RNA (2001), *7*:302–313. Cambridge University Press. Printed in the USA.
Copyright © 2001 RNA Society.

Phosphorylation by Sky1p promotes Npl3p shuttling and mRNA dissociation

WENDY GILBERT,[1] CHRISTIAN W. SIEBEL,[1,2] and CHRISTINE GUTHRIE[1]

[1]Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143-0448, USA

ABSTRACT

Mammalian SR proteins are currently thought to function in mRNA export as well as splicing. They contain multiple phosphorylated serine/arginine (RS/SR) dipeptides. Although SR domains can be phosphorylated by many kinases in vitro, the physiologically relevant kinase(s), and the role(s) of these modifications in vivo have remained unclear. Npl3 is a shuttling protein in budding yeast that we showed previously to be a substrate for the mammalian SR protein kinase, SRPK1, as well as the related yeast kinase, Sky1. Here we demonstrate that Sky1p phosphorylates only one of Npl3p's eight SR/RS dipeptides. Mutation of the C-terminal RS to RA, or deletion of SKY1, results in the cytoplasmic accumulation of Npl3p. The redistribution of Npl3p is accompanied by its increased association with poly(A)$^+$ RNA and decreased association with its import receptor, Mtr10p, in vivo. We propose that phosphorylation of Npl3p by the cytoplasmically localized Sky1p is required for efficient release of mRNA upon termination of export.

Keywords: phosphorylation; RNA binding protein; RNA export; serine and arginine-rich protein kinase SRPK; SR protein

INTRODUCTION

Eukaryotic gene expression demands bridging the distance that separates mRNA synthesis in the nucleus from mRNA translation in the cytoplasm. Newly synthesized pre-mRNAs must be processed in the nucleus via a number of covalent modifications, including splicing of noncoding intron sequences and polyadenylation of the 3′ end. Mature mRNAs are then transported, via an active and specific export process, through the nuclear pore complex (NPC) to the cytoplasm (Stutz & Rosbash, 1998). The substrates for these reactions and for export are RNA–protein complexes (RNPs; Dreyfuss et al., 1993; Daneholt, 1997).

The dynamic cycle of nuclear assembly and cytoplasmic disassembly of export RNPs raises enormous challenges for the orchestration of macromolecular transport through NPCs. Newly translated RNP proteins must be transported into the nucleus to package nascent mRNAs. Subsequently, at least a subset of these same RNP proteins must be transported in the opposite direction as part of mRNA–protein particles that deliver mRNAs to the cytoplasm (Stutz & Rosbash, 1998). Finally, these particles must be disassembled in the cytoplasm, to release the mRNA for translation and to liberate the proteins for nuclear reimport and use in subsequent rounds of mRNA export. To avoid reimporting RNPs in a futile cycle, the cell must coordinate disassembly with the completion of export and distinguish the RNA-binding proteins that are part of export RNPs from those that have been released.

The best candidates for mRNA export carriers are the shuttling RNA-binding proteins (Stutz & Rosbash, 1998). Two classes of RNA binding proteins known to shuttle in higher eukaryotes are the hnRNP (heterogeneous nuclear RNP) proteins and the SR proteins (Alzhanova-Ericsson et al., 1996; Nakielny & Dreyfuss, 1997; Nakielny et al., 1997; Caceres et al., 1998). Both contain one or more RRM-type RNA binding motifs in their N-termini. The C-terminal domain of hnRNP proteins is typically rich in arginine and glycine (RGG) and is the site of arginine dimethylation (Liu & Dreyfuss, 1995). The C-terminal domain of SR proteins contains multiple copies of serine-arginine (SR/RS) dipeptides, which are the sites of serine phosphorylation (Fu, 1995). Historically, hnRNP proteins have been studied as potential mRNA export factors. The prototype for this class is hnRNPA1, which accompanies mRNA from the nucleus to the cytoplasm (Visa et al., 1996; Daneholt,

Reprint requests to: Christine Guthrie, Department of Biochemistry and Biophysics, University of California, 513 Parnassus Avenue, San Francisco, California 94143-0448, USA; e-mail: guthrie@cgl.ucsf.edu.

[2]Present address: Deltagen, Inc., 1031 Bing Street, San Carlos, California 94070, USA.

1997). Export is terminated by the release of mRNA and reimportation of hnRNPA1 via its import receptor, Transportin (Pollard et al., 1996; Siomi et al., 1997). SR proteins were initially identified as essential splicing factors but the recent discovery that certain members of this family shuttle has suggested a broader role for these phosphoproteins in mRNA metabolism (Caceres et al., 1998). The role of posttranslational modifications in the functions of these two classes of proteins is only poorly understood.

Yeast has a large number of RRM-containing proteins, many of which carry an RGG domain. The best-studied protein of this class is Npl3, which shuttles between the nucleus and the cytoplasm, and is required for bulk mRNA export (Flach et al., 1994; Kadowaki et al., 1994; Lee et al., 1996). We and others have shown previously that the RGG domain of Npl3p is a substrate for the arginine dimethylase, Hmt1p (Henry & Silver, 1996; Siebel & Guthrie, 1996; Yun & Fu, 2000). On the other hand, the existence of SR proteins in budding yeast has been controversial; the genome contains no examples of the extensive stretches of SR/RS dipeptides that typify mammalian SR proteins (Birney et al., 1993). Interestingly, however, Npl3p contains eight such dipeptides within the C-terminal RGG domain. In support of the interpretation that Npl3 is a yeast SR-type protein, we demonstrated that Npl3p can be phosphorylated by the mammalian SR protein kinase, SRPK1 (Siebel et al., 1999). Using this information, we went on to identify a protein with significant sequence homology; indeed, Sky1p (SR kinase in yeast) phosphorylates Npl3p in vitro (Siebel et al., 1999).

Phosphorylation on serine residues within the RS-domain is a defining characteristic of SR proteins, but the physiological role(s) of RS-domain phosphorylation have remained uncertain. Mammalian cells contain at least two families of SR protein kinases, the Clk/Sty dual-specificity kinases that are nuclear at steady-state, and the SRPK family of serine kinases that are predominantly cytoplasmic (Fu, 1995). SRPK1 was purified based on its ability to phosphorylate SC35 and other SR proteins (Gui et al., 1994). A closely related mammalian kinase, SKPK2, displays similar activity in vitro (Wang et al., 1998). Clk/Sty was found to interact in a yeast two-hybrid screen with members of the SR protein family and was subsequently shown to phosphorylate RS domains in vitro (Colwill et al., 1996). In addition to these kinases, whose discovery depended on their interaction with SR proteins, various other proteins have been shown to phosphorylate RS domains in vitro, including protein kinases C and A (Colwill et al., 1996), p34cdc2, and DNA topoisomerase I (Rossi et al., 1996). Perhaps due to the repetitive nature of RS domain sequences, little attention has been focused on the potential for differential effects of phosphorylation at particular serines within these domains, despite the identification of such a wide variety of potential regulatory kinases.

Nonetheless, recent progress has been made in determining the site preferences of mammalian SRPK1,2. In vitro peptide selection experiments revealed sequence features in addition to RS dipeptides that contribute to recognition by this kinase (Wang et al., 1998). Importantly, these in vitro site preferences are consistent with in vivo binding studies using SF2/ASF and SRPK1,2 that showed that only a subset of SF2/ASF RS dipeptides were required for recognition by SRPK1,2 in vivo (Koizumi et al., 1999). Phosphorylation has been hypothesized to affect SR proteins' functions by modulating both protein–protein and protein–RNA interactions (Fu, 1995), and recent reports indicate that phosphorylation of metazoan SR proteins can influence their subcellular localization (Caceres et al., 1998; Misteli et al., 1998; Koizumi et al., 1999; Yeakley et al., 1999). There has been no evidence linking phosphorylation at a single RS dipeptide by a specific SR protein kinase to an in vivo function.

Here we demonstrate that Npl3p is phosphorylated on serine in vivo by Sky1p, further evidence that Npl3p is a bona fide yeast SR protein. We show that phosphorylation of Npl3p by Sky1p requires a single C-terminal RS dipeptide, and this specific phosphorylation is required for proper nuclear localization of Npl3p, corroborating similar results published while this manuscript was in preparation (Yun & Fu, 2000). Notably, we have found that the mislocalization of Npl3p in the absence of Sky1p-mediated phosphorylation is accompanied by Npl3p's increased association with poly$(A)^+$ RNA and decreased association with its import receptor, Mtr10p, in vivo. We propose that phosphorylation of Npl3p by cytoplasmic Sky1p could play a role in distinguishing nuclear and cytoplasmic compartments, thereby imparting directionality to the export of Npl3p-containing RNPs.

