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) 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; Kozhtz et al., 1994) to the conversion of the prespliceosome to the spliceosome (Roscigno and García-Blanco, 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 (Ayane 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 interactions (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...
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 (SA M; Sigma-Aldrich) or with the SA M analogue S-adenosyl-
L-homocysteine (SA H; 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 KPO 4, pH 6.5, 50 mM KCl, 0.5 mM EDTA, 1 mM DTT, Glu-
tathione sepharose (Amersham Pharmacia Biotech) bound with 1 µg
GST-Np1p3 was incubated with 2 µl in vitro-translated M tr10p (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 MgCl 2, and 1 mM DTT before SDS-PA GE. 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-Np1p3 were used for in vitro binding under the same conditions
described above for in vitro-translated Mtr10p. A 1:1 ratio and washing,
bound His-S-Mtr10p was detected by Western blotting using the S-tag
A P LumiBlot kit (Novagen).

Plasmid Shuffling and Synthetic Lethal Test

Strain M H Y 132 (NPL3-deleted strain covered with wild-type NPL3 in a
URA plasmid) was transformed with pM H Y 3, a CEN-LEU plasmid con-
taining wild-type and mutant npl3. These U le+ U ra+ transformants were
streaked on 5-FOA minus Leu plates, and growth was monitored at 25
and 37°C. To test synthetic lethality, Y C 52 (sky1 Δ ) was transformed with
pR S316-SKY1. M TR10 was then deleted from the strain using a kanamy-
cin resistance expression unit flanked by MTR10 genomic sequences
(Wach et al., 1994). MTR10 deletion was confirmed by PCR, and yeast
were streaked on 5-FOA and monitored for growth at 25 and 30°C.

Results

Sky1p Phosphorylates Npl3p at a Single Site in the
COOH Terminus

Np1p3 can be efficiently phosphorylated by Sky1p both in
vitro (Siebel et al., 1999) and in vivo (Siebel and Guthrie,
personal communication). Unlike mammalian SR pro-
teins, which are characterized by continuous SR/RS re-
deeses dispersed in its COOH-terminal RGG/RS domain (Fig. 1 a). We first
sought to determine the Sky1p phosphorylation site(s) in
Np1p3. Our previous peptide selection experiment (Wang
et al., 1998) indicated that human SRPK2 strongly selects
for arginine (R), histidine (H), glutamic acid (E), and pro-
ine (P), and against lysine (K), phenylalanine (F), and gly-
cine (G) around the phosphorylation site (Fig. 1 b). Be-
cause Sky1p displays identical substrate specificity to its
mammalian counterparts (Siebel et al., 1999), we applied
this rule to Np1p3 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
Np1p3 phosphorylation by Sky1p. These data indicate that
Sky1p phosphorylates a single site (Ser411) in Np1p3.

Phosphorylation Defects Cause Np1p3 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 stud-
ies of SR PK-mediated phosphorylation. In fact, an impor-
tant 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

Figure 1. Sky1p phosphorylates a single site in Np1p3. (a) Se-
quence of the RGG/RS domain in Np1p3. RGG repeats and SR/
RS dipeptides are underlined and in bold, respectively. Dele-
tion mutants are named according to the number of SR/RS dipeptide
removed, and specific point mutations in the deduced phosphory-
layers site are indicated. (b) Phosphorylation consensus deduced
from in vitro peptide selection using human SRPK2 (Wang et al.,
1998). (c) Wild-type and mutant Np1p3 were expressed as GST
fusion proteins (Coomassie stain, top) and tested for phosphor-
hylation with recombinant Sky1p ( Autoradiograph, bottom).

