Conserved Nuclear Export Sequences in Schizosaccharomyces pombe Mex67 and Human TAP Function in mRNA Export by Direct Nuclear Pore Interactions*

Received for publication, September 2, 2003, and in revised form, February 4, 2004
Published, JBC Papers in Press, February 12, 2004, DOI 10.1074/jbc.M309731200

Anjan G. Thakurta‡, Ganesh Gopal‡, Jin Ho Yoon§, Tapas Saha, and Ravi Dhar‡**

From the ‡Center for Cancer Research, NCI and §NICHD, National Institutes of Health, Bethesda, Maryland 20892, the ¶Department of Biology, Sungshin Women’s University, Dongsan-dong 3 ka 249-1, Seongbuk-gu, Seoul 136-742, South Korea, and the ¶¶Division of Biology, College of Natural Sciences, Kangnung National University, Gangneung 210-702, South Korea

Mex67, the homolog of human TAP, is not an essential mRNA export factor in Schizosaccharomyces pombe. Here we show that S. pombe encodes a homolog of the TAP cofactor that we have also named p15, whose function in mRNA export is not essential. We have identified and characterized two distinct nuclear export activities, nuclear export signal (NES) I and NES II, within the region of amino acids 434–509 of Mex67. These residues map within the known NTF2-like fold of TAP (amino acids 371–551). We show that the homologs of these two NESs are present and are functionally conserved in TAP. The NES I, NES II, and NES I + II of TAP and Mex67 directly bind with -phenylalanine-glycine (-FG)-containing sequences of S. pombe Nup159 and Nup98 but not with human p62. Mutants of NES I or NES II of Mex67/TAP that do not bind -FG Nup159 and Nup98 in vitro are unable to mediate nuclear export of a heterologous protein in S. pombe and in HeLa cells. Fused with the RNA recognition motifs (RRMs) of Crp79 and green fluorescent protein (GFP) (RRM-NES-GFP), the NES I and NES II of Mex67 or TAP can suppress the mRNA export defect of the Δp15 rae1-167 synthetic lethal S. pombe strain, suggesting that the NESs can function in the absence of p15. These novel nuclear export sequences may provide additional routes for delivering Mex67/TAP to the nuclear pore complex.

Evolutionarily conserved nuclear export factors (NXFs) transport eukaryotic messenger RNA as a ribonucleoprotein complex from the nucleus to the cytoplasm (1–3). NXF homologs such as Saccharomyces cerevisiae Mex67 or human TAP function in mRNA export by forming heterodimers with small cofactor proteins Mtr2p and p15, respectively (4, 5). NXF proteins have a modular organization encoding domains for cargo binding and nuclear export through the nuclear pore complex (NPC) (2, 3, 6). The C-terminal half of the NXF proteins contains multiple determinants for NPC targeting. In human TAP, they consist of two distinct domains: the NTF2-like scaffold resulting from dimerization with p15 and the UBA-like domain resembling the ubiquitin-associating fold in many enzymes in the ubiquitylation pathway, some kinases, and DNA repair factors (7–9). The NTF2-like or the UBA-like domains each contain a single binding pocket for -phenylalanine-glycine (-FG) repeat sequences along with the XFG repeat sequences present in many nuclear pore proteins (Nups) (8). Disruption of the NTF2 fold by mutations, e.g. Leu386 and Leu386 to Arg, reduces the nuclear rim association of TAP in vitro (8). A W594A mutation diminishes the ability of the UBA-like domain to bind -FG repeat sequence in vitro and abolishes nuclear rim association in vivo (7, 8, 10). Recent experiments suggest that the two -FG binding sites may act synergistically to promote translocation of the cargo-carrier complex through the nuclear pore (8). A minimum of two -FG-interacting sites provided by either NTF2-like or UBA-like domains, or a combination of the two, can suffice in mRNA export (11).

TAP could be also targeted to the NPC via its interaction with GLE2, a nuclear pore-associated protein whose Schizosaccharomyces pombe homolog Rael is essential for mRNA export (10, 12). A ternary complex of TAP-GLE2-NUP98 has been detected in the Xenopus oocyte extracts as a possible intermediate for targeting TAP to the NPC (13). Another nuclear protein, Sac3p of S. cerevisiae, with a proposed role in the nuclear export of nuclear export signal (NES)-bearing proteins, may be involved in localizing scMex67p to the NPC (14–16). scSac3p has been proposed to function like scYra1p in mRNA biogenesis and export. The middle and the N-terminal domain of scSac3p interacts with the scMex67p-Mtr2p heterodimer (14). Additionally, the C terminus of scSac3p can interact with nucleoporins scNup1p and scNup60p at the nuclear basket (14). Immunoprecipitation experiments have shown that scSac3p interacts with scNup116p and scDsp5p (16). Recently it was shown that Drosophila RanBP2/NUP358 provides a major docking site for TAP-p15 heterodimer at the cytoplasmic filaments of the NPC (17). Thus, a number of possible routes may operate in parallel to deliver Mex67p or TAP with their cargo to specific locations at the NPC.