RESULTS

Npl3p is serine phosphorylated in vivo by Sky1p

Serine phosphorylation within the C-terminal RS domain is a hallmark of the SR protein family in metazoans. As described in the Introduction, the Npl3 protein of budding yeast appears related to this family based on a number of criteria, including our observation that Npl3p is an efficient substrate for mammalian SRPK in vitro (Siebel et al., 1999). To determine whether this observation is biologically relevant, we asked whether Npl3p was phosphorylated in vivo. Crude cell extract was prepared from a yeast cell culture that had been labeled in vivo with ^{32}P orthophosphate. Npl3p was purified from this extract by immunoprecipitation. Anti-Npl3p antibodies specifically precipitated a radiolabeled

protein of the size expected for Npl3p (Fig. 1A, lane 2). Immunoblot experiments confirmed that this protein was in fact Npl3p (data not shown). As an additional control, preimmune sera failed to immunoprecipitate the radiolabeled protein (Fig. 1A, lane 1). These data demonstrate that Npl3p is a phosphoprotein.

To determine which amino acid(s) was phosphorylated, we performed phosphoaminoacid analysis. Npl3p that had been radiolabeled in vivo was immunoprecipitated, purified from a SDS gel, and then hydrolyzed to yield individual amino acids, which were then separated using two-dimensional electrophoresis. The radiolabel was detected exclusively on serine, and not on threonine or tyrosine (Fig. 1B). Thus, like metazoan SR proteins, Npl3p is a serine phosphoprotein.

The similarities between Npl3p and metazoan SR proteins led us to ask whether Sky1p is responsible for Npl3p phosphorylation in vivo. We radiolabeled cultures of a strain in which the SKY1 gene had been precisely deleted and an otherwise isogenic wild-type strain. Npl3p was immunoprecipitated from extracts made from each culture, and the incorporation of radiolabel into Npl3p was quantitated using a phosphorimager. In the strain lacking SKY1, Npl3p radiolabeling was reduced to approximately 50% of that observed in the isogenic wild-type strain (Fig. 1C, lower panel). Coomassie protein staining revealed that equal amounts of Npl3p were immunoprecipitated from the wild-type and Δsky1 extracts (Fig. 1C, upper panel). Thus SKY1 is required to fully phosphorylate Npl3p in vivo. The fact that the deletion of SKY1 does not eliminate Npl3p phosphorylation reveals the existence of at least one additional kinase capable of phosphorylating Npl3p in vivo.

In vitro phosphorylation of Npl3p by Sky1p requires RS8

We have previously shown that Sky1p phosphorylates purified recombinant HisNpl3p in vitro (Siebel et al., 1999). We have now tested the activity of a C-terminally truncated rHisNpl3p substrate that lacks all eight RS/SR dipeptides (rHisNpl3ΔRS) (Fig. 2A). As we predicted for an SR kinase, protein A-tagged Sky1p purified from yeast failed to phosphorylate rHisNpl3ΔRS (Fig. 2B, lane 4), but was active against a wild-type rHisNpl3p substrate (Fig. 2B, lane 2). rGSTSky1p purified from *Escherichia coli* displayed identical substrate specificity (data not shown). Mammalian SR protein kinases phosphorylate multiple serine residues within RS/SR dipeptides in the C-terminal RS domains of their substrates. Peptide selection experiments with human SRPK2 have identified additional sequence requirements for residues flanking the RS dipeptide (Wang et al., 1998). According to these rules, only the final

FIGURE 1. SKY1-dependent phosphorylation of Npl3p in vivo. **A**: In vivo phosphate labeling. Whole cell extract was prepared from yeast grown in the presence of ^{32}P orthophosphate. Protein was immunoprecipitated with anti-Npl3p polyclonal antibodies (lane 2) or preimmune sera (lane 1) and analyzed by SDS-PAGE and autoradiography. **B**: Phosphoaminoacid analysis. Gel-purified radiolabeled Npl3p was hydrolyzed, and individual amino acids separated by two-dimensional electrophoresis. **C**: In vivo phosphate labeling of Npl3p in a Δsky1 strain (lane 2) compared to a wild-type strain (lane 1).

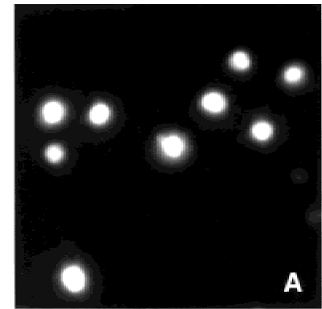
FIGURE 2. Phosphorylation of Npl3p by Sky1p requires RS8. **A**: Domain structure and sequence of Npl3p. SR/RS dipeptides are in bold and RGG repeats are underlined. The site of the RA8 point mutation is indicated with an asterisk. **B**: In vitro kinase assays. Recombinant His-tagged substrates, wild-type (lanes 1 and 2), Npl3RA8 mutant (lane 3), and Npl3ΔRS mutant (lane 4) were purified from *E. coli* (Coomassie-stained gel, upper panel) and tested in kinase reactions with Sky1-ProtAp purified from yeast (lanes 2–4) or a mock-purified fraction from an untagged yeast strain (lane 1).

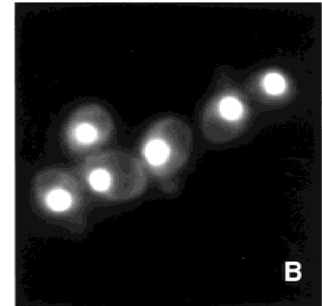
C-terminal RS dipeptide in Npl3p fits the mammalian consensus. To test the prediction that Sky1p phosphorylates this site, we prepared a mutant substrate in which the final serine was changed to an alanine (rHisNpl3RA8p) (Fig. 2A). Strikingly, Sky1p is completely unable to phosphorylate rNpl3RA8p (Fig. 2B, lane 3). The Npl3RA8 mutant protein is fully active in an RNA binding assay (data not shown); therefore its inactivity as a Sky1p substrate cannot be explained by gross misfolding of the protein. Thus the single serine in Npl3p's RS domain that is found within a mammalian SRPK consensus sequence is essential for phosphorylation by Sky1p in vitro.

Phosphorylation by Sky1p is required for proper nuclear localization of Npl3p

The shuttling of Npl3p between the nucleus and cytoplasm is a key feature of its proposed function as an mRNA carrier. Recent studies in mammals have suggested a role for phosphorylation in regulating the subcellular localization of shuttling SR proteins (Caceres et al., 1998). To test whether phosphorylation of Npl3p is important for its localization, we inserted the coding sequence for the green fluorescent protein (GFP) at the 5′ end of the NPL3 coding sequence under control of the NPL3 promoter. This construct fully complements an NPL3 deletion (data not shown). Wild-type cells expressing this GFP-NPL3 reporter displayed a bright fluorescent signal that localized exclusively to nuclei (Fig. 3A), consistent with previously published experiments (Lee et al., 1996). In contrast, GFP-Npl3p was readily detectable in both the cytoplasm and nucleus of cells lacking SKY1 (Fig. 3B). Immunoprecipitation and immunoblot experiments using anti-Npl3 antibodies demonstrate that GFP-Npl3p levels were not detectably altered in strains lacking SKY1, nor is there any evidence of cleavage of the GFP-tagged protein (Fig. 5A, and data not shown). Therefore, the increase in the cytoplasmic levels of GFP-Npl3p in cells lacking SKY1 indicates that Sky1p-catalyzed phosphorylation of Npl3p is important for proper nuclear localization of Npl3p.

To test directly whether the Sky1p phosphorylation site in Npl3p is required for proper nuclear localization, we constructed a GFP reporter in which the final serine was changed to alanine, the same mutation that abolished Sky1p-catalyzed phosphorylation of Npl3p in vitro (Fig. 2B). This mutant GFP-Npl3RA8p was observed in the cytoplasm of wild-type cells (Fig. 3C), mimicking the localization pattern observed for wild-type GFP-Npl3p in cells lacking SKY1. Other serine-to-alanine point mutations in the RS domain of Npl3p had no effect on GFP-Npl3p localization (data not shown). Taken together, our data support a model in which Sky1-catalyzed phosphorylation at RS8 is important for the nuclear localization of Npl3p.





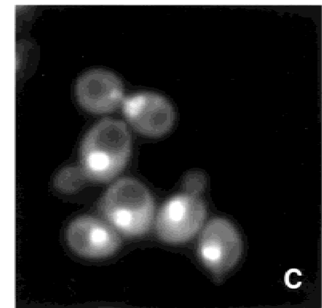


FIGURE 3. Loss of Sky1p-mediated phosphorylation causes cytoplasmic accumulation of Npl3p. Localization of wild-type GFP-Npl3p (**A**,**B**) and mutant GFP-Npl3RA8p (**C**) in SKY1 (**A**,**C**) and Δsky1 (**B**) strains.

