had been replaced with the TRP1 gene, amplification of this locus generated a product whose larger size distinguished it from the product amplified from the sc-NTF2 gene on a library plasmid. The scNTF2 ORF under the control of the MET3 promoter was not detected in this screen due to its lack of complementarity with the PCR primers used. A total of 480 isolates were examined by this procedure. Two-thirds of the colonies (322 of 480) were determined to be sc-NTF2 by this criteria, demonstrating the validity of our selection regimen. Furthermore, the strong selection for scNTF2 suppressors strongly suggests that the methionine-induced repression of transcription from the pMET-scNTF2 plasmid (together with cell doubling) leads to depletion of cellular scNTF2p.

Three of the 158 clones that did not contain the scNTF2 gene were analyzed further. We sequenced approximately 400 base pairs at each end of these clones and learned all three were derived from an approximately 7-kilobase segment of chromosome XII. This region of chromosome XII is notable as it includes the locus for GSP1, the S. cerevisiae homologue of Ran/TC4 (28). These three clones also contained the gene for a GTPase of unknown function, termed GUF1 (31). We determined by deletion analysis that the suppressor activity can be ascribed to the portion of the insert that includes the GSP1 gene. The BPY4 strain carrying the pMET-scNTF2 plasmid was transformed with the original library plasmid or the deletion variants as depicted in Fig. 4A. A dilution series of each culture was then applied to solid medium prepared without or with methionine. In this assay, we found that growth in the presence of methionine could be rescued by the full-length library insert or the portion containing GSP1, whereas a deletion construct containing the GUF1 locus failed to rescue (Fig. 4A).

We analyzed 27 additional candidate suppressor clone candidates from the 158 methionine-resistant colonies by Southern blotting and PCR and determined that 25 of these 27 clones contained the GSP1 gene. The remaining two clones failed to rescue growth on methionine when reintroduced into yeast and likely represent the background in this screen. These data indicate that the vast majority of the clones that rescued growth on methionine are GSP1.

Our finding that cells depleted of an essential gene product (scNTF2p) can be rescued by a gene that is unrelated in sequence (GSP1) is surprising. One explanation is that in the presence of methionine the scNTF2p was still expressed at low levels, and in this background overexpression of the GSP1p suppressed the growth defect. Alternatively, it was possible that the scNTF2p was virtually absent from the mutant strain, and overexpression of the GSP1p compensated for this loss. To
address this issue, we tested whether overexpression of GSP1p would allow a complete loss of the pMET-scNTF2 plasmid in the BY4 (ΔscNTF2) strain. We transformed the BY4 strain with pURA-GSP1 and maintained transformants in continuous culture for 48 h. This was done in the absence of uracil to maintain the pURA-GSP1 plasmid and in the presence of methionine and leucine to repress transcription and to allow for loss of the LEU plasmid pMET-scNTF2, respectively. The cultures were plated at low density on medium lacking uracil, grown for three days, and replica-plated onto media lacking tryptophan, threonine, and leucine to score for the presence/absence of the pMET-scNTF2 plasmid. Approximately one-third of the colonies were Ura− and Leu− (data not shown), indicating that overexpression of GSP1p can, in fact, compensate for the complete loss of scNTF2p in vivo. We also found that the growth rate of the strain carrying pGSP1 and lacking pMET-scNTF2 was essentially identical to the WT strain (Fig. 4B). GSP1p expression in this strain was elevated 1.73-fold relative to WT cells carrying only a plasmid to confer growth in the absence of uracil (Fig. 4C). It is not surprising that a higher level of GSP1p expression is not achieved, considering that GSP1p in WT cells is already a very abundant cellular protein. Taken together, these data establish unequivocally that modulating the level of GSP1p relieves the requirement for what is otherwise an essential gene.

**DISCUSSION**

Our initial characterization of scNTF2p in the budding yeast *S. cerevisiae* has provided valuable insight on the role of this protein in cell function. We have found that this protein is required for cell viability, in agreement with observations from other laboratories (19, 29). Moreover, we observed that depletion of scNTF2p leads to aberrant nuclear morphologies characterized by gross invaginations of the nuclear envelope and...
Functional Link of Ran/TC4 and NTF2

NTF2 binds directly to several NPC proteins of the NPC as well as to the GDP form of Ran, suggesting it could play a role in modulating the interaction of transport complexes with several discrete NPC sites (14, 91, 21). Our observations that a high level of Ran/TC4 can relieve the requirement for NTF2 in cell viability (in yeast) and nuclear protein import (in mammalian cells) suggests that NTF2 may regulate a protein-protein interaction(s) that involves Ran/TC4.

In summary, we have demonstrated that the scNTF2 gene encodes an essential protein that is linked to some function(s) of GSP1, the S. cerevisiae homolog of Ran/TC4. The ability of a high level of Ran/TC4 to relieve the requirement for an otherwise essential gene product in vivo as well as in vitro places constraints on possible functions for NTF2 in nuclear protein import. We favor a role for NTF2 in regulating protein-protein interactions that involves Ran/TC4 at the nuclear pore complex.

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