The small cofactor p15 belongs to a conserved class of proteins related to the Ran-GDP import factor NTF2 (18). In S. cerevisiae, its functional homolog Mtr2p is an essential pore-associated mRNA export factor (19, 20). In vitro binding studies indicate that Mtr2p or p15 is critical for the interaction of scMex67p or TAP with nuclear pore proteins. scMex67p and TAP can also interact directly with the -FG-containing nucleo-
porins (10, 21). TAP mutants deficient in forming a heterodimer with p15 are also unable to export mRNA (8). Unlike Mtr2p, p15 does not appear to bind the nuclear pore proteins directly but may stabilize the NTF2 fold and thus modulate the binding of TAP with -FG repeats at the pore, conferring directionality to the export process. One study showed that p15 significantly stimulates the binding of TAP with the -FG-containing nucleoporin p62, while another study showed that p15 alters the binding affinity of TAP to -FG-containing nucleoporins CAN4, hCG1, NUP98, and p62 (22, 23).

Although Mex67 is not essential in S. pombe, its function in mRNA export is essential in the rae1-167 mutant background (24). Overexpression of spMex67 proteins carrying internal deletions led to the identification of functional domains I (aa 434–468) and II (aa 469–509) (24). A triple mutation V460A/H461A/G462A (m9) in domain I completely abolished Mex67 function in mRNA export. Domain II appears to be required for mediating a direct interaction with Rae1 in vivo. Mutations R478A/T479A (m3) or N494A/D495A (m7) in domain II also mediated an indirect interaction with Rae1 function in mRNA export. Domain II appears to be required for the MED7-mediated nuclear export of poly(A)+ RNA in S. pombe (25). Here we show that S. pombe encodes a single, non-essential p15 gene that plays a conditional role in the mRNA export functions of spMex67. Extending our previous observation we show that human TAP (aa 473–546) also encodes a homologous NES. Both TAP and Mex67 nuclear export sequences can be further divided into NES I and NES II. These NESs can mediate the export of poly(A)+ RNA in S. pombe in the absence of p15 by interacting directly with nuclear pore proteins spNup159 and spNup98. These studies reveal the existence of nuclear export activities whose putative functions are conserved in TAP and spMex67.

**EXPERIMENTAL PROCEDURES**

**Strains and Culture**—Basic genetic and cell culture techniques have been described previously (26, 27). For constructing the p15 null strain, a PCR-amplified 3.4-kb BamHI genomic DNA fragment containing the p15 gene was cloned into pbluexpress SK+ strain. A derivative containing a deletion between amino acids 4 and 199 with a NotI site placed at the deletion junction was then constructed. A NotI fragment bearing uren+ or Kan was inserted in the deletion junction. The resulting plasmid was cut with BamHI, and the p15::uren or p15::kan fragment was transformed into the SP286 diploid strain. Stable Ura+ or G418-resistant transformants were screened for PCR for the replacement of one of the p15 genes. Analysis of 12 tetrads showed a 2:2 segregation pattern of the Ura+ or Kan marker. The p15 null colonies grow as well as p15 colonies. Other strains used were: wild-type h+ leu1-32 uren4D18, h− rae1-167 leu1-32 uren4D18 (12), h− rae1-167 Δp51, pREP81X-rae1− leu1-32 uren4D18 (this work), Δp51 ΔMex67 leu1-32 uren4D18 (this work), and ΔMex67 rae1-167/pREP81X-rae1− leu1-32 uren4D18 (24).

**Construction of Plasmids**—The construction of the vector (pAG177) for in vivo nuclear export assay in S. pombe has been described previously (25). It expresses two RNA recognition motifs (RRMs) of Crp79 fused to GFP at the C terminus (25). pAG188 was made by inserting a Xhol-BglII DNA fragment encoding an 434–509 of Mex67 between a Xhol and a BglII site engineered between the second RRM and the GFP-expressing sequences in pAG177. Similarly DNA fragments encoding Mex67 amino acids 434–468 (for NES I) or 469–509 (for NES II) were inserted into pAG177. Previously described plasmids expressing Mex67 mutants V460A/H461A/G462A (m9) and I492A/I493A (m6) were used to amplify DNA sequences representing NES I or NES II mutants. The corresponding DNA sequences were inserted into pAG177 at the Xhol and BglII sites. The plasmid pAG291 expressing the RRM-NES-GFP fusion of the double mutant NES I m9 + NES II m6 was constructed by fusing the DNA sequences of NES I m9 and NES II m6 in tandem by inserting a common KpnI restriction site between the two fragments and lighting the fragments to pAG177.
in *S. pombe* for growth or mRNA export (24). A double mutant strain of \( \Delta p15 \) \( \Delta m\text{ex}67 \) was viable and showed no accumulation of poly(A)' RNA in the nucleus (Fig. 1, A and B, panel b). Taken together, this indicates that neither Mex67 nor its cofactor p15 is a component of the essential mRNA export machinery in *S. pombe*.