Phosphorylation by Sky1p enhances binding of Npl3p to its import receptor Mtr10 in vivo

Phosphorylation could facilitate the steady-state nuclear localization of Npl3p by decreasing the rate of Npl3p export, increasing the rate of Npl3p import, or both. The fact that Sky1p is cytoplasmic is consistent with a model in which cytoplasmic phosphorylation of Npl3p actively promotes its import. We examined whether Sky1-catalyzed phosphorylation promoted Npl3p import by enhancing Npl3p's binding to its import receptor. Previous studies have demonstrated that Mtr10p, a member of the importinβ-family of nuclear transport receptors, functions as an import receptor for Npl3p in yeast. Specifically, mutations in MTR10 lead to accumulation of Npl3p in the cytoplasm (Pemberton et al., 1997; Senger et al., 1998). Moreover, Npl3p copurifies with protein A-tagged Mtr10 on IgG affinity resin, indicating that a fraction of Npl3p and Mtr10p exist in a complex together in vivo (Pemberton et al., 1997; Senger et al., 1998).

We exploited this affinity purification scheme to ask whether phosphorylation of RS8 by Sky1 is important for the Npl3p-Mtr10p interaction in vivo. Wild-type and Δsky1 strains were transformed with a plasmid expressing a protein A-Mtr10p fusion or an untagged Mtr10p as a negative control. Extracts were prepared from exponentially growing cultures of each strain, and protein A-Mtr10p was purified by binding to an IgG resin. ProtA-Mtr10p and interacting proteins were eluted and Npl3p binding was assessed by immunoblotting with an anti-Npl3p polyclonal antibody. Consistent with previous results, a fraction of wild-type Npl3p bound to the IgG resin in the presence of protein A-tagged Mtr10p (Fig. 4, lane 4), but not in extracts from the untagged strain (data not shown; Pemberton et al., 1997; Senger et al., 1998). In extracts from a strain lacking SKY1, the binding of Npl3p to protein A-Mtr10p was reduced (Fig. 4, lane 3). Mutation of the Sky1p phosphorylation site in Npl3p to alanine similarly reduced association of Npl3p with protein A-Mtr10p in vivo (data not shown). These results are particularly striking given the fact that loss of Sky1p-catalyzed phosphorylation increases the cytoplasmic pool of Npl3p (Fig. 3B,C), thereby increasing the fraction available to bind Mtr10p, which is predominantly cytoplasmic at steady state (Pemberton et al., 1997; Senger et al., 1998). These data show that phosphorylation of Npl3p at RS8 by Sky1p is required for efficient binding of Npl3p to Mtr10p in vivo.

Phosphorylation of Npl3p decreases its association with mRNA in vivo

We considered two hypotheses to explain the loss of Npl3p binding to Mtr10p in a Δsky1 strain: (1) unphosphorylated Npl3p is sequestered in a different complex, inaccessible to Mtr10p, and (2) loss of phosphorylation directly reduces Npl3p's binding affinity for Mtr10p. These mechanisms are not mutually exclusive. To test the first hypothesis, we guessed at the identity of a competitive binding partner. mRNA was an obvious candidate for the following reasons: (1) Npl3p displays a high level of non-sequence-specific RNA binding activity in vitro (W. Gilbert and C. Guthrie, unpubl. observations) and is bound to poly(A)$^+$ in vivo (Russell & Tollervey, 1995; Krebber et al., 1999); (2) poly(A)$^+$ RNA is a very abundant potential competitor for binding to Mtr10p in the cytoplasm (Groner & Phillips, 1975); and (3) phosphorylation has been shown to decrease the non-sequence-specific RNA-binding activity of some metazoan RS proteins in vitro (Tacke et al., 1997). We therefore set out to test the hypothesis that phosphorylation of Npl3p by Sky1p would act to dissociate Npl3p from mRNA in vivo.

To isolate Npl3p-poly(A)$^+$ RNA complexes formed in vivo, we first cross-linked proteins to RNA by irradiating living cells with UV and then purified the RNPs on oligo dT cellulose. We loaded equal A260 units of RNPs purified from wild-type and Δsky1 strains on a gel, and analyzed the Npl3p content by Western blotting with anti-Npl3p polyclonal antibodies. As shown by previous work, Npl3p crosslinks to poly(A)$^+$ RNA in wild-type cells (Fig. 5A, lane 4) (Russell & Tollervey, 1995; Krebber et al., 1999). The amount of Npl3p crosslinked to poly(A)$^+$ RNA in Δsky1 cells was strikingly increased approximately three- to fivefold compared to wild-type (Fig. 5A, compare lane 5 to lane 4). GFP-Npl3p behaved identically to endogenous Npl3p in this assay (Fig. 5A); thus we have made use of our plasmid-borne GFP-Npl3 constructs to examine the effects of various mutations on Npl3p crosslinking. Mutation of the Sky1p phosphorylation site in Npl3p from serine to alanine increased crosslinking of Npl3p to poly(A)$^+$ RNA approximately fivefold (Fig. 5B, compare lane 4 to lane 3), duplicating the effect of deletion of SKY1 on wild-type Npl3p (Fig. 5A). We have confirmed this result using integrated tagged Npl3p and RA8p (data not shown). Thus we conclude that loss of Sky1p-catalyzed phosphorylation at RS8 increases the association of Npl3p with poly(A)$^+$ RNA in living cells.

We have observed three effects of loss of phosphorylation on Npl3p: (1) mislocalization to the cytoplasm, (2) decreased binding to Mtr10p, and (3) increased binding to poly(A)$^+$ RNA. Any of these might be a secondary consequence of one of the other defects. To distinguish effects on localization and/or binding to Mtr10p from effects on RNA binding per se, we made use of a yeast strain in which the MTR10 gene was deleted. Wild-type GFP-Npl3p and mutant GFP-Npl3RA8p display identical localization in a Δmtr10 strain; both proteins are distributed throughout the cell (data not shown). If increased RNA binding were sim-

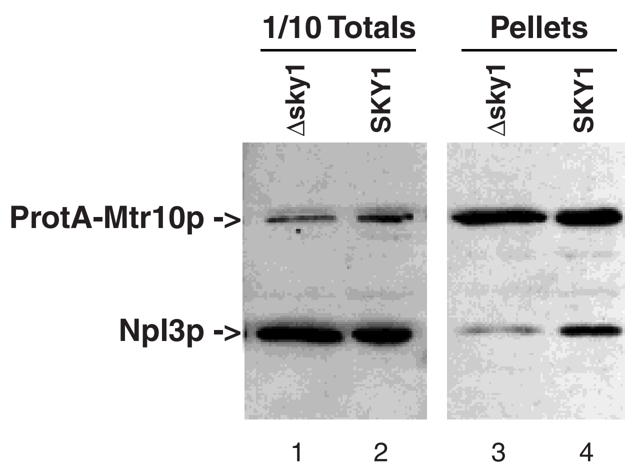


FIGURE 4. SKY1 enhances binding of Npl3p to Mtr10p in vivo. Whole-cell extracts were prepared from wild-type (lane 2) and Δsky1 (lane 1) strains expressing ProtA-Mtr10p. Npl3p was coimmunoprecipitated with ProtA-Mtr10p by binding to IgG Sepharose. Proteins were detected by Western blotting with anti-Npl3p polyclonal antibodies. Note that the polyclonal antibodies also recognize the protein A tag on Mtr10p. Binding of Npl3p to ProtA-Mtr10p was reduced in a Δsky1 strain (lane 3) compared to wild type (lane 4).

