Conserved SR Protein Kinase Functions in Nuclear Import and Its Action Is Counteracted by Arginine Methylation in *Saccharomyces cerevisiae*

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Abstract. Mammalian serine and arginine–rich (SR) proteins play important roles in both constitutive and regulated splicing, and SR protein–specific kinases (SRPKs) are conserved from humans to yeast. Here, we demonstrate a novel function of the single conserved SR protein kinase Sky1p in nuclear import in budding yeast. The yeast SR-like protein Npl3p is known to enter the nucleus through a composite nuclear localization signal (NLS) consisting of a repetitive arginine-glycine-glycine (RGG) motif and a nonrepetitive sequence. We found that the latter is the site for phosphorylation by Sky1p and that this phosphorylation regulates nuclear import of Npl3p by modulating the interaction of the RGG motif with its nuclear import receptor Mtr10p. The RGG motif is also methylated on arginine residues, but methylation does not affect the Npl3p–Mtr10p interaction in vitro. Remarkably, arginine methylation interferes with Sky1p-mediated phosphorylation, thereby indirectly influencing the Npl3p–Mtr10p interaction in vivo and negatively regulating nuclear import of Npl3p. These results suggest that nuclear import of Npl3p is coordinately influenced by methylation and phosphorylation in budding yeast, which may represent conserved components in the dynamic regulation of RNA processing in higher eukaryotic cells.

Key words: serine and arginine–rich protein kinase • RNA binding protein • nuclear transport • phosphorylation • arginine methylation

Introduction

RNA binding proteins containing one or two RNA recognition motifs (RRMs)\(^1\) and a signature arginine– and serine–rich (RS) domain are collectively referred to as serine and arginine–rich (SR) proteins (for reviews see Fu, 1995; Manley and Tacke, 1996; Valcárcel and Green, 1996). Biochemical studies demonstrate that SR proteins are essential factors for spliceosome assembly at multiple steps, from the formation of commitment complexes in early splice site recognition (Fu, 1993; Kohs et al., 1994) to the conversion of the prespliceosome to the spliceosome (Rossignol and Garcia-Bianco, 1995; Tarn and Steitz, 1995). Besides their roles in constitutive splicing, SR proteins are also known to affect alternative splicing in a concentration-dependent manner (for reviews see Fu, 1995; Manley and Tacke, 1996). Interestingly, in all examined cases, the activity of SR proteins in alternative splicing is antagonized by heterogeneous nuclear ribonucleoprotein (hnRNP) A/B proteins (for a review see Cáceres and Krainer, 1997). These observations have led to the current view that alternative splicing may be controlled by balanced activities of SR and hnRNP proteins in mammalian cells. In this regard, regulation of alternative splicing may be achieved by regulating SR and hnRNP proteins at transcriptional and posttranscriptional levels.

Regulation of SR and hnRNP proteins and the functional consequences associated with it are just beginning to be understood. Although most SR proteins are ubiquitously expressed, some are expressed in a tissue-specific manner (A yane et al., 1991), during T cell development (Lemaire et al., 1999), or induced in response to mitogens (Diamond et al., 1993; Screaton et al., 1995). All RS domain–containing proteins are posttranslationally modified by phosphorylation. Because RS domains are known to participate in protein–protein (Wu and Maniatis, 1993) and protein–RNA (Valcárcel et al., 1996) interactions during spliceosome assembly, phosphorylation may regulate the activity of SR proteins in splicing. Indeed, it has been shown that phosphorylation can modulate physical interactions involving SR proteins (Xiao and Manley, 1997; Wang et al., 1998; Yeakley et al., 1999), and is essential for

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\(^{1}\)Abbreviations used in this paper: GFP, green fluorescent protein; GST, glutathione S-transferase; hnRNP, heterogeneous nuclear ribonucleoprotein; NLS, nuclear localization signal; PrA, protein A; RGG, arginine-glycine-glycine; RRM, RNA recognition motif; RS, arginine- and serine-rich; SAH, S-adenosyl-L-homocysteine; SAM, S-adenosyl-L-methionine; SR, serine and arginine-rich; SR PK, SR protein-specific kinase.
their functions in splicing (Cao et al., 1997; Xiao and Mainley, 1997). A related, alternative splicing can be induced by SR protein-specific kinases (SR PKs) in transfected cells (Duncan et al., 1997; Prasad et al., 1999; Ding and Fu, unpublished observations). Finally, RS domains are also known to function as a nuclear localization signal (NLS) (Li and Bingham, 1991; Hedges et al., 1995; Cáceres et al., 1997). However, it is presently unclear whether nuclear translocation of SR proteins is subject to phosphorylation regulation in mammalian cells.