Previously we showed that the \( \Delta \text{mex}67 \) mutation is synthetically lethal with the *rae1-167* mutation (24). We found that the \( \Delta p15 \) mutation was also synthetically lethal with the *rae1-167* mutation. To assess mRNA export under the synthetic lethal conditions, Rae1 was conditionally expressed in the \( \Delta p15 \) *rae1-167* strain from a weak, B1-repressible promoter in a pREP81X-*rae1* plasmid (31). In the absence of B1, Rae1 was expressed, and the double mutant cells grew and mediated mRNA export out of the nucleus. When the expression of Rae1 was shut off in the presence of B1 creating a synthetic lethal condition, both growth (Fig. 1A) and nuclear export of mRNA was inhibited (Fig. 1B, compare panel g with panel h). However, the magnitude of mRNA export defect in \( \Delta p15 \) *rae1-167* pREP81X-*rae1* cells was not as severe as that seen in the \( \Delta \text{mex}67 \) *rae1-167* pREP81X-*rae1* cells grown in the presence of B1 (Fig. 1B, compare panels h and c). These results indicate that in the *rae1-167* mutant background, the functions of both Mex67 and p15 are required for mediating mRNA export. A comparison of the levels of poly(A)' RNA accumulation between \( \Delta p15 \) *rae1-167* and \( \Delta \text{mex}67 \) *rae1-167* mutant cells further raises the possibility that, in the absence of p15, genomic levels of Mex67 in *rae1-167* mutant strain background may partially function in mediating mRNA export. In that case, the role of p15 may be to simply stimulate Mex67 function.

**Mex67 Can Function in the Absence of p15**—We next tested whether excess Mex67 expressed from a multicopy plasmid (pMex67) could suppress the growth and mRNA export defects of the \( \Delta p15 \) *rae1-167* pREP81X-*rae1* strain under synthetic lethal (+B1) conditions. Results in Fig. 1A show that the double mutant cells carrying the pMex67 plasmid, but not the empty vector, grew in the presence of B1 in the medium (compare growth in –B1 and +B1). Fig. 1B shows that the double mutant cells carrying the pMex67 plasmid did not show accumulation of poly(A)' RNA in the nucleus (compare panels g, h, and i). Thus, in the absence of p15, a concentration of Mex67 higher than the genomic level could support the growth and mRNA export of the \( \Delta p15 \) *rae1-167* synthetic lethal strain. These results are consistent with an indirect stimulatory role of p15 in Mex67 function.

**pMex67 Contains Two NESs That Can Function without p15**—We previously showed that spMex67 sequences comprising amino acids 434–509 (Fig. 2A) encode a functional NES that was capable of mediating the nuclear export of a heterologous fusion protein in *HeLa* cells (24). We showed that the spMex67-NES could concurrently mediate the active nuclear export of an RRM-NES-GFP fusion protein and poly(A)' RNA in the *nup184-1 rae1-167* mutant *S. pombe* strain under synthetic lethal conditions (25). Two RRMAs were used here from Crp79, an mRNA export carrier of *S. pombe* (25). The NES dependence of the protein and poly(A)' RNA export was evident since an RRM-GFP fusion protein alone was unable to promote its own nuclear export as well the export of poly(A)' RNA under the synthetic lethal conditions (25). However, the RRM-NES-GFP fusion protein accumulated in the nucleus in *rae1-167* mutant cells at the restrictive temperature (25). Thus, the RRM-NES-GFP fusion could be used to further characterize the functions of the NES region of Mex67.

We first tested whether the Mex67-NES could mediate nuclear export of the RRM-NES-GFP fusion in the \( \Delta p15 \) background. For this, the \( \Delta p15 \) *rae1-167* synthetic lethal strain

**RESULTS**

**p15 Gene of *S. pombe* Is Not Essential for Growth or mRNA Export**—Data base searches of the *S. pombe* genome against the *S. cerevisiae* MTR2 and the human p15 revealed the presence of a gene (SPAPB1A10.03) whose translated product showed homology to an isoform of human p15, p15-2, but not to the *S. cerevisiae* Mtr2p. For convenience, we designated the *S. pombe* gene, p15 (annotated as *nxt1* in the *S. pombe* data base). The \( \Delta \text{p15} \) cells were viable and grew as well as the wild-type cells (Fig. 1A). Thus, unlike MTR2 of *S. cerevisiae*, p15 of *S. pombe* is not essential for growth. We next examined the distribution of poly(A)' RNA in \( \Delta \text{p15} \) cells. No detectable nuclear accumulation of poly(A)' RNA was observed in the nucleus of \( \Delta \text{p15} \) cells (Fig. 1B, panel a), also suggesting that the protein is not essential for mRNA export. The *mex67* gene is not essential.

