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Conservation in budding yeast of a kinase specific for SR splicing factors

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ABSTRACT **SR protein kinases (SRPKs) and their substrates, the SR family of serine/arginine-rich pre-mRNA splicing factors, appear to be key regulators of alternative splicing. Although SR proteins have been well characterized through biochemical experiments in metazoans, their functions *in vivo* are unclear. Because of the strict splice site consensus and near absence of alternative splicing in *Saccharomyces cerevisiae*, it had been thought that budding yeast would lack an SRPK and its substrates. Here, we present structural, biochemical, and cell-biological evidence that directly demonstrates an SR protein kinase, Sky1p, as well as a number of SRPK substrates in *S. cerevisiae*. One of these substrates is Npl3p, an SR-like protein involved in mRNA export. This finding raises the provocative possibility that Sky1p, and by extension metazoan SRPKs, regulates mRNA export or the nucleocytoplasmic shuttling of RS domain proteins. The unexpected discovery of an SR protein kinase in budding yeast provides a foundation for genetic dissection of the biological functions of SR proteins and their kinases.**

SR proteins, which contain a defining domain rich in SR/RS dipeptides, have received intensive experimental attention for their critical functions in metazoan cells (reviewed in refs. 1–3). *In vitro*, they are required at multiple steps in the assembly of the spliceosome, the dynamic RNA–protein complex that catalyzes intron removal. Most importantly, SR proteins are thought to play a key role in defining splice sites, which are characterized by degenerate consensus sequences in metazoans. Changes in SR protein concentrations can determine which competing splice sites are selected, suggesting that SR proteins function as key regulators of gene expression by controlling alternative splicing patterns.

SR proteins are characterized by one or two N-terminal RNA recognition motifs for RNA binding and a C-terminal RS domain, loosely defined as multiple continuous SR/RS repeats. Such RS domains have been noted in many other splicing factors and regulators and are thus thought to be indicative of an RNA processing function (1). In SR proteins, the RS domain is essential for splicing activity *in vitro*. However, unlike the RNA recognition motif domains, which appear to function in a coordinated manner to determine distinct splicing specificities of individual SR proteins (4), RS domains from different proteins can be interchanged with little effect on splicing activity (4) and cell viability (5). Together with the observation that RS domains function in part as interfaces for protein interactions (6–9), this result suggests that RS domains may be regarded as analogous to activation domains in transcription factors (10).

Extensive serine phosphorylation is a hallmark of RS domains, making SR proteins compelling potential targets for splicing regulation via reversible phosphorylation. Indeed, cycles of phosphorylation and dephosphorylation are required for splicing, and RS domains likely comprise at least some of the relevant phosphorylation substrates (11–16). Key to testing such models is a molecular understanding of RS domain functional mechanisms as well as of specific kinases and phosphatases that catalyze RS domain modifications.

Two mammalian kinase families [SR protein kinase (SRPK) and Clk/Sty] specifically phosphorylate RS domains (12, 17–19), with several consequences. First, phosphorylation by either kinase family has been shown to increase the affinity of some protein–protein interactions mediated by RS domains (16, 19). Second, RS domains bind RNA nonspecifically, likely because of their high content of positive charges (16, 20, 21), and phosphorylation appears to reduce such binding (16, 21). Third, certain RS domains possess intracellular localization signals that direct SR proteins to the nucleus and subnuclear speckles, where many splicing factors are concentrated. In addition, RS domains direct the nucleocytoplasmic shuttling of a subset of SR proteins (22–26). Supporting the notion that phosphorylation affects some of these localization signals, overexpression of SR protein kinases causes redistribution in the nucleus of SR proteins and other splicing factors (12, 17, 19), perhaps reflecting phosphorylation-dependent recruitment of SR proteins to nascent transcripts for cotranscriptional splicing (27). Together, these observations suggest that SR protein kinases may function through multiple mechanisms, including regulating the intracellular localization of RS domain-containing proteins as well as their interactions with each other or with RNA.