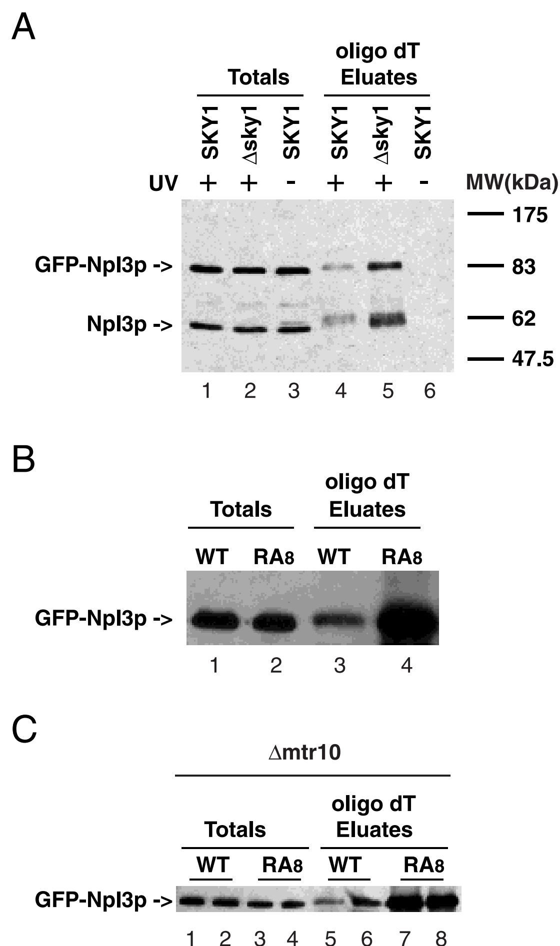


FIGURE 5. Loss of Sky1p-mediated phosphorylation increases binding of Npl3p to Poly(A)$^+$ RNA in vivo. Npl3p was crosslinked to RNA by mild UV treatment of living cells, and poly(A)$^+$ RNA–protein complexes were purified on oligo dT cellulose. One A260 unit of purified RNPs was used for each eluate gel lane. Total lanes contain less than 0.1% of the starting material. **A**: Δsky1 versus SKY1. Crosslinking to poly(A)$^+$ RNA was increased in a Δsky1 strain (lane 5) compared to SKY1 (lane 4). No Npl3p bound to the oligo dT cellulose in the absence of crosslinking (lane 6). GFP-Npl3p behaved identically to endogenous Npl3p. Deletion of the SKY1 gene had no effect on total protein levels (compare lane 2 to lane 1). **B**: The RA8 mutation increases crosslinking of Npl3p to poly(A)$^+$ RNA (lane 4) compared to wild-type Npl3p (lane 3). The GFP-tagged wild-type (lane 1) and mutant (lane 2) proteins were present at similar levels in total-cell extracts. **C**: Deletion of the MTR10 gene does not eliminate the effect of the RA8 mutation. RNPs were isolated from a Δmtr10 strain expressing either GFP-Npl3p or GFP-Npl3RA8p from a plasmid. Duplicate samples prepared from separate cultures are shown for each strain. The Npl3RA8 mutant protein was enriched in the oligo dT cellulose eluates (lanes 7 and 8) compared to wild-type GFP-Npl3p (lanes 5 and 6). Importantly, the variability between duplicate samples (and duplicate experiments) is small compared to the experimental difference (lanes 5–8).

ply a secondary effect of cytoplasmic accumulation, we predict that the RA8 mutation would have no effect on Npl3p–RNA crosslinking in this strain background. Similarly, if increased RNA binding were an indirect effect of decreased binding to Mtr10p, deletion of MTR10 would abolish the difference between wild-type Npl3p and Npl3RA8p. Mutation of the Sky1p phosphorylation site clearly increased crosslinking of GFP-Npl3RA8p to poly(A)$^+$ RNA compared to wild-type GFP-Npl3p in a Δmtr10 background (Fig. 5C, compare duplicate samples in lanes 7–8 to lanes 5–6). These results show that Sky1p-catalyzed phosphorylation decreases the binding of Npl3p to poly(A)$^+$ RNA in vivo, independent of effects on localization and independent of Mtr10p.

However, phosphorylation of Npl3p is probably not the sole determinant of the extent of RNA binding in vivo. Deletion of MTR10 dramatically increased RNA crosslinking of wild-type (phosphorylatable) Npl3p (Fig. 6A, compare lanes 3 and 4). mtr10-7ts, a mutant strain that localizes GFP-Npl3p to the nucleus and the cytoplasm at 25 °C (data not shown and Senger et al., 1998), similar to the localization of GFP-Npl3RA8p observed in wild-type cells (Fig. 3C), also shows increased crosslinking of Npl3p (Fig. 6B, compare lanes 4 and 5). We observed a modest increase in the total level of Npl3p protein in the mtr10 mutants (Fig. 6A,B, compare lanes 1 and 2). These crosslinking data are consistent with the hypothesis that binding of Mtr10p to Npl3p promotes dissociation of Npl3p from RNA, but do not rule out the possibility that simply increasing the cytoplasmic pool of Npl3 protein increases binding to poly(A)$^+$ RNA, the bulk of which is cytoplasmic in rapidly growing yeast cells (Groner & Phillips, 1975).

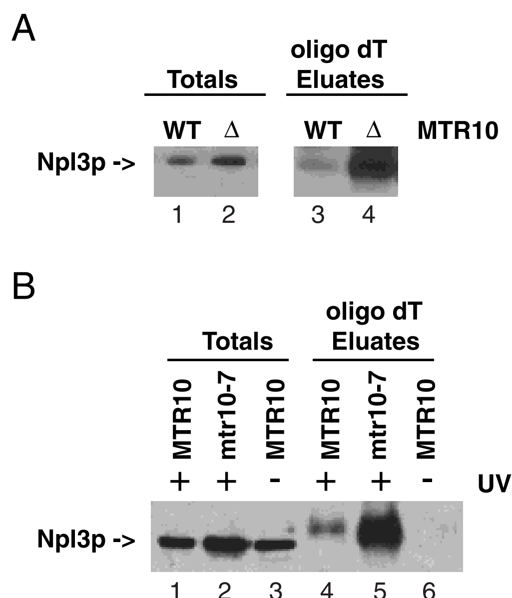


FIGURE 6. Mutations in MTR10 increase binding of Npl3p to Poly(A)$^+$ RNA in vivo. **A**: Crosslinking of Npl3p to poly(A)$^+$ RNA was increased in a Δmtr10 strain (lane 4) compared to wild type (lane 3). Deletion of MTR10 resulted in a modest increase in total Npl3p protein levels (lane 2) compared to wild type (lane 1). **B**: Crosslinking of Npl3p to poly(A)$^+$ RNA was increased in an mtr10-7 strain (lane 5) compared to wild type (lane 4). No Npl3p bound to oligo dT cellulose in the absence of crosslinking (lane 6). Npl3p protein levels were slightly elevated in an mtr10-7 strain (lane 2) compared to wild type (lane 1).

Phosphorylation by Sky1p does not affect binding of Npl3p to Mtr10p in a purified system

To test the hypothesis that Sky1p-catalyzed phosphorylation of Npl3p directly stimulates binding to Mtr10p, we performed in vitro binding studies using purified proteins. Full-length Sky1p tagged with protein A was purified from yeast by affinity purification on IgG Sepharose. In vitro kinase reactions were performed on these beads using recombinant HisNpl3p purified from *E. coli* as the substrate (Fig. 2B, lane 2). Careful quantitation of these kinase reactions determined that 0.9–1.0 mol of phosphate were incorporated per mole of rNpl3p (see Materials and Methods). Importantly, we observed no incorporation of phosphate into the otherwise active RA8 mutant substrate under these conditions (as shown in Fig. 2B). Thus we conclude that the rHisNpl3p used in subsequent binding reactions was 90–100% phosphorylated on Ser411. Mock-phosphorylated rHisNpl3p was prepared by incubating the substrate with a mock purification from a Δsky1 yeast strain (Fig. 2B, lane 1). Protein A-Mtr10p was purified from yeast by IgG-affinity chromatography and extensive washing with $MgCl_2$ (Fig. 7A). We have performed these experiments using recombinant HisMtr10p with similar results (data not shown); thus the binding is independent of the ProtA tag.

Purified ProtA-Mtr10p bound to phosphorylated and mock-phosphorylated rHisNpl3p with equal affinity (Fig. 7B, compare lanes 3 and 4). We reasoned that the effect of phosphorylation on binding to Mtr10p in vivo might be mediated by other proteins; therefore we assayed the effect of total yeast extract on the binding reaction. By increasing the concentration of yeast extract in the reaction, we were able to compete the binding of rHisNpl3p to ProtA-Mtr10p (Fig. 7C). Strikingly, there was no difference in the responses of phosphorylated and mock-phosphorylated rHisNpl3p to competition from yeast extract (Fig. 7C, compare lanes 1–3 to lanes 4–6). Thus, although phosphorylation of Npl3p clearly increases its association with Mtr10p in vivo, we find no evidence for a direct effect of phosphorylation on the binding affinity of Npl3p for Mtr10p in vitro. This finding conflicts with results recently reported (Yun & Fu, 2000), as we discuss below.