![Fig. 1. Growth and mRNA export of strains carrying the \( \Delta p15 \) mutation. A. upper panel, growth of wild-type, \( \Delta p15 \), and the \( \Delta p15 \) \( \Delta m\text{ex}67 \) cells grown in EMM. Two middle panels, growth of \( \Delta p15 \) *rae1-167* pREP81X-*rae1* and \( \Delta m\text{ex}67 \) *rae1-167* pREP81X-*rae1* cells grown in EMM in the absence of B1 (–B1) and in the presence of B1 (+B1). Lower panel, \( \Delta p15 \) *rae1-167* pREP81X-*rae1* cells containing either a vector or a multicopy plasmid (pMex67) expressing Mex67 grown in –B1 and in +B1. B, poly(A)' RNA localization in strains as indicated (panels a–i). The lower panels (d–l) shows their corresponding DAPI-stained nuclei.](image-url)

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containing a wild-type rae1 gene in pREP81X plasmid was used as before. The cells also carried a plasmid expressing either an RRM-GFP fusion or an RRM-Mex67-NES-(434–509)-GFP fusion. The RRM-GFP fusion localized predominantly in the cytoplasm in the absence of B1 but accumulated in the nucleus under the synthetic lethal (+H11001-B1) conditions (Fig. 2B, panels a and b). In contrast, the RRM-Mex67-NES-(434–509)-GFP fusion localized predominantly in the cytoplasm in the presence of B1 (see NES I + II in Fig. 2B, panel c). A part of the Δp15 rae1-167 culture grown in the presence of B1 was then shifted to 36 °C for 1 h to inactivate Rae1-167. The NES fusion protein was now localized in the nucleus (Fig. 2B, panel d) suggesting that under the synthetic lethal conditions (i) the cytoplasmic localization of the fusion protein was not due to a retention mechanism and (ii) its import into the nucleus was not impaired. These results show that the localization of RRM-GFP fusions of aa 434–509 of S. pombe Mex67 are consistent with the NES property originally demonstrated in HeLa cells (24). Therefore, Mex67-NES (amino acids 434–509) can function in the absence of p15 in S. pombe.

Based on the properties of internal deletions made in Mex67, we had previously shown that the region of amino acids 434–509 of Mex67 could be subdivided into functional domain I (aa 434–468) and domain II (aa 469–509) (24). RRM-GFP fusions...
containing either domain I or domain II sequences were found predominantly in the cytoplasm under synthetic lethal (+B1) conditions in the ∆p15 rae1Δ167 cells (Fig. 2B, panels i and k). Following heat inactivation of Rae1-167 as before, the fusion protein accumulated in the nucleus (data not shown). Thus, both domains could individually mediate the nuclear export of the RRM-GFP fusion protein. In this work, we use the terms “NES I” and “NES II” to describe the nuclear export activities associated with domain I and domain II sequences, respectively.

Amino Acids Critical for Mex67-NES I and -NES II Function—We next wanted to identify amino acid residues critical for the nuclear export activity of NES I and NES II. For this purpose, we analyzed the effects of some previously characterized mutations of Mex67 (that map within NES I or NES II) on the localization of the respective RRM-NES-GFP fusion protein by this assay. A triple mutation V460A/H461A/G462A inhibited the export activity of NES I; the fusion protein accumulated in the nucleus in the presence of B1 (Fig. 2B, panel k). Three separate clusters of mutations affected the NES II function. Mutations F476A/D477A (m2) and L481A/I482A (m4) affected the NES II moderately (data not shown), but I492A/ I493A (NES II”m6”) had a severe effect on NES II activity as judged by the nuclear accumulation of the mutant RRM-NES II”m6”/GFP fusion protein (Fig. 2B, panel l). In contrast, NES II mutant F464A/E465A/E466A (m10) or NES II mutants R478A/ T479A (m3), N494A/D495A (m7), and L496A/L497A (Fig. 2A; m8 and m10 data not shown) did not affect the cytoplasmic localization of the respective RRM-NES-GFP fusion under the synthetic lethal conditions (Fig. 2B, panels q and r).