Although studies of SR protein kinases have provided key insights into the functions and regulation of SR proteins, a thorough understanding of how SR proteins and their kinases function, particularly *in vivo*, has been restricted by the lack of genetic analyses. Some progress has been achieved by exploiting the fission yeast, *Schizosaccharomyces pombe*. The *S. pombe PRP*4 gene, which encodes a serine/threonine kinase that can phosphorylate a human RS domain *in vitro*, is thought to function in splicing because *prp*4 mutations reduced splicing efficiency (28, 29). Moreover, fission yeast expresses an SRPK family member, Dsk1 (30, 31), as well as a putative SR protein family member, Srp1 (28). The more extensive repertoire of biochemical and genetic reagents in *Saccharomyces cerevisiae* available to study splicing makes this organism a prime candidate for *in vivo* studies of SR proteins and their kinases. Yet, complete sequencing of the yeast genome has failed to reveal proteins with the explicit characteristics of the SR family described in numerous metazoan species. The conservation of the basic splicing machinery but not the SR protein family might be explained by the stringent consensus of splice site sequences and near absence of alternative splicing in budding yeast, rendering SR proteins dispensable. Alternatively, yeast

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Abbreviations: SRPK, SR protein kinase; NES, nuclear export signal.

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may express functional equivalents of metazoan SR proteins and kinases that are less conserved at the sequence level.

We reasoned that, if SR proteins and their modifying enzymes indeed function in fundamental aspects of eukaryotic mRNA processing, their functional relatives should be present in budding yeast. As a way to identify such proteins, we asked whether yeast contains substrates for human SRPK1, which phosphorylates RS domain-containing proteins in a highly specific manner (19, 32, 33). Our data show that yeast not only have a number of such substrates, including the Npl3 RNA binding protein previously implicated in mRNA export, but also express an SRPK family member. This discovery of a conserved SR protein kinase in budding yeast establishes a powerful genetic system to study the function of SRPKs and their substrates *in vivo* and suggests that they may function more broadly in mRNA maturation than previously anticipated.

MATERIALS AND METHODS

Cloning of *SKY1*. Two pairs of degenerate oligonucleotides [5′-AAGTTNGGNTGGGGNCAC(T)TTTNNNAC(T)GTNTGGTT-3′ and 5′-CCNAGNACTTCGAANACCATGCANAT(C)GTG-3′; 5′-CAC(T)GTNTGC(T)ATGGTNTTTGAGGTNTTNGG-3′ and 5′-AGNATGTTTTCNGGTTTNANGTCNGTGTG-3′] corresponding to kinase domains III and VI (see ref. 12) were used in the PCR reactions described in the text. The PCR fragment was used as a probe to screen a yeast genomic library (YCP50, a gift from S. Emr, Univ. of California, San Diego) by hybridization. Seven positive clones all matched an SRPK1-like gene found in cosmid 8216 from the *S. cerevisiae* genome database.

Expression of Sky1p. DNA encoding the FLAG epitope in-frame with the N terminus of Sky1p was generated by using a pair of oligonucleotides in PCR reactions (63-mer: 5′-GCGCGAAGCTTCACCATGGACTACAAAGACGATGACGATAAAGCCATGGGTTCATCAATTAAC-3′, which contains a *Hin*dIII site, FLAG epitope, and the N-terminal *SKY1* coding region, and a downstream primer 5′-CTCAACATCTCCAATCTCC-3′ from the internal *Hin*dIII site in *SKY1*). The PCR fragment was cut with *Hin*dIII and then was ligated with the *SKY1 Hin*dIII/*Nde*I fragment into pYES2 (Invitrogen) digested with *Hin*dIII and *Eco*RI (*Nde*I and *Eco*RI sites were filled). To express glutathione *S*-transferase–Sky1p in bacteria, two primers (5′-AACATTAATGGGTCGACCAATTAAC-3′ and 5′-TTATGATCGCG GAGCTCTTCAAACC-3′) containing *Sal*I and *Sac*I sites, respectively, were used to amplify *SKY1* by using PCR. This fragment was subcloned into the *Sal*I and *Sac*I sites in a modified pGEX-2T vector (Amersham Pharmacia). Glutathione *S*-transferase–Sky1p was expressed and purified according to previous protocols (Amersham Pharmacia).