Compared with SR proteins, the function of hnRNP proteins in RNA metabolism is not well understood. These abundant RNA binding proteins appear to have a variety of cellular activities, ranging from transcription and pre-mRNA processing to RNA transport and turnover (for a review see Krecic and Swanson, 1999). Some hnRNPs are modified by phosphorylation (Karn et al., 1977) or are glycosylated (Soulard et al., 1993). Interestingly, a large number of mammalian hnRNPs are proteins modified by arginine methylation (Liu and Dreyfuss, 1995), which accounts for 65% of the total N\textsubscript{G}-dimethylarginine found in the cell nucleus (Boffa et al., 1977). A arginine methylation appears to take place mainly in the RGG box (an arginine-glycine-glycine–rich region) involved in both RNA binding and protein–protein interactions. Because methylation would not alter the positive charge on arginine, this modification may affect functional properties of modified proteins by disrupting hydrogen bonds or introducing steric hindrance. In contrast to data on SR protein phosphorylation, no clear-cut biochemical evidence has been documented to show a major effect of arginine methylation on RNA–protein and protein–protein interactions, and thus, the functional significance of this modification remains largely elusive. Recently, an arginine methyltransferase HMT1/RMT1 was shown to be conserved in budding yeast (Gary et al., 1996; Henry and Silver, 1996; Siebel and Guthrie, 1996), which can be used for functional studies of arginine methylation using genetic approaches. Indeed, genetic studies from the Silver laboratory revealed a role of Hmt1p/Rmt1p in the regulation of hnRNP protein nuclear export in yeast (Shen et al., 1998). However, the biochemical basis for the cellular function of Hmt1p/Rmt1p remains unknown.

We have been characterizing a family of SR PKs that regulate the interaction and nuclear distribution of SR proteins in mammalian cells (Gu et al., 1994a,b; Colwill et al., 1996; Wang et al., 1998). Recently, we demonstrated that a single SR PK family member, named Sky1p, is conserved in Saccharomyces cerevisiae (Siebel et al., 1999). This finding is surprising because generally it is assumed that budding yeast do not have an SR system based in part on the observations that alternative splicing does not occur in yeast and that their genome does not encode proteins containing a continuous stretch of SR/R S dipeptide repeats. Interestingly, we found that one of the endogenous substrates for Sky1p is the well-studied RNA binding protein Npl3p, which has been implicated in both pre-mRNA splicing (Siebel and Guthrie, unpublished results) and mRNA transport (Kadowaki et al., 1994; Lee et al., 1996; Krebber et al., 1999). Npl3p has two RRM S and a COOH-terminal RGG/R S domain containing multiple RGG repeats and distributed R S or SR dipeptides. Therefore, Npl3p resembles both hnRNP and SR proteins in mammalian cells, as previously indicated by sequence alignment (Birney et al., 1993). Here, we present biochemical and genetic evidence revealing that Sky1p regulates nuclear import of Npl3p by promoting the interaction between Npl3p and its nuclear import receptor Mtr10p. Strikingly, we found that arginine methylation also regulates nuclear import of Npl3p by using an unprecedented mechanism: hypermethylation of the RGG box by Hmt1p/Rmt1p interferes with Sky1p-mediated phosphorylation, thereby indirectly blocking complex formation between Npl3p and Mtr10p both in vitro and in vivo. Therefore, nuclear import of Npl3p may be regulated by both phosphorylation and methylation. These findings have significant implications on the regulation of SR and hnRNP proteins in mammalian cells.

Materials and Methods

Strains and Plasmids

Wild-type (Y C519) and sky-1Δ (Y C522) strains used for green fluorescent protein (GFP)-Npl3p localization and for synthetic lethality were described previously (Siebel et al., 1999). Strains M HY 132 (Lee et al., 1996), and DFSA containing Mtr10-Protein A (PrA) (Pemberton et al., 1997) were used for plasmid shuffling and coimmunoprecipitation experiments, respectively. M T R10 and SKY1 were deleted from these strains by standard recombination (Wach et al., 1994). PCR–amplified full length Npl3p and its mutants were subcloned into pGEX-KG for glutathione S-transferase (GST) fusion protein production. Wild-type and mutant npl3p were expressed as GFP fusion proteins from the GAL-inducible plasmid pSB11 for localization or expressed from its own promoter in a CEN/EU plasmid pMH3 for plasmid shuffling experiments (Lee et al., 1996). MTR10 was PCR–amplified from genomic DNA and subcloned into pSP72 for transcription translation reactions in vitro, or into the GAL-inducible plasmid p415 for overexpression in yeast. The genetic M T R10 fragment was also cloned into pET30a to express His- and S-tagged recombinant protein in bacteria. Plasmids prS316/SKY1 for expression of Sky1p in yeast (Siebel et al., 1999), and pGEX-X-RMT1/HMT1 for preparation of recombinant methyltransferase (Gary et al., 1996) were described previously. A BamHI-EcoRI fragment of HMT1/RMT1 from pGEX-X-RMT1/HMT1 was subcloned into p415 for Hmt1p overexpression in yeast. His-tagged ixb/R S fusion protein was obtained from B. Nolen and G. Gosh (University of California at San Diego).