The physical contiguity of the two NESs raised the question whether a loss of function in one NES could affect the functioning of the other NES. For this purpose, the effect of two key mutations, m9 and m6, that abrogated the activity of NES I or NES II, respectively, were tested separately or together in the context of RRM-NES I + II-GFP fusion proteins (Fig. 2B). Mutations in either NES I (m9) or in NES II (m6) did not affect the extent of cytoplasmic localization of the fusion protein (data not shown). But when the two mutations were combined, the RRM-NES I”m9” + II”m6”/GFP fusion was seen predominantly localized in the nucleus of ∆p15 rae1Δ167/pREP81X-rae1 cells when grown in the presence of B1, demonstrating a loss of nuclear export activity by NES I + II (Fig. 2C, panel l). An NES II double mutant, carrying mutations R478A/T479A and N494A/D495A (NES II”m3m7”) fused to RRM-GFP localized to the nuclear rim in ∆p15 rae1Δ167/pREP81X-rae1 cells grown in the absence (data not shown) or the presence of B1 (Fig. 2C, panel s and inset) or in the wild-type (not shown) cells. A full-length RRM-NES I”m9” + II”m3m7”/GFP also showed similar rim localization in both strains (data not shown). These results suggest first that the m3m7 mutant NES II protein can interact with the pore components directly or via an intermediate receptor in the absence of p15. Second, NES I and NES II alone can mediate the nuclear export of a heterologous protein in the absence of p15. Moreover amino acids Val466-His467-Gly468 are critical for NES I activity, while amino acids Ile492-Ile493 appear critical for NES II-mediated nuclear export activity.

Mex67-NES I and -NES II Also Mediate Nuclear Export in HeLa Cells—We analyzed the ability of NES I and NES II to mediate the nuclear export of a heterologous protein in HeLa cells. DNA sequences for the wild-type or mutant NESs were fused between Gr and GFP gene sequences, and plasmids expressing the fusion proteins (Gr-NES-GFP) were used to transfect HeLa cells. All fusion proteins accumulated in the cytoplasm of the transfected HeLa cells (Fig. 2C, Untreated panels). Upon treatment with the steroid hormone (corticosterone, 30 min) the fusion proteins translocated into the nucleus (Fig. 2C, Import panels). 30 min following removal of the hormone and addition of cycloheximide to prevent new protein synthesis, the localization of the fusion proteins was monitored. As shown before, the NES I + II fusion of Gr-GFP (amino acids 434–509 of Mex67) relocated to the cytoplasm (Fig. 2C, panel o). Individually NES I and NES II were also able to mediate the nuclear export of the Gr-GFP fusion recapitulating the results obtained in the S. pombe assay (Fig. 2C, panels c and i). Consistently NES I”m9” and NES II”m6” as well as the NESs I”m9” + II”m6” double mutants were unable to mediate the nuclear export of the Gr-GFP fusions (Fig. 2C, panels f, i, and r). Thus, both NES I and NES II can function in mediating the nuclear export of a heterologous protein in HeLa cells. Furthermore, unlike in S. pombe, the NES-mediated protein export in HeLa cells was not linked to mRNA export. These results confirm that in vivo nuclear export activity in S. pombe was the cause of the observed mRNA localization.

A Region of TAP Homologous to Mex67 NES I + II Sequences Contains Two NESs That Mediate the Nuclear Export of Heterologous Proteins in S. pombe and HeLa Cells—Amino acids 473–546 of the human TAP align with amino acids 434–509 of spMex67 (Fig. 2A). Further amino acids 473–505 correspond to the NES I of Mex67, and amino acids 506–546 correspond to NES II of Mex67. No nuclear export activity within amino acids 473–546 of TAP has been reported before. We analyzed the ability of TAP amino acid sequences 473–546, 473–505, and 506–546, represented as TAP-NES I + II, TAP-NES I, and TAP-NES II, respectively, in Fig. 3A, to mediate the nuclear export of the Gr-GFP fusion protein in S. pombe by using the same experimental procedure described earlier for Mex67- NESs. TAP-NES I + II exhibited a strong nuclear export capability in S. pombe cells, and the fusion protein localized to the cytoplasm both in the absence and in the presence of B1 (Fig. 3A, compare panels a and b). Following inactivation of Rae1-167 at 36 °C for 1 h, the RRM-TAP-NES I + II-GFP fusion protein accumulated in the nucleus (Fig. 3A, panel c). Cells expressing TAP-NES I or TAP-NES II fusion proteins also localized predominantly to the cytoplasm in the ∆p15 rae1Δ167/pREP81X-rae1 strain under the synthetic lethal conditions (Fig. 3A, compare panels d and g). On the other hand, when the equivalent of m9 and m6 mutations were constructed in TAP, the corresponding RRM-GFP fusions (Fig. 3A, TAP-NES I”m9”, TAP-NES II”m6”, and TAP-NES I”m9” + II”m6”) all accumulated in the nucleus in the presence of B1 (Fig. 3A, panels h–j). These results show that TAP-NES I and TAP-NES II can also mediate the nuclear export of a heterologous protein and that they can do so in the absence of p15 in S. pombe cells. The ability of the analogous mutations to block the NES activity in both proteins indicates a common mode of function.