Drosophila SRp55/B52 (a gift from Mark Roth, Fred Hutchinson Cancer Research Center, Seattle) was expressed in yeast from the galactose-inducible *GAL1* promoter. SRp55/B52 was purified from yeast-splicing extract by using precipitation with ammonium sulfate and magnesium chloride as described for mammalian extracts (34). Yeast extracts without SRp55/B52 also were fractionated by using the magnesium chloride precipitation alone.

Mutagenesis. *Construction of the ATP binding site mutant (*sky1 K-M*).* A pair of primers (5′-C AAT CAT CAT AGC CAC GTG AGT GTT GTT T-3′ and 5′-A AAC AAC ACT CAC GTG GCT ATG ATG ATT G-3′) were designed for PCR-based site-directed mutagenesis. Both primers contain a Lys-to-Met mutation in the predicted ATP binding site (amino acid 187) as well as a Pml I site for cloning and diagnosis. These two primer were used in PCR reactions in conjunction with an upstream and a downstream primer, respectively.

*Construction of the spacer deletion mutant (*sky1Δ*).* The amino half (F1) of the *SKY1* kinase domains from both wild-type *SKY1* and the *sky1 K-M* mutant from pYES2-*SKY1* was generated by PCR amplification with the 63-mer N-terminal oligo described above and a primer (5′-GAGATTGTCGACGTTGAG-3′) corresponding to the kinase–spacer junction region. PCR products were digested with *Kpn*I and *Sal*I and were subcloned into pSP73. Similarly, two oligos (5′-ACTCCCTCGAGAATTTG-3′ and 5′-CGG CCCTCTAGATGCATG-3′) were used to amplify the carboxyl half (F3) of the *SKY1* kinase domain from pYES2-*SKY1*. The PCR fragment was digested with *Xho*I and *Sph*I and was inserted into *Sal*I/*Sph*I-cut pSP73-F1. Finally, the *Kpn*I fragment from pSP73-F1-F3 was subcloned into the *Kpn*I-linearized pYES2-*SKY1* plasmid.

Yeast Strains. YCS2A (*MAT a*; *ura3–52*; *his3*; *trp1*; *ade2–101*; *lys2–801*) is a haploid derivative of the TR1 strain (gift from the lab of P. Hieter, Univ. of British Columbia, Vancouver). YCS19 (*MATa*; *ura3–52*; *his3–11, 15*; *leu2–3, 112*; *trp1–1*; *ade2–1*; *ade3*; *can1–100*) was a gift from the lab of I. Herskowitz (Univ. of California, San Francisco), equivalent to strain IH3004 (W303 background). YCS22 is identical to YCS19 except for the deletion of *SKY1*, constructed as follows: The entire coding region of *SKY1* was precisely replaced with the *TRP1* insert from plasmid pRS304 (35). PCR was used to amplify this *TRP1* insert flanked by 60-base pair overhangs corresponding to the noncoding sequences that immediately flank the *SKY1* coding sequence using the following primers: SKY1TRPC, 5′-ATAAATAGACACCCCCTTTTGAGGTTGAAGAGATAGAGTAAAGAAGAAGTGTAGACATTACACACCGCATAGGCAAGTGCA-3′; and SKY1TRPNC, 5′-AGAGGTTAAACAGAAAAAAAAGTAAAAGGCAAGGGCAAAATAAAGGTATAAAGGTAATCAA-G-ATTGTACTGAGAGTGCACC-3′. The PCR product then was transformed into the indicated yeast strains. Homologous recombination at *SKY1* was confirmed by using PCR and DNA blot hybridization as well as by assaying for SRp55/B52 phosphorylation (data not shown).