Immunoprecipitation and Immunoblotting

IgG-Sepharose beads (J ackson Immunoresearch Laboratories) were used to immunoprecipitate M T R10-PrA from either cytoplasmic or whole cell extracts as described previously (Pemberton et al., 1997). We used 2 μl beads which is sufficient to capture most M tr10-PrA from 100 μg total yeast protein. The use of a minimum amount of beads is critical, because some IgG heavy chain was released during incubation with yeast extract and the released heavy chain comigrated with Npl3p in our gels, interfering with quantitation of coimmunoprecipitated Npl3p by Western blot analysis. Npl3p and M tr10-PrA in the immunocomplexes were detected with rabbit polyclonal antibodies against Npl3p (a gift from C.W. Siebel and C. Guthrie, University of California at San Francisco) and rabbit IgG (J ackson Immunoresearch Laboratories), respectively.

Expression of GFP-Npl3p and HA-tagged Hmt1p/Rmt1p was confirmed by Western blotting using a rabbit polyclonal anti-GFP antibody (a gift from K.ahana and Silver), J. Kahan and P. Silver, Dana Farber Cancer Institute, Boston, MA) and mouse anti-HA mAb 12CA5 (Eastman Kodak Co.). The methylation state of Npl3p was determined by immunoblotting with the methylation-specific mAb IE4 (a gift from Swanson, University of Florida, Gainesville, FL).

In Vitro Modification and Binding

GST-Npl3p phosphorylation using Sky1p (Siebel et al., 1999) and methylation using Hmt1p/Rmt1p with methylation donor S-adenosyl-L-methio-
nine (SAM; Sigma-Aldrich) or with the SAM analogue S-adenosyl-L-homocysteine (SAH; Sigma-Aldrich) (Gary et al., 1996) were done as described. GST pull-down assays were performed as described (Yakley et al., 1999) with the following modifications: binding was done in 200 μl of 30 mM KPO4, pH 6.5, 50 mM KCl, 0.5 mM EDTA, 1 mM DTT. Glutathione sepharose (Amersham Pharmacia Biotech) bound with 1 μg GST-Npl3p was incubated with 2 μl in vitro–translated Mtr10p (Promega) in the presence or absence of sky1Δ cytosolic extract (10 μg total protein). Beads were washed three times in 0.5 mM Hepes, pH 7.6, 200 mM NaCl, 2 mM MgCl2, and 1 mM DTT before SDS-PAGE. For binding studies using purified components, bacterially produced His-S-Mtr10p was purified on a Ni column and equal amounts (~1 μg) of recombinant His-S-Mtr10p and GST-Npl3p were used for in vitro binding under the same conditions described above for in vitro–translated Mtr10p. A filter binding and washing, bound His-S-Mtr10p was detected by Western blotting using the S-tag AP LumiBlot kit (Novagen).

**Results**

**Sky1p Phosphorylates Npl3p at a Single Site in the COOH Terminus**

Npl3p can be efficiently phosphorylated by Sky1p both in vitro (Siebel et al., 1999) and in vivo (Siebel and Guthrie, personal communication). Unlike mammalian SR proteins, which are characterized by continuous SR/RS repeats, Npl3p contains eight SR/RS dipeptides dispersed in its COOH-terminal RGG/RS domain (Fig. 1 a). We first sought to determine the Sky1p phosphorylation site(s) in Npl3p. Our previous peptide selection experiment (Wang et al., 1998) indicated that human SR PK2 strongly selects for arginine (R), histidine (H), glutamic acid (E), and proline (P), and against lysine (K), phenylalanine (F), and glycine (G) around the phosphorylation site (Fig. 1 b). Because Sky1p displays identical substrate specificity to its mammalian counterparts (Siebel et al., 1999), we applied this rule to Npl3p and found that only the most COOH-terminal RS dipeptide fits this consensus (Fig. 1 a). This prediction was tested by mutagenesis. As shown in Fig. 1 c, deletion of the most COOH-terminal sequence (Δ1) or point mutations of the predicted phosphorylation site (S411A) or the consensus sequence (E409K) abolished Npl3p phosphorylation by Sky1p. These data indicate that Sky1p phosphorylates a single site (Ser411) in Npl3p.

**Phosphorylation Defects Cause Npl3p to Accumulate in the Cytoplasm**

This simple pattern of phosphorylation is in contrast to the numerous SR PK sites present in mammalian SR proteins, making budding yeast an ideal system for functional studies of SR PK-mediated phosphorylation. In fact, an important clue to the function of Sky1p in vivo emerged from this mapping study because npl3p(E409K) corresponds to a previously isolated npl3p mutant which mislocalized in the cytoplasm at steady state (Lee et al., 1996; see Fig. 2 b). Thus, Sky1p may function in the regulation of nucleocytoplasmic shuttling and the mutant phenotype associated with npl3p(E409K) may be attributable to a defect in Sky1p-mediated phosphorylation. To further test this hypothesis, we used the GFP-Npl3p fusion protein as described previously (Lee et al., 1996) to examine the localization of additional npl3p phosphorylation mutants. In contrast to the exclusively nuclear localization of GFP-tagged wild-type Npl3p, both phosphorylation mutants GFP-npl3p(S411A) and GFP-npl3p(E409K) mislocalized in the cytoplasm (Fig. 2 b). In the converse experiment, we took advantage of SKY1 being nonessential (Siebel et al., 1999) and found that wild-type GFP-Npl3p was localized in the cytoplasm of sky1Δ yeast (Fig. 2 b). Together, these results demonstrate an important role for Sky1p in the regulation of Npl3p nuclear localization in budding yeast.