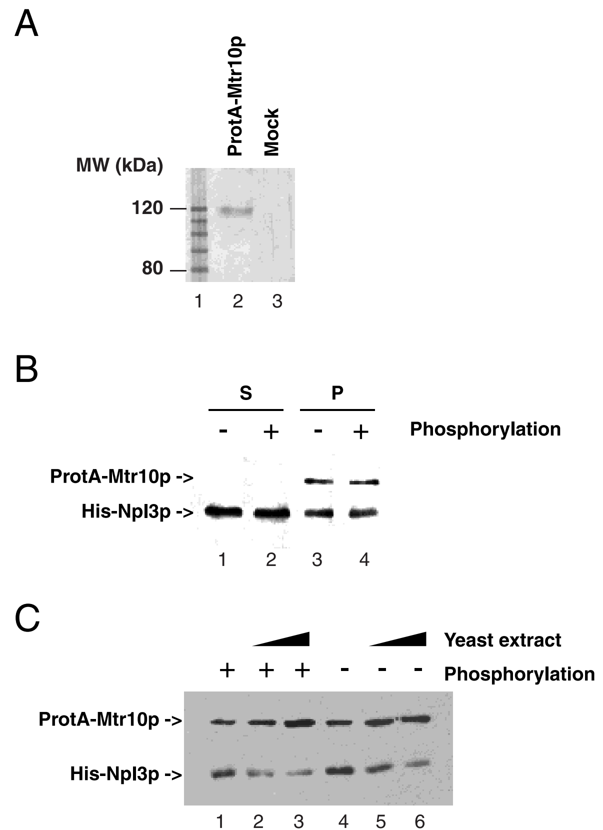


FIGURE 7. Phosphorylation of Npl3p does not affect binding to Mtr10p in vitro. **A**: ProtA-Mtr10p expressed from a low-copy plasmid was purified from yeast extract. A single band of ~120 kDa was detected on a silver-stained gel (lane 2). No band was detected in the same fraction purified from a yeast strain carrying an untagged MTR10 gene on a plasmid (lane 3). Molecular weight standards (10-kDa ladder) are resolved in lane 1. **B**: In vitro binding assay. Phosphorylated rHisNpl3p (lanes 2 and 4) and mock-phosphorylated rHisNpl3p (lanes 1 and 3) were incubated with purified ProtA-Mtr10p at 4 °C. Complexes were precipitated with IgG Sepharose, and the pellets (lanes 3 and 4) and supernatants (lanes 1 and 2) were analyzed by Western blotting with anti-Npl3p antibodies. **C**: Yeast extracts compete binding of ProtA-Mtr10p to phosphorylated and mock-phosphorylated rHisNpl3p alike. Addition of yeast protein from whole-cell extract (15 μg, lanes 2 and 5; 60 μg, lanes 3 and 6) resulted in decreased binding of rHisNpl3p (compare lanes 2 and 3 to lane 1, lanes 5 and 6 to lane 2). Phosphorylated rHisNpl3p (lanes 1–3) and mock-phosphorylated rHisNpl3p (lanes 4–6) were competed equally.

DISCUSSION

We have presented evidence that phosphorylation of Npl3p, a yeast shuttling SR protein, functions to promote the dissociation of Npl3p-RNA complexes in vivo. Sky1p, the yeast SRPK homolog, catalyzes Npl3p phosphorylation in vitro and in vivo, at a unique serine within Npl3p's RS domain. Loss of phosphorylation by Sky1p causes the mislocalization of Npl3p to the cytoplasm, and reduced association of Npl3p with its import receptor Mtr10p. Below we propose that phosphorylation of Npl3p by cytoplasmic Sky1p might serve as a molecular switch signaling the disassembly of mRNA–protein particles after export, thereby providing a potential mechanism for imparting directionality to the export process.

Mechanisms for dissociation of Npl3p–RNA complexes

Loss of phosphorylation by Sky1p resulted in a three- to fivefold increase in Npl3p's association with poly(A)$^+$ RNA in vivo and a decrease in Npl3p's association with Mtr10p in vivo. The inverse correlation between binding to RNA and binding to Mtr10p in vivo suggests that

formation of these Npl3p-containing complexes may be mutually exclusive. Previous studies of an Npl3p-Mtr10p complex purified from yeast extracts found that RNA could dissociate it in the presence of RanGTP, presumably reflecting a postnuclear import event (Senger et al., 1998). Conversely, we have obtained preliminary data showing that purified Mtr10p can dissociate a preformed Npl3p–RNA complex in vitro (W. Gilbert and C. Guthrie, unpubl. observations). Consistent with a model in which Mtr10p stimulates dissociation of Npl3p from RNA in the cytoplasm, we observe that mutations in MTR10 increase the amount of Npl3p that crosslinks to poly(A)$^+$ RNA in vivo. Notably, however, the mechanism by which Sky1p-catalyzed phosphorylation of Npl3p dissociates Npl3p from poly(A)$^+$ RNA does not require the presence of Mtr10 protein; we observed a similar three- to fivefold effect of phosphorylation on RNA binding in a Δmtr10 background. The converse is also true: Mutation of MTR10 increases Npl3p-RNA crosslinking in the absence of phosphorylation by Sky1p. Thus our data suggest that at least two mechanisms exist to promote the dissociation of Npl3p from poly(A)$^+$ RNA in the cytoplasm: (1) phosphorylation by Sky1p, and (2) binding of Mtr10p (see Fig. 8). The fact that deletion of the SKY1 gene is synthetically lethal with deletion of MTR10 (Yun & Fu, 2000) is consistent with our interpretation that both mechanisms contribute to the same essential process, the termination of messenger RNA export in the cytoplasm. This genetic interaction would not be expected if the function of SKY1 were simply to stimulate binding of Npl3p to Mtr10p; deletion of SKY1 would then have no effect in a Δmtr10 background. Npl3p, like most shuttling proteins described to date, is predominantly nuclear at steady state. If, in fact, the bulk of Npl3p is continuously cycling between the nucleus and the cytoplasm, the release and