Immunoprecipitation and *in Vitro* Phosphorylation. Ten milliliters of log-phase yeast cells were harvested and resuspended in 100 μl of cold PBS supplemented with protease and phosphatase inhibitors. Cells were vortexed for 1 min with an equal volume of glass beads in 1.5-ml microfuge tubes. Whole cell extract was clarified by centrifugation for 5 min in a microfuge, and the protein concentration was determined by using the Bradford assay with BSA as the standard. One-hundred micrograms of the extract was incubated with 1 μl of anti-FLAG tag monoclonal antibody (M2, Eastman Kodak) for 1 hour on ice. Ten microliters of protein G beads were added and incubated on ice for 30 min. Antigen–antibody complex was collected by centrifugation and was washed three times with PBS. *In vitro* phosphorylation assays were performed on beads by using ASF/SF2 as described (33).

Immunolocalization of Sky1p. Yeast cells were grown to an OD_{600} of 0.1 in 5 ml of synthetic dextrose media supplemented with Ade, Leu, Trp, and His at 30°C. Cells were washed in H_2O and then were grown for 8 hours in 5 ml of synthetic galactose medium supplemented with Ade, Leu, Trp, and His to induce wild-type and mutant *sky1* expression. Cells were fixed for 1.5 hours at room temperature by adding 0.6 ml of 37% formaldehyde to the culture. Indirect immunofluorescence analysis was conducted according to Koepp *et al.* (36). Monoclonal anti-FLAG and Rhodamine-labeled donkey anti-mouse IgG (The Jackson Laboratory) were used as primary (1:300 dilution) and secondary (1:200 dilution) antibodies, respectively.

RESULTS

SRPK1 Substrates and an SR Protein Kinase Activity Are Conserved in Budding Yeast. To probe for candidate yeast SR proteins, we followed two empirical criteria established for

metazoan SR proteins. First, many metazoan SR proteins are quantitatively precipitated by 20 mM magnesium chloride (34). Second, all SR proteins tested are readily detectable substrates for SRPK1, which displays a pronounced specificity *in vitro* for the SR family of splicing factors (19, 32, 33). We tested whether yeast whole-cell extracts contain magnesium-insoluble proteins that are also efficient substrates for SRPK1 *in vitro*. The phosphorylation of three polypeptides of ≈43, 85, and 100 kDa depended highly on the addition of SRPK1 (Fig. 1*A*). These polypeptides thus met both criteria for candidate SR-like proteins; it is not yet known whether any of these candidates function as yeast counterparts of metazoan SR proteins. In contrast, a triplet of polypeptides migrating near 63 kDa was phosphorylated in the absence of SRPK1, presumably by endogenous kinases or autophosphorylation, and was further phosphorylated in the presence of SRPK1.

Consistent with the idea that yeast express substrates for human SRPK1, we also detected an SR protein kinase activity in yeast whole-cell extracts (data not shown). We used mAb104, which recognizes a phosphoepitope in metazoan RS domains and has been established as a standard reagent for detecting RS domain phosphorylation (34). In immunoblotting experiments, mAb104 efficiently detected Drosophila SRp55/B52 protein expressed in yeast and purified by using magnesium precipitation (Fig. 1*B*); as a control, dephosphorylation of SRp55/B52 with calf intestinal phosphatase prevented recognition by mAb104 but not by a monoclonal antibody that recognizes a nonphosphoepitope on SRp55/B52 (Fig. 1*B*). Calf intestinal phosphatase treatment also increased the mobility of SRp55/B52, as expected for dephosphorylation (34). Taken together, these results demonstrate that yeast express an SR protein kinase activity that correctly phosphorylates a heterologously expressed metazoan SR protein.