the cytoplasm at steady state (Lee et al., 1996; see Fig. 2 b).
Thus, Sky1p may function in the regulation of nucleiocyto-
plasmic shuttling and the mutant phenotype associated with
npl3p(E409K) may be attributable to a defect in Sky1p-mediated phosphorylation. To further test this hy-
thesis, we used the GFP-Np1p3 fusion protein as de-
scribed previously (Lee et al., 1996) to examine the local-
ization of additional npl3p phosphorylation mutants. In
contrast to the exclusively nuclear localization of GFP-
tagged wild-type Np1p3, both phosphorylation mutants
GFP-npl3p(S411A) and GFP-npl3p(E409K) mislocalized in
the cytoplasm (Fig. 2 b). In the converse experiment, we
ook advantage of SKY1 being nonessential (Siebel et al.,
1999) and found that wild-type GFP-Np1p3 was localized in
the cytoplasm of sky1 Δ yeast (Fig. 2 b). Together, these
results demonstrate an important role for Sky1p in the
regulation of Np1p3 nuclear localization in budding yeast.

Sky1p Is Required for Efficient
Np1p3–Mtr10p Interaction

Because Np1p3 shuttles between the nucleus and the cyto-
plasm, 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. 2 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, c and d). 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 reproduced 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.

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 and sky1Δ yeast strains, and observed the same effect. Thus, it is unlikely that endo-
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 non-repetitive 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 Δ1 bound Mtr10p, but further deletion of the RGG domain gradually decreased binding (Fig. 4a, lanes 2–7). In the presence of yeast extract, however, both unphosphorylated Npl3p and Δ1 mutant retained a low level binding and further deletions eliminated binding (Fig. 4a, 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 Sky1p-mediated phosphorylation at Ser411.

**Figure 3.** Sky1p-mediated phosphorylation plays an important role in facilitating Npl3p–Mtr10p interaction. (a) Coimmunoprecipitation between Npl3p and Mtr10p. Immunoprecipitation (IP) was done with IgG-Sepharose beads and analyzed by Western blotting using rabbit polyclonal anti-Npl3p antibodies and IgG to detect Npl3p and Mtr10-PrA, respectively. Quantification of these coimmunoprecipitation results revealed a threefold reduction of Npl3p binding to Mtr10p-PrA in sky1Δ yeast. (b) In vitro GST binding assay. Wild-type and mutant (S411A) GST-npl3p were phosphorylated (+P) using Sky1p or mock phosphorylated (−P), and used to pull down in vitro–translated 35S-labeled Mtr10p in the absence (lanes 2–5) or presence (lanes 6–9) of cytosolic yeast extract. (c) In vitro binding using purified components. The binding was done as in b, except that equal amounts (1 μg) of purified recombinant GST-Npl3p and His-S-Mtr10p were used. Bound His-S-Mtr10p was detected with alkaline phosphatase–conjugated S-protein.

**Figure 4.** Mtr10p interacts with the RGG box in Npl3p. (a) Deletion mapping of the Mtr10p interaction domain in Npl3p. Binding was done as in the legend to Fig. 3b using deletion mutants illustrated in Fig. 1a. (b) Plasmid shuffling experiments. An npl3Δ strain covered by wild-type NPL3 (wt) on a Ura+ plasmid was transformed with Leu+ plasmids containing wild-type or mutant npl3. Transformants were streaked on 5-FOA minus Leu plates and incubated at 25 or 37° C.
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 M tr10p in nuclear import, we reasoned that if the absence of Sky1p-mediated phosphorylation impaired but did not eliminate the Npl3p–M tr10p interaction as seen in the coimmunoprecipitation 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 into 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 (Krebber 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°C (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 box 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/Rmt1p 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/Rmt1p in nuclear import and/or a positive function of the arginine methyltransferase in export. Hmt1p/Rmt1p 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/Rmt1p-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 \textit{HMT1} 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 \textit{HMT1} on Npl3p nuclear import (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 methyla-
tion 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 \Delta6 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 indistinguishably (lanes 3 and 4), suggesting that methylation alone does not affect the Npl3p–Mtr10p interaction. We noted, however, that the interaction of Npl3p with Mtr10p was significantly reduced in the presence of yeast extract, regardless of its methylation state (Fig. 7 b, lanes 6 and 7), indicating that phosphorylation might be a prerequisite for a potential methylation effect on Npl3p binding to Mtr10p.