We next tested whether the TAP NESs could function in HeLa cells. TAP-NES I + II, TAP-NES I, and TAP-NES II directed nuclear export of the Gr-GFP fusion (Fig. 3B, compare panels i, l, and o). The fusions localized in the cytoplasm in untreated cells and upon treatment with the steroid hormone translocated into the nucleus (Fig. 3B, see Untreated and Import panels). On the other hand, TAP-NES I”m9” + II”m6”, TAP-NES I”m9”, and TAP-NES II”m6” were unable to mediate export (Fig. 3B, panels r, u, and x). As controls for the nuclear export in HeLa cells, Gr-GFP (vector) and Gr-Rev-NES-GFP were used as negative and positive controls, respectively (Fig. 3B, panels a–f). Taken together, our results show that both spMex67 and human TAP contain two contiguous, non-overlapping, conserved nuclear export activities. The sequences that encode the export activities in the two domains in Mex67 and in TAP are short like those seen for NESs. Their export through
Fig. 3. Analyses of NESs of TAP in S. pombe and HeLa cells. A, localization of the RRM-GFP fusion containing human TAP-NES sequences in Δp15 rae1-167/pREP81X-rae1 in the absence and presence of B1 as in Fig. 2B is indicated. TAP-NES I + II corresponds to TAP aa 473–546, TAP-NES I corresponds to aa 473–505, and TAP-NES II corresponds to human TAP aa 506–546. Corresponding NES mutations in NES II, TAP-NES I, and NES I + II are shown as indicated. Lower panels show the same cells stained with DAPI. B, NES characterization by HeLa cell nuclear export assay of TAP. The TAP NES regions or their mutants were fused to a Gr-GFP chimeric protein and expressed in HeLa cells. In the absence of corticosteroid (Untreated panels a–c) the fusion proteins were mainly cytoplasmic. Import panels, treatment with 2 μM corticosteroid for 30 min at 37°C (panels b–w). Export panels, hormone-washed cells incubated for 30 min in the presence of cycloheximide (10 μg/ml) (panels c–x). Rev-NES-Gr-GFP fusion is shown as a positive control, and empty vector (Gr-GFP) served as a negative control for export (panel f).

the NPC could be mediated by export receptors. Alternatively they could directly interact with the nuclear pore proteins, eliminating the need for export receptor(s).

NES I and II of spMex67 and TAP Can Mediate mRNA Export in S. pombe—RRM-NES I + II-GFP fusion expressed from a multicopy plasmid could mediate poly(A)⁺ RNA export from the nucleus of nup184-1 rae1-167/pREP81X-rae1 cells under the synthetic lethal conditions, presumably by acting as an NES-dependent mRNA carrier (25). Since neither NES of TAP and Mex67 showed dependence on p15 for protein export, we tested the ability of NES I + II, NES I, or NES II of Mex67 and TAP to mediate mRNA export in the Δp15 rae1-167/pREP81X-rae1 strain under the synthetic lethal (+B1) conditions. In cells expressing the RRM-GFP fusion protein (control without an NES), poly(A)⁺ RNA localized to the cytoplasm in the absence of B1 (Fig. 4A, panel a) but accumulated in the nucleus in the presence of B1 (18–20 h) (Fig. 4A, panel b). In the cells expressing the RRM-Mex67-NES I + II-GFP or RRM-TAP-NES I + II-GFP, poly(A)⁺ RNA was found predominantly in the cytoplasm in the presence of B1 (Fig. 4A, panel b, and B, panel a). In cells expressing either NES I or NES II fusion of Mex67 or TAP, poly(A)⁺ RNA accumulated in the nucleus of some cells (Fig. 4, A, panels d and e, and B, panels b and c). These results suggest that NES I and NES II can export poly(A)⁺ RNA but with reduced efficiency compared with NES I + II. To confirm that the poly(A)⁺ RNA export was directly linked to the nuclear export activity of NES I or NES II, the poly(A)⁺ RNA localization was analyzed in cells expressing the mutant fusion proteins. RRM-GFP fusion of the mutant proteins of Mex67-NES I⁺⁻, -NES II⁺⁻, or -NES I⁺⁻ + II⁺⁻ were unable to mediate poly(A)⁺ RNA export from the nucleus under synthetic lethal (+B1) conditions (Fig. 4, panels f, g, and h). Similarly RRM-GFP fusion proteins of TAP-NES I⁺⁻, -NES II⁺⁻, or -NES I⁺⁻ + II⁺⁻ were unable to mediate poly(A)⁺ RNA export from the nucleus under the synthetic lethal (+B1) conditions (Fig. 4B, panels d, e, and f). Taken together, these results show that both TAP and Mex67 NES or their NES I and NES II sequences are functional in vivo in mediating the export of poly(A)⁺ RNA from the nucleus of S. pombe cells under the conditions of our assay. Mutations that abolish NES activity of TAP or Mex67 also inhibit poly(A)⁺ RNA export. The simplest interpretation of these results is that the nuclear export activity-dependent export of the protein directly exports bound poly(A)⁺ RNA. The results further suggest that the functions of NES I and II could be additive or synergistic.