**Sky1p Is Required for Efficient Npl3p–Mtr10p Interaction**

Because Npl3p shuttles between the nucleus and the cytoplasm, its accumulation in the cytoplasm could be due to...
impaired nuclear import and/or accelerated export. Although we cannot rule out a potential function of Sky1p in export, an active role for the kinase in nuclear import is consistent with the primary localization of this kinase and all other SRPK family members in the cytoplasm (Takeuchi and Yanagida, 1993; Wang et al., 1998; Siebel et al., 1999). Recently, the nuclear import receptor for Npl3p was identified as Mtr10p, a member of the importin b superfamily (Pemberton et al., 1997; Senger et al., 1998), and deletion of MTR10 resulted in cytoplasmic accumulation of Npl3p. Thus, the simplest explanation for the mislocalization of Npl3p in sky1Δ yeast is that Sky1p-mediated phosphorylation is important for efficient interaction between Npl3p and its nuclear import receptor Mtr10p. We tested this hypothesis by conducting coimmunoprecipitation experiments to compare the association of Npl3p with Mtr10p in wild-type and sky1Δ yeast strains. To facilitate detection, MTR10 was genomically tagged with PrA in these strains (Pemberton et al., 1997). The Mtr10p-PrA fusion protein from yeast cytoplasmic extracts was captured by IgG-Sepharose and associated Npl3p was detected by Western blotting using polyclonal anti-Npl3p antibodies. Npl3p bound Mtr10p-PrA in wild-type yeast as previously reported (Pemberton et al., 1997; Senger et al., 1998), but binding was less efficient in sky1Δ cells (Fig. 3 a; see also Fig. 6 d). Such a reduction in binding between Mtr10p and unphosphorylated Npl3p is significant, considering that more Npl3p molecules are available for Mtr10p binding in the cytoplasm of sky1Δ cells (Fig. 2 b, data not shown). These results demonstrate that Sky1p plays an important role in facilitating the interaction between Npl3p and Mtr10p in vivo and provide a molecular basis for the observation that Sky1p-mediated phosphorylation is required for efficient nuclear localization of Npl3p.

To determine whether Sky1p mediates a direct interaction between the cargo and its import receptor, we carried out in vitro binding studies using a GST-Npl3p fusion protein to pull down in vitro-translated 35S-labeled Mtr10p. We found that phosphorylated Npl3p bound Mtr10p directly (Fig. 3 b, lane 4) with a high affinity (KD = 30 nM, data not shown). Such a high affinity was also reflected by the nearly quantitative capture of input Mtr10p in the binding reactions (Fig. 3, b and c). Similar to the coimmunoprecipitation results, both unphosphorylated GST-Npl3p and the S411A mutant interacted less efficiently with Mtr10p than Sky1p-phosphorylated GST-Npl3p (Fig. 3 b, compare lane 4 with lanes 3 and 5). However, the difference was reproducibly smaller than that observed in vivo, suggesting that an additional mechanism may operate in cells to discriminate between Npl3p phosphorylation states. Indeed, we observed that binding of unphosphorylated GST-Npl3p to Mtr10p was significantly reduced in the presence of a yeast extract while binding of phosphorylated GST-Npl3p remained efficient (Fig. 3 b, lanes 6–9). To provide further evidence that Npl3p interacts directly with Mtr10p, we expressed and purified recombinant histidine- and 5 peptide–tagged Mtr10p, which was then used to interact with GST-Npl3p in different phosphorylation states. As shown in Fig. 3 c, recombinant His-S-Mtr10p bound GST-Npl3p. The binding was modestly affected by phosphorylation (Fig. 3 c, compare lanes 3 and 4) in the absence of the yeast extract, and significantly diminished in the presence of the yeast extract (Fig. 3 c, compare lanes 6 and 7). Together, these results demonstrate that Sky1p plays an important role in facilitating the interaction between Npl3p and Mtr10p. However, our binding reactions were routinely carried out in the presence of excess total yeast RNA, suggesting that an additional mechanism may be responsible for the observed effect of yeast extract on Sky1p-mediated phosphorylation both in vivo and in vitro in the presence of a yeast extract.

The mechanism for the observed effect of yeast extract requires further investigation, although we ruled out several simple scenarios. First, RNA and DNA in the yeast extract might block unphosphorylated Npl3p through ionic interactions, thereby preventing its interaction with Mtr10p. However, our binding reactions were routinely carried out in the presence of excess total yeast RNA, and treatment of the yeast extract with RNase and DNase did not improve the interaction of Mtr10p with unphosphorylated Npl3p (data not shown). Thus, the effect does not seem to be due to RNA or DNA in the yeast extract. Second, a protein factor might bind specifically to unphosphorylated Npl3p to sequester it from contacting Mtr10p. We deleted the COOH-terminal domain containing the Sky1p phosphorylation site, and the mutant protein behaved the same way as unphosphorylated Npl3p (see below). This observation suggests that the sequestering scenario may not be true. Third, we tested both whole cell and cytosolic extracts from wild-type or sky1Δ yeast strains, and observed the same effect. Thus, it is unlikely that endoge-
nous phosphorylated Npl3p in the extracts was in competition with unphosphorylated Npl3p in the in vitro binding assays. Furthermore, we tested E. coli extract or included NP-40 in washing buffer, and under either condition, we detected some reduction of nonspecific binding through free GST, but no effect on phosphorylation-dependent binding (data not shown). Future studies will test whether other Mtr10p cargos unaffected by Sky1p in the extracts compete out unphosphorylated Npl3p, or more interestingly, whether a cofactor cooperates with Mtr10p to enhance and/or stabilize its interaction with phosphorylated Npl3p.