To test this possibility, we investigated the effect of both modifications. When Npl3p was phosphorylated first with Sky1p, methylation had no effect on its efficient interaction with Mtr10p either in the presence or absence of yeast extract (Fig. 7 c, lanes 4 and 7). Surprisingly, when Npl3p was first methylated with Hmt1p/Rmt1p, further treat-
ment with Sky1p failed to enhance its interaction with Mtr10p (Fig. 7 c, lane 9). These results suggest that Hmt1p/Rmt1p-mediated methylation may interfere with the Npl3p–Mtr10p interaction through an indirect mechanism.

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/Rmt1p 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/Rmt1p 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 Iκb (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/Rmt1p (Fig. 8 c, lanes 3 and 6). We conclude that Hmt1p/Rmt1p-mediated methylation in the RGG domain interferes allosterically with Sky1p-mediated phosphorylation at the COOH-terminal sequence, which in turn regulates the binding of Mtr10p 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 Mtr10p, 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/Rmt1p 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
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(E409K) 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 (hnRNPs and SR proteins) in mammalian cells (Burd and Dreyfuss, 1994; Fu, 1995). Many hnRNPs 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). A though hnRNPs 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 RGG 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 Mr10p-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 Mr10p) and regulators (SRPKs), are conserved between yeast and humans. However, the yeast and metazoan receptors have evolved distinct substrate specificities because Mr10p 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 (Mermod 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 Morell, 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 demonstrate 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, hnRNP A1 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 hnRNP and SR proteins in the nucleus may be critical for specific alternative splicing events. Modification of hnRNP 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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References

A yane, M., U. Preuss, G. Kohler, and P. J. Nielsen. 1991. A differentially expressed murine RNA encoding a protein with similarities to two types of nucleic acid binding motifs. Nucleic Acids Res. 19:1273–1278.

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

Cao, W., S. F. Jamison, and M. A. Garcia-Blanco. 1997. Both phosphorylation and dephosphorylation of an SF/SF 2 are required for pre-mRNA splicing in vitro. Nucleic Acids Res. 3:1456–1467.

Colwill, K., T. Pawson, B. A d w e r d s, J. P r a s a d, J. L. M a n l e y, J. C. Bell, and P. J. Dunc an. 1996. The C l k 1/S k y protein kinase phosphorylates SR splicing factors and regulates their intracellular distribution. EMBO J. 15:265–275.

Desjardins, K. C., and P. M o r e l. 1983. Phosphate groups modifying myelin basic proteins are metabolically labile, methyl groups are stable. J. Biol. Chem. 258:438–446.

Diamond, R. H., K. D u, V. M. L e e, K. L. M o o n, B. A. H a b e r, D. S. T e w a r i, and R. Taub. 1993. Novel delayed-early and highly insulin-induced growth response genes. Identification of H R S, a potential regulator of alternative pre-mRNA splicing. J. Biol. Chem. 268:15185–15192.

Duncan, P. J., D. F. Stojdl, R. M. M a r i u s, and J. C. Bell. 1997. In vivo regulation of alternative pre-mRNA splicing by the Clk1 protein kinase. Mol. Cell. Biol. 17:5996–6001.

Fu, X.-D. 1995. Specific commitment of different pre-mRNA splicing factors by single SR proteins. Nature. 365:82–85.

Fu, X.-D. 1995. The superfamily of arginine-serine rich splicing factors. RNA. 1:63–68.

Gary, J. D., J. W. L i n, M. C. Y a n g, H. R. H e r s c h m a n, and S. C l a r k e. 1996. The predominant protein–arginine methyltransferase from Saccharomyces cerevisiae. J. Biol. Chem. 271:12585–12594.

Gui, J. F., W. S. L a n e, and X.-D. Fu. 1994a. A serine kinase regulates intracellular localization of splicing factors in the cell cycle. Nature. 369:678–682.

Gui, J. F., H. Tronchere, S. D. C h a n d l e r, and X.-D. Fu. 1994b. 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.