Structural and Functional Criteria Define a Yeast Member of the SRPK Family. To determine whether the yeast SR protein kinase described above came from a protein that was structurally related to members of the SRPK family, we sought to clone and sequence the gene encoding this activity. Using PCR with degenerate oligonucleotide primers corresponding to conserved SRPK regions, we amplified a specific DNA fragment from yeast genomic DNA (data not shown). The complete gene, isolated from a genomic library, matched a single ORF identified by the yeast genome project. Database searching revealed that this ORF is most related to all established SRPK family members (data not shown). The predicted kinase catalytic core domains encoded by this ORF and human SRPK1 are 50% identical and 72% similar, and, like all metazoan SRPKs, these kinase domains are interrupted by a unique spacer sequence (Fig. 2*A*). Previous studies suggest that, although less conserved, the spacer sequences may play an important role in the localization of SRPKs in cells (ref. 30; see below). Based on these structural features as well as the biochemical characterizations described below, we have named this yeast kinase gene *SKY1*, for the SR protein-specific kinase from budding yeast.

To determine whether Sky1p is also functionally related to human SRPKs, we tested Sky1p for the stringent substrate specificity characteristic of this family. For example, SRPK-catalyzed phosphorylation depends on the presence of SR/RS dipeptides within an RS domain substrate whereas Clk/Sty kinases phosphorylate both SR/RS and SK/KS dipeptides (19, 32). Purified glutathione *S*-transferase–Sky1p that had been expressed in bacteria exhibited a substrate specificity very similar to that of SRPK1 (Fig. 2*B*). Both kinases efficiently phosphorylated the mammalian SR protein ASF/SF2 (Fig. 2*B*, WT lane), and phosphorylation required the RS domain (Fig. 2*B*, ΔRS lane). Conservative substitutions for arginines (Fig. 2*B*, GS and KS lanes) or serines (Fig. 2*B*, RG and RT lanes) within the RS/SR dipeptides prevented or severely reduced phosphorylation, indicating that arginines and serines are both critical for recognition by these kinases. Note that RT/TR dipeptides were weakly phosphorylated by both kinases and that the low level of phosphorylation observed in the RG mutant represents weak phosphorylation at serine residues that remain outside of the mutant dipeptides, as described (33). These data demonstrate that Sky1p functions as an SR protein kinase *in vitro* with the same substrate specificity as mammalian SRPK1.

The Yeast RNA-Binding Protein Npl3p Is a Substrate for Metazoan and Yeast SRPKs *in Vitro*. The yeast Npl3 protein, previously implicated in mRNA export (38), contains two RNA recognition motifs that are similar in sequence and position to two metazoan SR proteins (39) as well as a C-terminal RGG-RS domain that includes eight dispersed SR/RS dipeptides. Thus

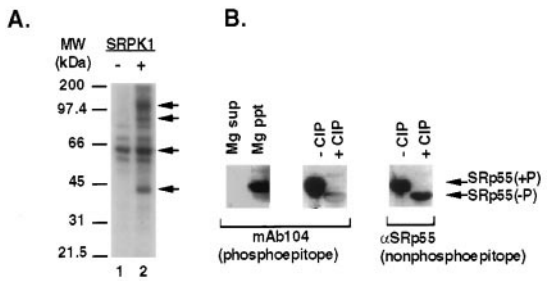

FIG. 1. *S. cerevisiae* express both SR-like proteins and an SR protein kinase. (*A*) Proteins were precipitated with 20 mM magnesium from a whole-cell yeast extract and then were tested in a kinase assay without (lane 1) or with (lane 2) purified SRPK1. Proteins were resolved by SDS/PAGE and were detected by autoradiography. Arrows indicate prominent phosphorylated proteins. Molecular weight standards are shown on the left. (*B*) A yeast extract from cells expressing SRp55/B52 was fractionated by using precipitation with 20 mM magnesium (sup, supernatant; ppt, precipitate) and then was analyzed on immunoblots probed with mAb104, which recognizes a phosphoepitope in SR proteins (*Left*), or anti-SRp55, which does not recognize a phosphoepitope (*Right*). CIP indicates incubation in the absence (−) or presence (+) of calf intestinal phosphatase before immunoblot analysis.