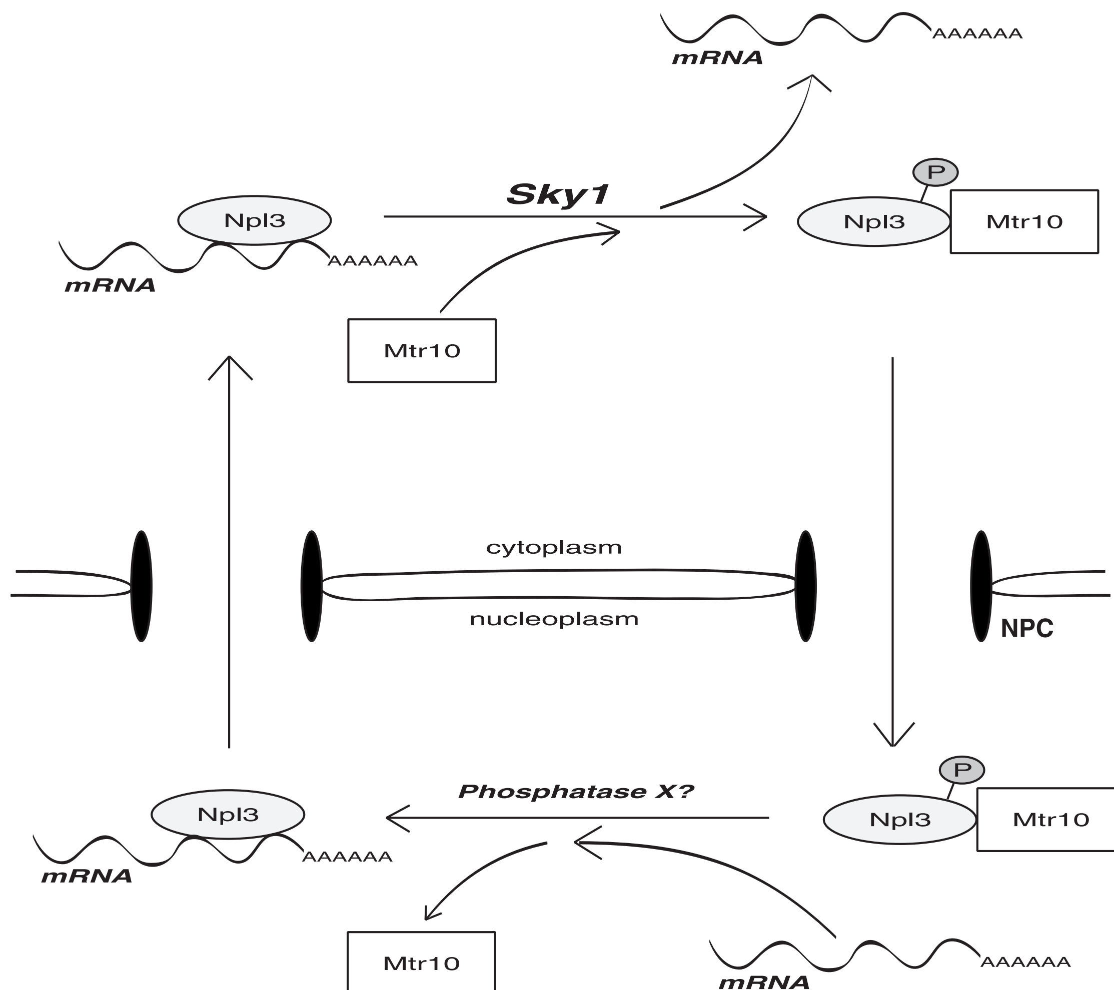


FIGURE 8. Model: the role of phosphorylation in Npl3p's nuclear transport cycle. In the cytoplasm, phosphorylation of Npl3p by Sky1p promotes the dissociation of Npl3p from exported RNPs. The disassembly of RNPs is facilitated by the binding of Mtr10p to Npl3p. Phosphorylated Npl3p is then targeted to the nucleus by Mtr10p. In the nucleus, Npl3p binds to newly transcribed mRNA and dissociates from Mtr10p. This exchange of Npl3p's binding partners in the nucleus may be stimulated by dephosphorylation of Npl3p.

reimport steps must be very rapid. Direct coupling between release of Npl3p from RNA and binding to Mtr10p could increase the efficiency of reimport.

An important question for the future will be to determine where Npl3p normally dissociates from exported mRNA, and how phosphorylation affects the location and timing of this event. In a preliminary experiment to determine whether phosphorylation by Sky1p reduced the affinity of Npl3p for synthetic polynucleotides, phosphorylation at RS8 had no effect. The possibility remains that phosphorylation would influence binding to specific mRNA sequences. In addition, it seems likely that phosphorylation may influence RNA binding via effects on protein–protein interactions within the RNP. A single Balbiani ring messenger RNP contains a variety of RNA-binding proteins, including cap-binding complex, hnRNP proteins, and shuttling SR proteins (Daneholt, 1997). Notably, the composition of this RNP changes several times during transit from the site of transcription to the ribosomes, with some shuttling proteins dissociating at the cytoplasmic face of the NPC whereas others remain associated with the mRNA in polysomes (Daneholt, 1997). We have obtained preliminary evidence that the presence of RA8 mutant Npl3p in a cell dominantly increases the crosslinking of Nab2p, another shuttling hnRNP protein (Lee & Aitchison, 1999; Duncan et al., 2000), to poly(A)$^+$ RNA in vivo (W. Gilbert and C. Guthrie, unpubl. observations). This is consistent with a model in which phosphorylation of Npl3p by Sky1p functions as a trigger for cytoplasmic remodeling of mRNPs.

Conservation of Sky1/SRPK function in yeast and mammals

What are the physiological roles of phosphorylation of SR proteins by SRPKs? Both the yeast kinase and its mammalian homologs are predominantly cytoplasmic (Wang et al., 1998; Siebel et al., 1999). Studies of mammalian SRPKs have emphasized the potential effects of phosphorylation of SR proteins on nuclear pre-mRNA splicing, the process in which SR proteins' role is best appreciated. Our data do not exclude the possibility that cytoplasmic phosphorylation of SR proteins may be an important mechanism for regulating the nuclear concentration and/or activity of SR proteins involved in pre-mRNA splicing. However, our studies of yeast Sky1p raise the possibility that a conserved function of SRPKs may be to regulate the nucleocytoplasmic shuttling of SR proteins. In recent studies of SF2/ASF, overexpression of an SRPK2 kinase-inactive mutant caused SF2/ASF to accumulate in the cytoplasm (Koizumi et al., 1999). It will be interesting to determine whether SRPK-mediated phosphorylation promotes the dissociation of mammalian SR proteins from RNA, as we have shown for Sky1p phosphorylation of Npl3p.

A mammalian homolog of Mtr10p, TRN-SR, was recently identified as a cytosolic factor capable of mediating the import of recombinant RS-domain containing proteins in a permeabilized cell assay (Kataoka et al., 1999). The effects of phosphorylation were not determined in this article. However, a recent study of TRN-SR2, which differs from TRN-SR by the absence of two short regions of ~30 amino acids, found that phosphorylation of RS domains was required for efficient binding to the import receptor in vitro (Lai et al., 2000). Further experiments are needed to clarify the relationship between these two proteins, and to determine the effects of RS-domain phosphorylation on mammalian SR-protein import in vivo. Our in vitro binding studies with purified Mtr10 and Npl3 proteins indicate that phosphorylation of Npl3p by Sky1p does not significantly increase Npl3p's affinity for Mtr10p in vitro. This contradicts a recent report from Fu and colleagues (Yun & Fu, 2000). One potential difference may be the specificity of the in vitro kinase reactions. The extent of Npl3p phosphorylation in our in vitro reactions is 0.9–1.0 mol of incorporated phosphate per mole of rNpl3p, consistent with quantitative phosphorylation of a single site. In contrast to the full-length Sky1p used in our studies, Yun and Fu (2000) employed a form of Sky1p lacking the amino terminus and the spacer region, together comprising >50% of the coding sequence; conceivably the specificity of this kinase is compromised.

Only one of eight RS/SR dipeptides is required for phosphorylation by Sky1p

Npl3p was identified by our laboratory and others as a likely yeast SR protein based on the presence of eight SR/RS dipeptides in the C-terminal domain, in addition to homology within the RRM domains. In this article, we show that only one of Npl3p's SR/RS dipeptides is essential for phosphorylation by Sky1p in vitro. It is intriguing to note that six of the remaining seven RS/SR dipeptides in Npl3p that do not appear to be sites for Sky1p phosphorylation are in the context SRGG. This sequence is predicted to be a site for arginine methylation by Hmt1p (Henry & Silver, 1996; Siebel & Guthrie, 1996; Shen et al., 1998; Yun & Fu, 2000). Additionally, the serine that is required for phosphorylation by Sky1p in vitro is the only one found within the preferred sequence for human SRPK2 (Wang et al., 1998). Our results clearly show that all RS/SR dipeptides are not equivalent and emphasize the importance of analyzing specific phosphorylation events when attempting to draw conclusions about the effects of RS-domain phosphorylation. This may be particularly important to consider in the interpretation of in vivo studies involving the overexpression of SR protein kinases; it will be important to determine that the sites phosphorylated under such conditions in fact reflect the normal sites of these kinases.

Implications

Whatever the specific mechanism by which Sky1p-mediated phosphorylation destabilizes Npl3p's association with RNA in vivo, this activity has the potential to perform two important functions. First it could be a signal to terminate mRNA export in the cytoplasm, thereby imparting directionality to the process. The model that cytoplasmic phosphorylation of Npl3p functions to terminate mRNA export predicts a nuclear Npl3p phosphatase, and a cycle of phosphorylation and dephosphorylation with each round of RNA export. We are currently investigating whether there is a correlation between Npl3p's phosphorylation state and its subcellular localization. Direct testing of this model awaits the identification of an Npl3p phosphatase. Mammalian PP1A phosphatase has been shown to antagonize the activity of SRPKs in vitro (Fu, 1995); it will be interesting to see whether the yeast PP1A homolog GLC7 reverses Sky1p-catalyzed phosphorylation of Npl3p in vivo. A second function of Sky1p could be to prevent sequestering of newly synthesized Npl3p protein in the cytoplasm by inhibiting inappropriate binding to abundant cytoplasmic poly(A)$^+$ RNA. This could be a role for SRPK-catalyzed phosphorylation of nonshuttling nuclear SR proteins such as SC35. Our discovery of a function for Sky1p unrelated to splicing invites further study of the functions of SR proteins and their regulators in all aspects of mRNA metabolism.

MATERIALS AND METHODS

Yeast strains and plasmids

Δsky1::TRP1 (YCS22) and the parent SKY1 strain (YCS19: Mata; ura3-52; his3-11,15; leu2-3,112; trp1-1; ade2-1; ade3; can1-100) were described previously (Siebel et al., 1999). MTR10 (RS453), mtr10-7, and Δmtr10::HIS3 were described previously (Senger et al., 1998). The SKY1-ProtA strain (YWG35) was generated by integrating the protein A coding sequence into YCS19 at the C-terminus of the SKY1 open reading frame using the method of Longtine et al. (1998). The integrating SKY1TEVProtA-kan plasmid was constructed by replacing the GFP coding sequence in pRA6a-GFP(S65T)-kanMX6 (Longtine et al., 1998) with a PCR product encoding protein A flanked by *Pac*I (5′) and *Asc*I (3′) sites. The presence of the protein A tag was confirmed by whole-cell PCR and Western blotting.