Hedley, M. L., H. A m r e i n, and T. M a n i a t i s. 1995. A named acid sequence motif sufficient for nuclear localization of an arginine-serine rich splicing factor. Proc. Natl. Acad. Sci. USA. 92:11524–11528.

Henry, M. F., and P. A. S i l v e r. 1996. A novel methyltransferase (H m t1p) modi-
flies poly(A)-RNABinding proteins. Mol. Cell. Biol. 16:3668–3678.
Kadowaki, T., S. Chen, M. Hiromi, E. Jacobs, C. Kumagai, S. Liang, R.
Schneider, D. Singleton, J. Wisniewska, and A.M. Tartakoff. 1994. Isolation and
characterization of Saccharomyces cerevisiae mRNAs transport-defective (mtr)
mutants. J. Cell Biol. 126:649–659.
Karn, J., G. Vidal, L.C. Beffa, and V.G. A lifeff. 1977. Characterization of the
non-histone nuclear proteins associated with rapidly labeled heterogeneous
nuclear RNA. J. Biol. Chem. 252:7307–7322.
Kataoka, N., J.L. Bachorik, and G. Dreyfuss. 1999. Transportin-SR, a nuclear
import receptor for SR proteins. J. Cell Biol. 145:1145–1152.
Kohut, J.D., S.F. Jamison, C.L. Will, Z. Zuo, R. Luhrmann, M.A. Garcia-
Blanco, and J.L. Manley. 1994. Protein-protein interactions and 5′-splice-
site recognition in mammalian mRNAs precursors. Nature. 368:119–124.
Krebber, H., T. Taura, M.S. Lee, and P.A. Silver. 1999. Uncoupling of the
hnRNPNpl3p from mRNAs during the stress-induced block in mRNA ex-
port. Genes Dev. 13:1994–2004.
Krejc, A.M., and M.S. Swanson. 1999. hnrNPC complexes: composition, structure,
and function. Curr. Opin. Cell Biol. 11:363–371.
Lai, M.-C., R.-I. Lin, S.-Y. Huang, C.-W. Tsai, and W.-Y. Tarn. 2000. A human
importin-β family protein, Transportin-SR2, interacts with the phosphory-
lated RS domain of SR proteins. J. Biol. Chem. 275:7950–7957.
Lee, M.S., M.F. Henry, and P.A. Silver. 1996. A protein that shuttles between
the nucleus and the cytoplasm is an important mediator of RNA export.
Genes Dev. 10:1233–1246.
Lemaire, R., A. Winne, M. Sarkissian, and R. Lafyatis. 1999. SF2 and SRp55
regulation of CD45 exon 4 skipping during T cell activation. Eur. J. Immu-
nol. 29:823–837.
Li, H., and P.M. Bingham. 1991. Arginine/serine-rich domains of the su(wa)
and tra RNA processing regulators target proteins to a subnuclear compart-
ment implicated in splicing. Cell. 67:335–342.
Liu, Q., and G. Dreyfuss. 1995. In vivo and in vitro arginine methylation of
RNA binding proteins. Mol. Cell. Biol. 15:2800–2808.
Manley, J.L., and R. Tacke. 1996. SR proteins and splicing control. Curr. Opin.
Mol. Biol. 9:420–429.
M. Bellini, J.C. Lacroix, G. Monod, and G. Dreyfuss. 1993. hnRNPG: se-
quence and characterization of a glycosylated RNA-binding protein.
Mol. Cell. Biol. 13:1994–2004.
McBride, A.E., V.H. Weiss, H.K. Kim, J.M. Hogle, and P.A. Silver. 1999. Analysis of
the yeast arginine methyltransferase Hmt1p/Rmt1p and its in vivo function.
EMBO J. 18:6991–7000.
Roscigno, R.F., and M.A. Garcia-Blanco. 1995. SR proteins escort the U4/
U6.U5 tri-snRNP to the spliceosome. RNA. 1:692–706.
Screaton, G.R., J.F. Caceres, A. Mayeda, M.V. Bell, M. Plebanski, D.G. Jack-