Mtr10p Contacts the RGG Box in Npl3p

The RS domain of mammalian SR proteins is believed to directly engage in protein-protein interactions, and phosphorylation of the RS domain can therefore modulate the affinity between two interacting RS domains. It is unclear, however, whether the COOH-terminal region harboring the last RS dipeptide in Npl3p directly interacts with Mtr10p, which does not contain an RS domain itself. Both the repetitive RGG domain and the COOH-terminal nonrepetitive sequence are required for Npl3p to localize in the nucleus, but neither functions as an autonomous NLS (Senger et al., 1998). How these two types of sequences act together as a functional NLS remains obscure. To determine which of these sequences directly contacts Mtr10p, we carried out in vitro binding studies using a series of npl3p COOH-terminal deletion mutants. In the absence of yeast extract, both unphosphorylated Npl3p and the COOH-terminal deletion mutant Δ11 bound Mtr10p, but further deletion of the RGG domain gradually decreased binding (Fig. 4 a, lanes 2–7). In the presence of yeast extract, however, both unphosphorylated Npl3p and Δ11 mutant retained a low level binding and further deletions eliminated binding (Fig. 4 a, lanes 10–15). These biochemical experiments, together with the genetic evidence described below, suggest that the RGG domain serves as the binding site for Mtr10p and that binding is allosterically regulated by Sky1-mediated phosphorylation at Ser411.

Significantly, our binding studies revealed that the interaction of Mtr10p with the RGG domain of Npl3p decreased gradually as this domain was truncated (Fig. 4 a, lanes 2–7). To determine whether this gradual decrease in binding correlated with a gradual loss of function, we performed a plasmid shuffling experiment using individual deletion mutants to substitute for wild-type Npl3p. Fig. 4 b shows that a point mutation (S411A) and deletion (Δ11) of the Sky1p phosphorylation site in Npl3p had little effect on growth. Although these mutations significantly reduced Mtr10p binding in the presence of yeast extract (Fig. 4 a, lanes 10 and 12), the residual binding was nevertheless sufficient for growth. This observation is also consistent with SKY1 being a nonessential gene under laboratory conditions. However, deletion of some RGG repeats (Δ3) se-
verely impaired growth, but surprisingly, cells were still able to grow slowly at 25°C although not at 37°C, and further deletions (Δ6 and ΔRS) completely inactivated Npl3p function. Therefore, a gradual decrease in M tr10p binding is correlated with a gradual loss of Npl3p function in supporting growth, although the growth defect may result from pleiotropic effects of Npl3p as the function of the RGG domain may not be restricted to nuclear import.

**SKY1 and MTR10 Show a Genetic Interaction**

To provide further in vivo evidence for the functional importance of the interaction between the RGG box and Mtr10p in nuclear import, we reasoned that if the absence of Sky1p-mediated phosphorylation impaired but did not eliminate the Npl3p–Mtr10p interaction as seen in the coinmunoprecipitation and in vitro binding experiments, overexpression of M tr10p might compensate for both inefficient M tr10p binding and nuclear import caused by phosphorylation defects. To examine this possibility, we conducted an overexpression suppression experiment, and found that both the npl3p(S411A) mutant in wild-type yeast and wild-type Npl3p in sky1Δ cells were driven back to the nucleus when M tr10p was overexpressed (Fig. 5 a). A similar observation was also made with the npl3p(E409K) mutant by the Silver laboratory (Kreber et al., 1999). These experiments clearly illustrate the importance of M tr10p binding in Npl3p nuclear import and the regulatory nature of Sky1p-mediated phosphorylation in this process.

Both published work (Pemberton et al., 1997; Senger et al., 1998) and our current results demonstrate that M tr10p functions as the major nuclear import pathway for Npl3p. However, deletion of MTR10 generated a temperature sensitive rather than lethal phenotype (Pemberton et al., 1997). In our hands, deletion of MTR10 resulted in no growth at 37°C (data not shown) but had little effect on growth at 30°C (Fig. 5 b, top right). These observations suggest that Npl3p may also be imported through a separate pathway. Sky1p may also function in this parallel import pathway and/or downstream from the nuclear import step. In either case, simultaneous deletion of both SKY1 and MTR10 would result in a synthetic lethal phenotype. This prediction was confirmed as deletion of both SKY1 and MTR10 prevented cell growth at both 25 (data not shown) and 30°C (Fig. 5 b, top left). These results, coupled with those of the overexpression suppression experiment, clearly establish a genetic interaction between SKY1 and MTR10, and are complementary to the biochemical evidence for the function of Sky1p in nuclear import.