To visualize wild-type and mutant Npl3p in living cells, we inserted the GFP open reading frame at the 5′ end of the NPL3 coding sequence within a 3,880 bp *Hae*II/*Afl*III blunt-ended genomic fragment cloned into the pRS315 CEN, ARS vector previously cut with *Sma*I. DNA encoding GFP flanked by *Stu*I sites was generated by PCR and cloned into an *Stu*I site that was generated at the ATG of NPL3 by PCR. This pRS315-GFP-NPL3 fusion (pCS38) complemented a Δnpl3 strain for growth at all temperatures tested from 16 °C to 37 °C. The RS → RA mutant reporter plasmid, pRS315-GFP-NPL3RA8 (pCS55), was generated by PCR of the C-terminus of NPL3 with a mutagenic oligo, followed by subcloning of the mutant fragment into pCS38. All PCR products were confirmed by sequencing.

The protein A-tagged (pNOPPAMTR10) and untagged control (pUN100MTR10) plasmids for the production of ProtA-Mtr10p in yeast were described previously (Senger et al., 1998).

Expression and purification of *E. coli* recombinant proteins

The NPL3 gene (from ATG to stop codon) was inserted into pRSET,A (Invitrogen) by PCR amplification using *Bam*HI (5′) and *Dra*I (3′) primers and insertion of the *Bam*HI-*Dra*I fragment into pRSETA cut with *Bam*HI and *Pvu*II, to make pHis-Npl3. pHisNpl3ΔRS was constructed by deleting the entire RS domain (see Fig. 2A for domain boundaries) using two rounds of PCR. pHisNpl3RA8 was generated by inserting the *Hin*dIII fragment from pCS55 containing the RA8 mutation into pHisNpl3 cut with *Hin*dIII. All expression constructs were confirmed by sequencing. The vectors containing the wild-type and mutant NPL3 genes were transformed into *E. coli* BL21(DE3)plysS cells and 1-L cultures were grown in Luria broth + ampicillin (100 μg/mL) + CAM (25 μg/mL) at 30 °C to OD 0.6 and induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) in the presence of 1 mM PMSF for 3.5 h. The bacterial cell pellet was lysed by sonication in 12.5 mL of 50 mM NaH_2PO4, pH 8, 300 mM NaCl, 0.05% Triton X-100, 10 mM imidazole. The lysate was cleared by centrifugation at 10,000 × *g* for 20 min, and bound in batch to 1.25 mL Ni-NTA Agarose (Qiagen) at 4 °C for 1 h. The resin was poured into a column and washed extensively with 80 mM imidazole in lysis buffer. Bound proteins were eluted with 250 mM imidazole in lysis buffer and dialyzed against 20 mM HEPES, 25 mM KCl, 10% glycerol, 0.5 mM DTT, pH 7.9.

Affinity purification of ProtA-fusions from yeast

The vectors containing the protein A-tagged (pNOPPAMTR10) and untagged (pUN100MTR10) genes were transformed into YCS22 cells and 1-L cultures were grown in SD-Leu at 30 °C to OD 1.0. Cells were lysed in IgG buffer [50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, 5 mM $MgCl_2$, 2 mM β-mercaptoethanol, 1 mM PMSF, 1 μg/mL pepstatin, 10 mM NaF, 10 mM β-glycerophosphate-HCl, pH 7.5, and a cocktail of protease inhibitors (Boehringer Mannheim)] by shaking with 0.5 mm glass beads in a multi-bead-beater (Biospec) at 4 °C for three 2-min cycles with >2 min on ice in between cycles. The lysate was cleared by centrifugation and the supernatant (whole-cell extract) was mixed with 100 μL IgG-Sepharose beads (Pharmacia) and incubated at 4 °C for 2 h. To check for associated Npl3p, 1% of the IgG-Sepharose bound lysate was washed in IgG buffer, boiled in SDS loading buffer, and analyzed by SDS-PAGE and Western blotting with anti-Npl3p polyclonal antibodies. ProtA-Mtr10p was purified from the remaining fraction by extensive washing with IgG buffer + 500 mM $MgCl_2$, followed by equilibration in 600 μL IgG buffer. To analyze the purified protein, 1/10th of the IgG-Sepharose beads were eluted with 80 μL of acetic acid (0.5 M, pH 3.4), neutralized with 20 μL 2 M Tris base, and boiled in

25 μL 4× SDS loading buffer. Twenty microliters were analyzed by SDS-PAGE and silver staining.

Sky1-ProtA was purified as described above for ProtA-Mtr10p, with the following modifications: 1-L cultures (YWG35 and YCS22) were grown in YPD at 30 °C to OD 1.0. After binding in IgG buffer, the IgG-Sepharose bound protein was washed extensively in 1× PBS + 0.1% NP-40, 10 mM NaF, 10 mM β-glycerophosphate-HCl, pH 7.5, at 4 °C, followed by equilibration in 1 mL kinase wash buffer (40 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 100 mM NaCl, 0.01% NP-40, 1 mM PMSF, 10 mM NaF, 10 mM β-glycerophosphate-HCl, pH 7.5) at 22 °C. One liter of starting culture yielded ~2 μg purified Sky1-ProtAp.

In vitro phosphorylation and binding

Recombinant Npl3p was purified from *E. coli* as described above. Five hundred microliter in vitro kinase reactions contained 50 μg purified Npl3p substrate and ~1 μg Sky1-ProtAp (bound to IgG-Sepharose), or an equivalent fraction of IgG-Sepharose from a mock purification, in kinase reaction buffer (40 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 100 mM NaCl, 0.01% NP-40, 10 mM NaF, 10 mM β-glycerophosphate-HCl, pH 7.5, 1 mM ATP) with 20 μCi gamma-^{32}P-ATP. The reactions were incubated at 30 °C with gentle mixing for 1 h. The extent of phosphorylation was determined by quantitating the incorporation of gamma ^{32}P ATP into Npl3p on a phosphorimager and comparing the signal to a standard curve obtained by scanning a dilution series of the gamma^{32}P ATP label. The phosphorylated or mock-phosphorylated Npl3p was recovered from the supernatant of the kinase reactions and frozen in liquid nitrogen in the presence of 20% (v/v) glycerol for use in subsequent experiments.

Four-hundred-microliter binding reactions containing ~3 μg ProtA-Mtr10p bound to IgG beads, 3 μg phosphorylated or mock-phosphorylated rHisNpl3p, and 0, 15, or 60 μg total yeast protein from whole-cell extract were incubated in IgG buffer +0.5 mg/mL BSA at 4 °C for 4 h. The pellet and supernatant fractions were analyzed by SDS-PAGE and Western blotting with anti-Npl3 antibodies, previously described (Siebel & Guthrie, 1996).

Purification of UV-crosslinked RNPs

One-liter yeast cultures were grown in YPD to OD 0.6–1.0. Cells were treated with UV light, as described (Anderson et al., 1993). Lysates in RNP lysis buffer [20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 50 mM LiCl, 1% SDS, 1% β-mercaptoethanol, 1 mg/mL heparin, 10 mM vanadyl adenosine, 1 mM PMSF, and a cocktail of protease inhibitors (Boehringer Mannheim)] were made by shaking with glass beads as described above. Subsequent purification of proteins UV crosslinked to poly(A)+ RNA was performed essentially as described (Anderson et al., 1993). Resuspended poly(A)+ RNA samples were normalized by A260, treated with nuclease, and loaded for SDS-PAGE and immunoblotting with anti-Npl3p polyclonal antibodies (Siebel & Guthrie, 1996) followed by HRP-conjugated secondary antibody (Bio-Rad) and detection with Super Signal West Femto substrate (Pierce). One A260 unit of each eluate was loaded per gel lane. A detailed protocol is available at http://www.metazoa.com/upl2887.

ACKNOWLEDGMENTS

We thank Ed Hurt for providing strains and plasmids; Anne de Bruyn Kops, Amy Kistler, Julianne Dunphy, and Doug Jefferey for helpful discussions; Chi Yun and Xiang-Dong Fu for communicating results prior to publication; Kent Duncan, Stephen Rader, and Erin O'Shea for critical comments on the manuscript; and Lucita Esperas and Carol Pudlow for excellent technical support. This work was supported by a grant from the National Institutes of Health to C.G. W.G. is a Howard Hughes Medical Institute Predoctoral Fellow. C.G. is an American Cancer Society Research Professor of Molecular Genetics.