son, J.J. Bell, and A.R. Kainer. 1995. Identification and characterization of
three members of the human SR family of pre-mRNA splicing factors.
EMBO J. 14:4336–4346.
Senger, B., G. Simos, F.R. Bischoff, A. Podtelejnikov, M. Mann, and E. Hurt.
1998. Mtr10p functions as a nuclear import receptor for the mRNAs binding
protein Npl3p. EMBO J. 17:2196–2207.
Shen, E.C., M.F. Henry, V.H. Weiss, S.R. Valentin, P.A. Silver, and M.S. Lee.
1996. An arginine methylation facilitates the nuclear export of hnrNP proteins.
Genes Dev. 12:679–691.
Siebel, C.W., and C. Guthrie. 1996. The essential yeast RNA binding protein
Npl3p is methylated. Proc. Natl. Acad. Sci. USA. 93:13641–13646.
Siebel, C.W., L. Feng, C. Guthrie, and X.-D. Fu. 1999. Conservation of a yeast
protein kinase specific for the SR family of splicing factors. Proc. Natl. Acad.
Sci. USA. 96:5440–5445.
Souland, M., B. Dela Valle, M.C. Siomi, S. Pinol-Roma, C. Bauvy, M. Bellini, J.C.
Lacroix, G. Monod, and G. Dreyfuss. 1993. hnRNPG: sequence and characteriza-
tion of a glycosylated RNA-binding protein. Nucleic Acids Res. 21:4210–4217.
Takeuchi, M., and M. Yagamida. 1993. A mitotic role for a novel fission yeast
protein kinase in cell cycle stage dependent phosphorylation and local-
ization. Mol. Biol. Cell. 4:247–260.
Tarn, W.Y., and J.A. Steitz. 1995. Modulation of 5′ splice site choice in pre-
messenger RNA by two distinct steps. Proc. Natl. Acad. Sci. USA. 92:2504–
2508.
Valcarcel, J., and M.R. Green. 1996. The SR protein family: pleiotropic func-
tions in pre-mRNA splicing. Trends Biochem. Sci. 21:296–301.
Valcarcel, J., R.K. Gaur, R. Singh, and M.R. Green. 1996. Interaction of
U2A F65 RS region with pre-mRNA branch point and promotion of base pari-
ing with U2 snRNA. Science. 273:1706–1709.
Wach, A., A. Brachat, R. Pohlmann, and P. Philippson. 1994. New heterologous
modules for classical or PCR-based gene disruptions in Saccharomyces cere-
visiae. Yeast. 10:1793–1808.
Wang, H.-Y., W. Lin, J.A. Dyck, J.M. Y. Oakley, Z. Songyang, L.C. Cantley, and
X.-D. Fu. 1998. SRPK2: a differentially expressed SR protein-specific kinase
involved in mediating the interaction and localization of pre-mRNA splicing
in mammalian cells. J. Cell Biol. 140:737–750.
Wilson, S.M., K.V. Datar, M.R. Paddy, J.R. Swedlow, and M.S. Swanson.
1994. Characterization of nuclear polyadenylated RNA-binding proteins in
Saccharomyces cerevisiae. J. Cell Biol. 127:1173–1184.
Wu, J.Y., and T. Maniatis. 1993. Specific interactions between proteins impli-
cated in splice site selection and regulated alternative splicing. Cell. 75:1061–
1070.
Xiao, S.-H., and J.L. Manley. 1997. Phosphorylation of the ASF/SF2 RS domain
affects both protein-protein and protein-RNA interactions and is necessary
for splicing. Genes Dev. 11:334–344.
Yakley, J.M., H. Tronchere, J. Olesen, J.A. Dyck, H.-Y. Wang, and X.-D. Fu.
1999. Phosphorylation regulates in vivo interaction and molecular targeting of
serine/arginine-rich pre-mRNA splicing factors. J. Cell Biol. 145:447–455.
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