NES Sequences of Mex67 and TAP Bind -FG-containing Regions of S. pombe Homologs of scNup116p and scNup159p in Vitro—Structural and functional studies of TAP-p15 heterodimer indicate a single -FG binding pocket within the NTF2-like fold of TAP (12). These studies also showed that the NTF2-like fold is functional in vivo in mRNA export only in the presence of p15. Mutations that inhibit heterodimer formation between TAP and p15 reduced nuclear rim association of TAP (12). We wanted to know whether the NES I and NES II regions could directly interact with -FG-containing nucleoporins. For this purpose, we used -FG repeat-containing fragments of S. pombe homologs of S. cerevisiae Nup116p (spNup98) and S. cerevisiae Nup159p (spNup159). The -FG regions chosen...
were homologous to the regions in human NUP98 and scNup159p that have been used by others to study nuclear export receptor-NPC interactions (10, 21, 23). For binding experiments, individual GST- and His-tagged proteins were purified from Escherichia coli. Bound complexes were purified from unbound fractions on a Talon affinity column. His-tagged spNup98 (amino acids 142–612) or spNup159 (amino acids 480–850) -FG repeat-containing regions, respectively, showed no interaction with GST alone but showed stable interactions with a purified GST fusion of Mex67 NES I + II (Fig. 5, A and B, compare lane 4 with lane 5), NES I (Fig. 5, C and D, lanes 4 and 5), and NES II (Fig. 5, E and F, lanes 4 and 5). Thus, in contrast to the p15-dependent functions of the NTF2-like fold of TAP described previously, the binding of the short NES I, NES II, and NES I + II sequences to the -FG-containing nucleoporins does not require p15. The GST fusion of NES I<sup>1st</sup> + II<sup>1st</sup> did not bind either spNup98 or spNup159 -FG regions (Fig. 5, G and H, lane 4). The lack of binding for the double mutant NES was confirmed by Western blot analysis using polyclonal anti-GST antibody (Fig. 5, G and H, lower panel, see lane 4 in both gels). GST fusion of the human TAP-NES I + II sequence between amino acids 473–546 but not the double mutant TAP-NES I<sup>1<sub>st</sub></sup> + II<sup>1<sub>nd</sub></sup> was able to bind spNup159 -FG sequence (Fig. 5I, lanes 7 and 8). Binding of GST-TAP-NES I + II to spNup98 -FG sequences was not tested. These results show a correlation between binding to nucleoporins and nuclear export activity of Mex67 and TAP NESs.

A nuclear pore-associated non-FG repeat carrying mRNA export factor Npp106 was also tested for the ability to bind the NES. The GST-NES I + II was not able to bind His-Npp106 (Fig. 5I, lane 4). Thus, it appears that the NES-Nup binding is specific for the spNup98 and spNup159 (-FG repeat-containing regions).

To test whether the interactions described above could be extended to other -FG-containing proteins also, we purified an N-terminal His-tagged fragment containing amino acids 1–300 of the human p62 and tested its ability to bind GST fusions of Mex67 and TAP NESs. The C-terminal domain of TAP was previously shown to bind this region of p62 (10, 22). GST fusion of neither Mex67 NES I + II nor TAP NES I + II showed any detectable binding to p62 sequences (Fig. 5K, compare lanes 6 and 7). However, a GST-Mex67 fusion was able to bind p62 protein (Fig. 5K, lane 8), demonstrating that other regions of Mex67 were able to bind p62. These results suggest that NES sequences discriminate among different -FG-containing regions. These results are consistent with previous binding studies where it was shown that TAP (aa 61–610) or a derivative containing an internal deletion between amino acids 567–613 bound Nup98, demonstrated di-
minished binding to CAN, but did not bind p62 or Nup153, respectively (10).

We also tested a GST fusion of NES I\textsuperscript{m9/H11001} IIm3m7 (whose RRM-GFP fusion localized to the nuclear pores \textit{in vivo}) for its ability to bind His-Nup98 and His-Nup159 -FG regions. Both nucleoporins bound the mutant protein but not GST alone (Fig. 5, L and M, compare lanes 4 and 5 in both gels).

**DISCUSSION**

Extending from our previous demonstration of an NES activity in spMex67, here we identify its counterpart in TAP and show that the two NESs are organizationally and functionally similar. Furthermore each NES can be subdivided into two short export sequences, NES I and NES II. Individually or together, these NESs can bind -FG-containing spNup159 and spNup98 (TAP NES-spNup98 binding was not tested). The inability of the mutant NESs to bind the spNup159 and spNup98 correlates strongly with a corresponding loss of function in \textit{S. pombe} and in HeLa cells, suggesting a possible common mechanism of pore interaction by the NESs. While several NPC targeting activities have been reported in the NXF proteins, this report demonstrates the existence of two new nuclear export/pore targeting activities whose functions are conserved between \textit{S. pombe} and human cells.