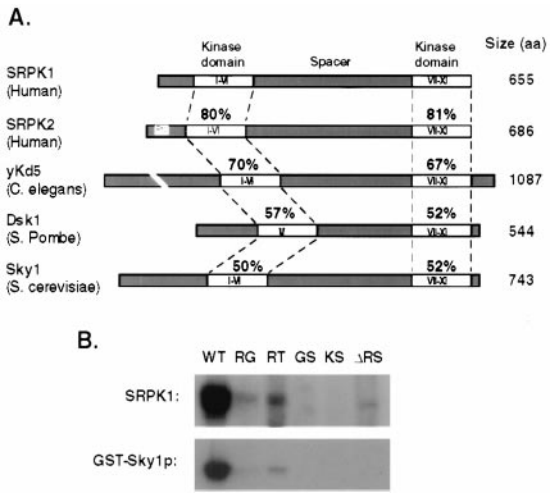

FIG. 2. Sky1p is structurally and functionally related to the metazoan SRPK family. (*A*) Sequence comparison of the SRPK family members, including Sky1p (SGDID L0003941). The kinase domains are highly conserved, as indicated by the percentages of amino acids identical to those in SRPK1. C. elegans, *Caenorhabditis elegans*. (*B*) Shared substrate specificity between Sky1p and human SRPK1. Equal masses of purified forms (WT, wild-type; RG, RT, GS, and KS, mutant RS/SR dipeptides containing the indicated amino acid changes; ΔRS, RS domain deletion) of the human SR protein ASF/SF2 were phosphorylated with purified human SRPK1 (*Upper*) and glutathione *S*-transferase–Sky1p (*Lower*).

Npl3 is structurally related to metazoan SR proteins (ref. 39; C.W.S. and C.G., unpublished results). Moreover, Npl3p also shares functional properties with metazoan SR proteins. Specifically, immunodepletion of Npl3p from splicing extracts inhibits RP51A intron splicing, and this defect is complemented by the addition of purified bacterially expressed Npl3p (C.W.S. and C.G., unpublished results). To determine whether Npl3p might be an endogenous substrate for Sky1p, we tested whether Sky1p phosphorylated purified, bacterially expressed Npl3p. Sky1p phosphorylated Npl3p (Fig. 3, lane 4) at least as efficiently as ASF/SF2 (lane 2) and at levels comparable to SRPK1 phosphorylation of either substrate (lanes 1 and 3). These results suggest that Npl3p is an endogenous substrate for Sky1p and indicate that Npl3p possesses the hallmark biochemical characteristics of an SR protein.

Sky1p Is the only Kinase in Vegetatively Growing Budding Yeast that Can Phosphorylate Mammalian SR Proteins. To begin studies of *SKY1* function *in vivo*, we precisely replaced only the *SKY1* coding sequence with the *TRP1* gene, creating *sky1*Δ strain. In three different strain backgrounds, such *sky1*Δ cells grew on plates similarly to wild-type parent cells at temperatures ranging from 16 to 37°C. However, the *SKY1* deletion slowed growth in one strain background (Fig. 4*A*; data not shown). We exploited the former *sky1*Δ strains to ask whether Sky1p was required for phosphorylation of metazoan SR proteins expressed in yeast. Strikingly, immunoblot analysis revealed that SRp55/B52 expressed in a *sky1*Δ strain completely lacked the mAb104 phosphoepitope (Fig. 4*B*; compare with Fig. 1*B*). In addition, SRp55/B52 from the *sky1*Δ strain migrated with increased mobility relative to SRp55/B52 expressed in the wild-type parent strain, further indicating the lack of RS domain phosphorylation (Fig. 4*B*). Expression of *SKY1* from a plasmid (p*SKY1*) fully restored SRp55/B52 phosphorylation in the *sky1*Δ strain (data not shown), confirming that SRp55/B52 phosphorylation reflected Sky1p activity alone. We have obtained similar results by using the mammalian SR proteins ASF/SF2 and SC35 in place of SRp55/B52 (46). Given that Sky1p directly and efficiently phosphorylates RS domains *in vitro* (Fig. 2*B*), the dependence of RS domain phosphorylation *in vivo* on *SKY1* expression likely reflects the direct phosphorylation of RS domains by Sky1p, as opposed to a phosphorylation cascade in which Sky1p activates an SR protein kinase. Hence, *SKY1* is absolutely required for proper phosphorylation of metazoan RS domains *in vivo*. These results also suggest that, unlike mammalian cells that express multiple SRPK and Clk/Sty family members, yeast cells may express only a single SRPK.