Received November 22, 2000; accepted without revision November 27, 2000

REFERENCES

Alzhanova-Ericsson AT, Sun X, Visa N, Kiseleva E, Wurtz T, Daneholt B. 1996. A protein of the SR family of splicing factors binds extensively to exonic Balbiani ring pre-mRNA and accompanies the RNA from the gene to the nuclear pore. *Genes & Dev 10*:2881–2893.

Anderson JT, Wilson SM, Datar KV, Swanson MS. 1993. NAB2: A yeast nuclear polyadenylated RNA-binding protein essential for cell viability. *Mol Cell Biol 13*:2730–2741.

Birney E, Kumar S, Krainer AR. 1993. Analysis of the RNA-recognition motif and RS and RGG domains: Conservation in metazoan pre-mRNA splicing factors. *Nucleic Acids Res 21*:5803–5816.

Caceres JF, Screaton GR, Krainer AR. 1998. A specific subset of SR proteins shuttles continuously between the nucleus and the cytoplasm. *Genes & Dev 12*:55–66.

Colwill K, Pawson T, Andrews B, Prasad J, Manley JL, Bell JC, Duncan PI. 1996. The Clk/Sty protein kinase phosphorylates SR splicing factors and regulates their intranuclear distribution. *EMBO J 15*:265–275.

Daneholt B. 1997. A look at messenger RNP moving through the nuclear pore. *Cell 88*:585–588.

Dreyfuss G, Matunis MJ, Pinol-Roma S, Burd CG. 1993. hnRNP proteins and the biogenesis of mRNA. *Annu Rev Biochem 62*:289–321.

Duncan K, Umen JG, Guthrie C. 2000. A putative ubiquitin ligase required for efficient mRNA export differentially affects hnRNP transport. *Curr Biol 10*:687–696.

Flach J, Bossie M, Vogel J, Corbett A, Jinks T, Willins DA, Silver PA. 1994. A yeast RNA-binding protein shuttles between the nucleus and the cytoplasm. *Mol Cell Biol 14*:8399–8407.

Fu XD. 1995. The superfamily of arginine/serine-rich splicing factors. *RNA 1*:663–680.

Groner B, Phillips SL. 1975. Polyadenylate metabolism in the nuclei and cytoplasm of *Saccharomyces cerevisiae*. *J Biol Chem 250*:5640–5646.

Gui JF, Tronchere H, Chandler SD, Fu XD. 1994. Purification and characterization of a kinase specific for the serine- and arginine-rich pre-mRNA splicing factors. *Proc Natl Acad Sci USA 91*:10824–10828.

Henry MF, Silver PA. 1996. A novel methyltransferase (Hmt1p) modifies poly(A)+-RNA-binding proteins. *Mol Cell Biol 16*:3668–3678.

Kadowaki T, Chen S, Hitomi M, Jacobs E, Kumagai C, Liang S, Schneiter R, Singleton D, Wisniewska J, Tartakoff AM. 1994. Isolation and characterization of *Saccharomyces cerevisiae* mRNA transport-defective (mtr) mutants [published erratum appears in *J Cell Biol* 1994 *126*:1627]. *J Cell Biol 126*:649–659.

Kataoka N, Bachorik JL, Dreyfuss G. 1999. Transportin-SR, a nuclear import receptor for SR proteins. *J Cell Biol 145*:1145–1152.

Koizumi J, Okamoto Y, Onogi H, Mayeda A, Krainer AR, Hagiwara M. 1999. The subcellular localization of SF2/ASF is regulated by direct interaction with SR protein kinases (SRPKs). *J Biol Chem 274*:11125–11131.

Krebber H, Taura T, Lee MS, Silver PA. 1999. Uncoupling of the hnRNP Npl3p from mRNAs during the stress-induced block in mRNA export. *Genes & Dev 13*:1994–2004.
Lai MC, Lin RI, Huang SY, Tsai CW, Tarn WY. 2000. A human importin-beta family protein, transportin-SR2, interacts with the phosphorylated RS domain of SR proteins. *J Biol Chem 275*:7950–7957.
Lee DC, Aitchison JD. 1999. Kap104p-mediated nuclear import. Nuclear localization signals in mRNA-binding proteins and the role of RAN and RNA. *J Biol Chem 274*:29031–29037.
Lee MS, Henry M, Silver PA. 1996. A protein that shuttles between the nucleus and the cytoplasm is an important mediator of RNA export. *Genes & Dev 10*:1233–1246.
Liu Q, Dreyfuss G. 1995. In vivo and in vitro arginine methylation of RNA-binding proteins. *Mol Cell Biol 15*:2800–2808.
Longtine MS, McKenzie A 3rd, Demarini DJ, Shah NG, Wach A, Brachat A, Philippsen P, Pringle JR. 1998. Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast 14*:953–961.
Misteli T, Caceres JF, Clement JQ, Krainer AR, Wilkinson MF, Spector DL. 1998. Serine phosphorylation of SR proteins is required for their recruitment to sites of transcription in vivo. *J Cell Biol 143*:297–307.
Nakielny S, Dreyfuss G. 1997. Nuclear export of proteins and RNAs. *Curr Opin Cell Biol 9*:420–429.
Nakielny S, Fischer U, Michael WM, Dreyfuss G. 1997. RNA transport. *Annu Rev Neurosci 20*:269–301.
Pemberton LF, Rosenblum JS, Blobel G. 1997. A distinct and parallel pathway for the nuclear import of an mRNA-binding protein. *J Cell Biol 139*:1645–1653.
Pollard VW, Michael WM, Nakielny S, Siomi MC, Wang F, Dreyfuss G. 1996. A novel receptor-mediated nuclear protein import pathway. *Cell 86*:985–994.
Rossi F, Labourier E, Forne T, Divita G, Derancourt J, Riou JF, Antoine E, Cathala G, Brunel C, Tazi J. 1996. Specific phosphorylation of SR proteins by mammalian DNA topoisomerase I. *Nature 381*:80–82.
Russell I, Tollervey D. 1995. Yeast Nop3p has structural and functional similarities to mammalian pre-mRNA binding proteins. *Eur J Cell Biol 66*:293–301.
Senger B, Simos G, Bischoff FR, Podtelejnikov A, Mann M, Hurt E. 1998. Mtr10p functions as a nuclear import receptor for the mRNA-binding protein Npl3p. *EMBO J 17*:2196–2207.
Shen EC, Henry MF, Weiss VH, Valentini SR, Silver PA, Lee MS. 1998. Arginine methylation facilitates the nuclear export of hnRNP proteins. *Genes & Dev 12*:679–691.
Siebel CW, Feng L, Guthrie C, Fu XD. 1999. Conservation in budding yeast of a kinase specific for SR splicing factors. *Proc Natl Acad Sci USA 96*:5440–5445.
Siebel CW, Guthrie C. 1996. The essential yeast RNA binding protein Np13p is methylated. *Proc Natl Acad Sci USA 93*:13641–13646.
Siomi MC, Eder PS, Kataoka N, Wan L, Liu Q, Dreyfuss G. 1997. Transportin-mediated nuclear import of heterogeneous nuclear RNP proteins. *J Cell Biol 138*:1181–1192.
Stutz F, Rosbash M. 1998. Nuclear RNA export. *Genes & Dev 12*:3303–3319.
Tacke R, Chen Y, Manley JL. 1997. Sequence-specific RNA binding by an SR protein requires RS domain phosphorylation: Creation of an SRp40-specific splicing enhancer. *Proc Natl Acad Sci USA 94*:1148–1153.
Visa N, Alzhanova-Ericsson AT, Sun X, Kiseleva E, Bjorkroth B, Wurtz T, Daneholt B. 1996. A pre-mRNA-binding protein accompanies the RNA from the gene through the nuclear pores and into polysomes. *Cell 84*:253–264.
Wang HY, Lin W, Dyck JA, Yeakley JM, Songyang Z, Cantley LC, Fu XD. 1998. SRPK2: A differentially expressed SR protein-specific kinase involved in mediating the interaction and localization of pre-mRNA splicing factors in mammalian cells. *J Cell Biol 140*: 737–750.
Yeakley JM, Tronchere H, Olesen J, Dyck JA, Wang HY, Fu XD. 1999. Phosphorylation regulates in vivo interaction and molecular targeting of serine/arginine-rich pre-mRNA splicing factors. *J Cell Biol 145*:447–455.
Yun CY, Fu XD. 2000. Conserved SR protein kinase functions in nuclear import and its action is counteracted by arginine methylation in *Saccharomyces cerevisiae*. *J Cell Biol 150*:707–718.