**Arginine Methylation Affects the Npl3p Import Pathway In Vivo**

In addition to Sky1p-mediated phosphorylation, Npl3p is also modified in the RGG domain by arginine methylation (Henry and Silver, 1996; Siebel and Guthrie, 1996), and this modification is mediated by the predominant arginine methyltransferase Hmt1p/Rmt1p in budding yeast (Gary et al., 1996; Henry and Silver, 1996). Interestingly, cytoplasmic localization of Npl3p could be induced by overexpressing HMT1/RMT1 (Fig. 6 a) even though overexpressed Hmt1p/R mt1p was exclusively localized in the nucleus (Henry and Silver, 1996). Because Npl3p continuously shuttles between the nucleus and the cytoplasm, the observed steady state localization of Npl3p in the cytoplasm of sky1Δ yeast could be explained by a positive role of Sky1p in nuclear import and/or a negative function of the kinase in nuclear export. The former appears to be the case based on the evidence presented in this report, and the latter remains a possibility to be addressed. Similarly, the localization of Npl3p in the cytoplasm of HMT1/RMT1-overexpressing cells is consistent with a negative role of Hmt1p/R mt1p in nuclear import and/or a positive function of the arginine methyltransferase in export. Hmt1p/R mt1p was previously shown to play a positive role in Npl3p export (Shen et al., 1998; McBride et al., 2000), but its potential function in nuclear import has not been examined.

Because the import pathway for Npl3p is well characterized, we asked whether overexpression of HMT1/RMT1 could induce Npl3p hypermethylation and whether Hmt1p/R mt1p-induced hypermethylation could exert a negative influence on Npl3p nuclear import through Mtr10p. For these purposes, we transformed wild-type yeast with the HMT1/RMT1 gene under the GAL1 promoter and examined Npl3p methylation using the methylation-specific mAb 1E4 (Wilson et al., 1994; Siebel and Guthrie, 1996). When the expression of HMT1/RMT1 was
induced with galactose (Fig. 6 b), methylation of Npl3p increased over time (Fig. 6 c), indicating that Npl3p became hypermethylated. Consequently, the Npl3p-Mtr10p complexes formed in cells overexpressing Hmt1p/Rmt1p were reduced (Fig. 6 d). These data strongly suggest that arginine methylation plays a negative role in Npl3p nuclear import. It should be pointed out that these data do not argue against a positive role for arginine methylation in Npl3p export, and it is quite plausible that the effect of overexpressed Hmt1p on Npl3p localization (Fig. 6 a) results from a combination of negative impact on import and positive influence on export (see Discussion).

**Arginine Methylation Indirectly Interferes with the Npl3p–Mtr10p Interaction**

To understand the mechanism by which arginine methylation interferes with the Npl3p-Mtr10p interaction in vivo, we asked whether the reduced interaction between Npl3p and Mtr10p was a direct consequence of Hmt1p/Rmt1p-catalyzed arginine methylation of the RGG motifs in Npl3p. In vitro methylation of a series of COOH-terminal deletion mutants revealed that arginine methylation of Npl3p took place in the middle of the RGG domain (Fig. 7 a). Although half of the RGG repeats remain in the Δ6 mutant, it is possible that these repeats are methylated in the full length protein but not in the mutant due to a conformational change. We then tested whether arginine methylation directly interfered with Mtr10p binding. As shown in Fig. 7 b, both unmethylated control and Npl3p treated with saturating methylation conditions bound Mtr10p in the absence (lanes 2-4) or presence (lanes 5-7) of yeast extract. (c) Effect of double modification on Npl3p binding to Mtr10p. GST-Npl3p was either phosphorylated using Sky1p (lanes 4 and 7) or mock phosphorylated (lanes 3 and 6) and then methylated using Hmt1p/Rmt1p. Conversely, the fusion protein was mock methylated (lane 8) or methylated (lane 9) and then phosphorylated. These modified proteins were used in the GST binding assay as in b.
Methylation Antagonizes Phosphorylation

To investigate the mechanism by which Hmt1p/Rmt1p-mediated methylation interferes with phosphorylation-dependent binding, we carried out sequential modifications using the proteins illustrated in Fig. 8 a. Consistent with the binding results, prior phosphorylation with Sky1p or deletion of the Sky1p phosphorylation site (Δ1) had no effect on Hmt1p-mediated methylation of Npl3p (Fig. 8 b, lanes 1 and 3). In contrast, prior methylation with Hmt1p/R mt1p significantly attenuated Sky1p-mediated phosphorylation of Npl3p (Fig. 8 c, lanes 1 and 4). Mock methylation with either Hmt1p (Fig. 8 c, lane 4) or the methylation donor SAM (data not shown) alone did not interfere with Npl3p phosphorylation. To rule out the possibility that Hmt1p/R mt1p binds to Npl3p in the presence of a methylation donor, thereby sequestering Sky1p from binding to Npl3p, we carried out mock methylation in the presence of a SAM analogue, SAH. Clearly, Npl3p mock methylated with SAH was phosphorylated by Sky1p as efficiently as untreated Npl3p (Fig. 8 d). Thus, the effect of methylation on phosphorylation was due to modification on arginine residues.