Mislocalization of Sky1p to the Nucleus Inhibits Growth Independently of Kinase Activity. The nonconserved spacer sequence separating the conserved catalytic kinase domains is a defining feature of the SRPK kinase family (Fig. 2*A*). These spacers in the fission yeast kinase Dsk1 (30) and the human kinases SRPK1 and SRPK2 (L.F. and X.-D.F., unpublished

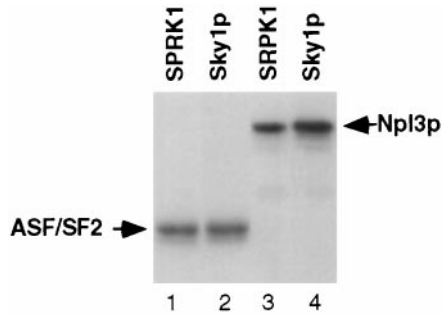

FIG. 3. Npl3p is a substrate for SRPK1 and Sky1p. Kinase assays were performed by using purified kinases (SRPK1 or Sky1p) and purified substrates (recombinant Npl3p or ASF/SF2) and were analyzed by SDS/PAGE followed by autoradiography.

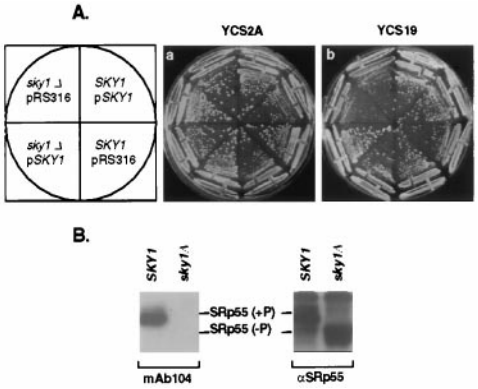

FIG. 4. SKY1 is not essential for vegetative growth but is required for phosphorylation of metazoan SR proteins expressed in yeast. (*A*) Effect of *SKY1* deletion (*sky1*Δ) on cell growth. *SKY1* was precisely deleted from two yeast strains, YCS2A (*a*) or YCS19 (*b*). As shown in the diagram on the left, the wild-type parent and *sky1*Δ strains were transformed with either the p*SKY1* plasmid expressing a genomic copy of the *SKY1* gene in the pRS316 vector or the pRS316 vector alone. Note that the slow growth (small colony size in *b*) caused by the *SKY1* deletion in the YCS19 strain was specifically complemented by p*SKY1*. (*B*) SRp55/B52 was expressed in a wild-type or *sky1*Δ strain and was analyzed as in Fig. 1*B*.

results) are required for normal steady-state localization of these kinases to the cytoplasm (during interphase), and their deletion results in exclusive nuclear localization of the mutant kinases (ref. 30; L.F. and X.-D.F., unpublished results). Recently, a leucine-rich nuclear export signal (NES) was identified in Dsk1 (40), suggesting that at least one mechanism for the normal localization of SRPK family members may be through the active export to the cytoplasm of SRPKs that enter the nucleus; however, the Dsk1 NES is not obviously conserved in the spacer domains of SRPKs from other species, and whether this mechanism generally applies to other SRPKs is not known.