**Mex67-p15 Is Not a Component of the Essential mRNA Export Pathway in \textit{S. pombe}**—This work demonstrates that \textit{S. pombe} encodes a single p15 gene, which is not essential for growth or for mRNA export. This implies that the Mex67-p15 heterodimer is not a component of the essential mRNA export pathway.
mRNA Export in *S. pombe*

machinery in *S. pombe*. Similar to spMex67, the function of p15 is essential in a *rpl1-167* mutant background. However, the requirement of p15 is not absolute since a multicopy level expression of Mex67 in the Δr15 *rpl1-167* mutant background can mediate mRNA export. The role of p15 may be to stimulate Mex67 function. Indeed binding to p15 appears to stimulate mRNA export activity of TAP (8).

Novel NESs in spMex67 and TAP—NXF proteins have been shown to contain several nuclear export/pore targeting activities (8, 10, 13, 32, 33). The two NESs that we have described here are new. Based upon *in vitro* binding studies and *in vivo* functional assays, the simplest explanation of our results is that Nup98 and Nup159 binding is directly and specifically required by the Mex67-NES I and -NES II sequences for mediating export through the NPC in *S. pombe*. Although we have not tested direct binding between TAP-NES and human Nup98 or CAN, the comparative analyses of TAP and Mex67 NESs suggest that the NESs of TAP may function in an analogous manner.

What are the biological implications of so many pore targeting/nuclear export activities in NXF proteins? One possibility is that multiple export activities increase the efficiency of the mRNA export process. Studies show that two -FG-interacting sites on NXF proteins are necessary and sufficient for mRNA export. The identity of the -FG-interacting domains appears less critical than two TFIID-like folds, two UBA domains, or one of each can provide pore targeting and mRNA export functions to TAP. Conversely different nuclear export activities could have specialized roles, which could involve the export of different kinds of cargo and/or targeting of the cargos to different locations in the NPC.

In the case of spMex67-NES I and -NES II, originally described as domain I and domain II, respectively, several observations suggest that in addition to nuclear activities they provide unique and essential non-export functions. First, the overexpression of Mex67 carrying a deletion in domain II but not in domain I was able to inhibit growth and mRNA export of the wild-type *S. pombe* cells (24). The mechanism of this inhibition could be the titration of some essential soluble export factors or the saturation of essential interacting sites by Mex67. Both NES I and NES II bound -FG-containing regions in spNup98 and spNup159. *A priori*, we expected that, irrespective of the actual mechanism, the behavior of Mex67 deletions in either domain to be similar. The differences between the two NESs in this regard indicate that their mode of interactions with the Nups may be subtly different. Second, NES II but not NES I is involved in indirect interactions with Rae1 *in vivo* (24). Finally we found that mutations m3 and m7 did not inhibit either the binding of the NES II protein to the -FG regions *in vitro* (Fig. 5, L and M) or its nuclear export activity *in vivo* (Fig. 2B, panels q and r). But in the context of the full-length protein, these mutations abolished the functions of spMex67 (24).

Nature of the NES—What is the nature of the NES described in this work? First, the nuclear export activity is included within the TFIID-like fold of TAP and is not part of the C-terminal UBA-like domain where a similar activity was previously identified (32). Second, in contrast to the p15 dependence of the interaction of the TFIID-like domain with -FG repeat-containing fragments (such as human Nup98), the NESs described here do not require the physical association with p15 for their export functions. Third, the two NESs are too short to contain a functional TFIID-like fold. Also a pair of mutations (Leu236 and Leu236 to Arg) that were shown to disrupt the TFIID-like fold is not contained within the two NESs (8). The TFIID-like fold contains a p15-dependent pore targeting function, but no NES has been reported within this region of TAP. The presence of the two short, functionally equivalent NESs in TAP and spMex67 within the TFIID-like fold suggests that they possess pore targeting as well as additional interactions to negotiate their passage through the NPC.

Acknowledgments—We thank Dr. J. Landry and Dr. A. Mir for critically reading the manuscript.

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Conserved Nuclear Export Sequences in *Schizosaccharomyces pombe* Mex67 and Human TAP Function in mRNA Export by Direct Nuclear Pore Interactions

Anjan G. Thakurta, Ganesh Gopal, Jin Ho Yoon, Tapas Saha and Ravi Dhar

*J. Biol. Chem.* 2004, 279:17434-17442.
doi: 10.1074/jbc.M309731200 originally published online February 12, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M309731200

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