Because arginine is critical for substrate recognition by the SRPK family of kinases (Colwill et al., 1996b; Wang et al., 1998; Siebel et al., 1999), we investigated the possibility that methylation on arginine residue 410 directly blocked Sky1p-mediated phosphorylation at serine 411. We fused the Sky1p phosphorylation site to Ikβ (Fig. 8 a) so that we could carry out phosphorylation without the adjacent RGG box. We found that phosphorylation of this fusion protein was unaffected by treatment with Hmt1p/R mt1p (Fig. 8 c, lanes 3 and 6). We conclude that Hmt1p/R mt1p-mediated methylation in the RGG domain interferes allosterically with Sky1p-mediated phosphorylation at the COOH-terminal sequence, which in turn regulates the binding of M10p to the RGG domain.

Discussion

Our current results suggest that nuclear import of Npl3p is affected by juxtaposed methylation and phosphorylation (Fig. 9 a). Our finding that Sky1p-mediated phosphorylation plays an important role in nuclear import is consistent with the localization of the kinase in the cytoplasm. Thus, newly synthesized Npl3p may be readily phosphorylated by Sky1p in the cytoplasm for efficient interaction with its nuclear import receptor M10p, which leads to efficient nuclear import, and this modification may also be important for the function of Npl3p in the nucleus. Upon translocation to the nucleus, RanGTP and RNA release Npl3p from the import complex, as previously demonstrated (Senger et al., 1998). The arginine methyltransferase Hmt1p/R mt1p is localized in the nucleus even when it is overexpressed (H enry and Silver, 1996), which may mediate multiple steps important for Npl3p export with RNA as proposed based on genetic studies (Shen et al., 1998). Because Npl3p is localized in the nucleus at steady state, its import
Figure 9. A model for Npl3p nuclear import and export controlled by Sky1p-mediated phosphorylation and Hmt1p-mediated methylation. (a) In wild-type cells, Sky1p in the cytoplasm phosphorylates Npl3p, which is required for its efficient nuclear import by Mtr10p. Imported Npl3p is methylated by Hmt1p/Rmt1p in the nucleus, which may facilitate Npl3p export. Methylation of Npl3p interferes with Sky1p-mediated phosphorylation, thereby indirectly inhibiting Npl3p import. The size of the arrows indicates relative transport efficiency, and shaded regions indicate Npl3p localization at steady state. (b) Impaired nuclear import of wild-type Npl3p in the nucleoporin mutant nup49–313 strain (based on data from Lee et al., 1996). (c) Impaired nuclear import of the phosphorylation mutant npl3p(E409K) in wild-type yeast. (d) Deletion of HMT1/RMT1 improves import and impairs export of Npl3p in the nup49–313 strain. (e) Import and export are affected by Npl3p mutation in the phosphorylation site and inactivation of Hmt1p/Rmt1p, respectively (our observation, and that reported by McBride et al., 2000).

Our results also extend previous genetic data regarding the function of methyltransferase Hmt1p/Rmt1p in Npl3p nucleocytoplasmic shuttling. As reported previously (Lee et al., 1996), wild-type GFP-Npl3p accumulated in the cytoplasm of nucleoporin mutant (nup49–313) cells when nuclear import was impaired (Fig. 9 b, small arrow) while nuclear export proceeded normally (Fig. 9 b, medium arrow). In the present studies, we found that phosphorylation defects also caused the accumulation of mutant npl3p in the cytoplasm of wild-type yeast (see Fig. 2; illustrated in Fig. 9 c) due to its inefficient interaction with Mtr10p. The function of Hmt1p/Rmt1p became evident when the HMT1/RMT1 gene was deleted or inactivated in these genetic backgrounds (Fig. 9 d and e). When HMT1/RMT1 was deleted in the nup49–313 strain, wild-type GFP-Npl3p quantitatively relocated to the nucleus, as reported previously (Shen, et al., 1998). The observation was previously interpreted to indicate that nuclear export of GFP-Npl3p was impaired in the absence of Hmt1p/Rmt1p (Fig. 9 d, small arrow). However, considering the possibility that nuclear import in nup49–313 cells was impaired but not completely blocked at the restrictive temperature, redistribution of Npl3p to the nucleus of hmt1/rmt1Δ cells could also be contributed by the removal of the methylation interference of phosphorylation as shown in this study. As a result, improved import (Fig. 9 d, medium arrow) was concomitant with impaired export in the absence of Hmt1p/Rmt1p, which together caused efficient shift of Npl3p back into the nucleus.

According to this model, one would predict that the improvement of nuclear import could not occur if the Sky1p phosphorylation site is mutated. Indeed, we observed that GFP-npl3p(S411A) expressed from a plasmid under the GAL1 promoter remained in the cytoplasm of hmt1/rmt1Δ cells (data not shown). Consistently, endogenously expressed npl3p(E409K) only partially shifted back to the nucleus when Hmt1p/Rmt1p was inactivated (McBride et al., 2000). Thus, the observed distribution of the mutant npl3p in both the cytoplasm and the nucleus is likely due to defects in both import and export pathways (indicated by small arrows in both directions in Fig. 9 e). Together, these observations in combination with biochemical evidence presented in this report lend strong support for a positive role for the major arginine methyltransferase in Npl3p export and a negative role for this enzyme in Npl3p import in budding yeast.

The model presented in Fig. 9 raises several fundamental questions with regard to the functional consequences of phosphorylation and arginine methylation: (a) Which components of this regulatory pathway are conserved in mammalian cells? (b) Are phosphatases and demethylases involved in the pathway? and (c) Does this regulatory pathway represent some of the key steps in controlling the function of shuttling RNA binding proteins? Below, we discuss the significance of our novel findings in the context of these global questions.

The NLS in Npl3p is composed of both repetitive (RGG repeats) and nonrepetitive (Sky1p phosphorylation site) sequences. Interestingly, these sequence features are conserved in two separate classes of RNA binding proteins (hnRNP and SR proteins) in mammalian cells (Burd and Dreyfuss, 1994; Fu, 1995). Many hnRNP proteins shuttle between the nucleus and the cytoplasm, but their transport appears to be mediated by separate signal sequences adjacent to their RGG domains (Nakielny and Dreyfuss, 1997). Although hnRNP proteins are extensively modified by arginine methylation in mammalian cells (Liu and Dreyfuss, 1995), how methylation might modulate their transport signals remains to be addressed. The R5 domain in SR proteins is critical for nuclear and subnuclear localization (Li and Bingham, 1991; Hedley et al., 1995; Cáceres, et al., 1997). It was recently shown that SR pro-
proteins in mammalian cells interact with two Mtr10p-related receptors called transportin-SR (Kataoka et al., 1999) and transportin-SR 2 (Lai et al., 2000). Thus, the transport machinery for SR and SR-like proteins, including nuclear import receptors (transportin-SR, transportin-SR 2 and Mtr10p) and regulators (SRPKs), are conserved between yeast and humans. However, the yeast and metazoan receptors have evolved distinct substrate specificities because Mtr10p contacts the RGG box in Npl3p whereas transportin-SR interacts with the RS domain containing SR/RS instead of RGG repeats. Furthermore, it appears that transportin-SR can interact with unphosphorylated SR proteins in vitro, although potential phosphorylation regulation of the interaction was not addressed (Kataoka et al., 1999). On the other hand, the interaction between SR proteins and transportin-SR 2 was shown to be dependent on SRPK-mediated phosphorylation (Lai et al., 2000). Future studies will define the signal sequences for nuclear import of SR proteins and address how their nuclear import might be regulated by SRPKs in mammalian cells.

Phosphorylation of SR proteins is required for spliceosome assembly and dephosphorylation is critical for the splicing reaction to occur (Mermoud et al., 1994). Npl3p and other Sky1p substrates may play a role in pre-mRNA splicing in budding yeast, but it is not known whether a phosphorylation–dephosphorylation cycle accompanies their function in the nucleus. In contrast to reversible phosphorylation, the reversibility of arginine methylation remains highly controversial. It is clear that arginine methylation is rather stable (Desjardins and Morrell, 1983). It has been argued that demethylation may be absent because the reaction to break the N–C bond would be energetically unfavorable and a potential arginine demethylase has not been found. In the absence of a specific arginine demethylase, we speculate that the stable arginine modification may serve as a mechanism to allow shutting Npl3p sufficient time in the cytoplasm to unload its RNA cargo. Whether Sky1p-mediated phosphorylation, although inefficient on methylated Npl3p, serves as a molecular switch for unloading and reimport is another interesting possibility to be investigated. Considering the irreversibility of this modification, our data clearly demonstrated that methylation is not saturated on Npl3p, indicating that arginine methylation may be more dynamic than previously assumed. Since Npl3p is largely cytoplasmic when hypermethylated, it is possible that nuclear import of Npl3p may gradually decrease as methylation gradually accumulates. Thus, methylation may function as a molecular device to determine how many cycles each Npl3p molecule can shuttle between the nucleus and cytoplasm before it is degraded in the cytoplasm. Further experiments will test this interesting possibility.

Continuous shuttling of RNA binding proteins may not only reflect their function in transporting materials out of the nucleus, but also represent a mechanism to regulate their function in the nucleus. For example, hnRNAP A 1 and SR proteins can switch splice site selection in opposite ways in a concentration-dependent manner (for reviews see Fu, 1995; Manley and Tacke, 1996; Cáceres and Krainer, 1997). Therefore, the ratio of hnRNAP and SR proteins in the nucleus may be critical for specific alternative splicing events. Modification of hnRNAP proteins by arginine methylation and SR proteins by phosphorylation may effectively control their trafficking and therefore their ratio in the nucleus during development or in response to external stimuli. Our results using yeast as a genetic system have revealed a novel function of the conserved SRPK in regulated nuclear import and established a framework to investigate the function of the SRPK family members in mammalian cells.

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