To examine the intracellular localization of Sky1p and compare it to that of other SRPKs, we fused the FLAG epitope to the Sky1p N terminus and used immunofluorescence microscopy with anti-FLAG antibodies to localize wild-type and mutant forms of Sky1p (Fig. 5*A*). Similar to other SRPKs, wild-type Sky1p localized primarily to the cytoplasm, although a fainter nuclear signal was also evident in some cells (Fig. 5*B*). In contrast, a Sky1p mutant carrying a precise deletion of the spacer domain [sky1(Δ spacer)p] was localized exclusively in the nucleus (Fig. 5*B*). Therefore, similar to other SRPK spacer domains, the Sky1p spacer sequence is required for the steady-state localization of the kinase to the cytoplasm.

Of importance, mislocalization of Sky1p to the nucleus inhibited growth in a genetically dominant manner (Fig. 5*C*). Although overexpression of full-length, wild-type Sky1p had only a modest effect, if any, on growth (Fig. 5*C Left*), overexpression of sky1(Δ spacer) protein severely inhibited growth (Fig. 5*C Right*). A number of experiments showed that this growth inhibition was independent of Sky1p kinase activity. First, a K-M ATP binding site mutation eliminated kinase activity, as shown by *in vitro* kinase assays using immunoprecipitated Sky1p. In contrast, the spacer deletion mutation alone retained significant kinase activity (Fig. 5*D*). Second, immunofluorescence experiments revealed that the addition of the K-M mutation did not affect the nuclear mislocalization of sky1(Δ spacer) protein; indeed, both inactive and active Sky1p kinases lacking the spacer domain mislocalized to the nucleus (Fig. 5*B*). Third, overexpression of nuclear-localized Sky1p kinases, whether inactive or active, inhibited growth, although inhibition was most severe after overexpression of the inactive kinase (Fig. 5*C*). These results clearly establish that the constitutive mislocalization of Sky1p to the nucleus is toxic to cell growth. The fact that the complete absence of *SKY*1 expres-

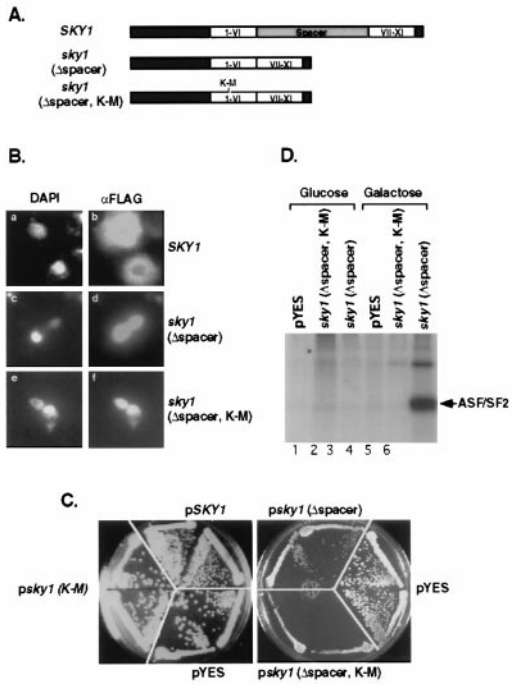

FIG. 5. Mislocalization of Sky1p to the nucleus inhibits growth. (*A*) Domain arrangement of wild-type *SKY1*, mutant sky1 with the spacer domain precisely deleted [*sky1(Δspacer)*], and *sky1(Δspacer)* that also carries a K-to-M point mutation (kinase-inactivating) at the ATP binding site. (*B*) Immunofluorescent localization of the indicated kinases, expressed as N-terminal fusion proteins with the FLAG epitope, using a monoclonal anti-FLAG antibody (*b*, *d*, and *f*). DNA was stained with 4′,6-diamidino-2-phenylindole to reveal the positions of the nuclei (*a*, *c*, and *e*). (*C*) Growth phenotypes associated with Sky1p overexpression. Overexpression from the *GAL1* promoter of the indicated wild-type or mutant forms of Sky1p was induced in wild-type cells streaked onto plates containing galactose. Cells containing the plasmid vector alone (pYES) served as a control. (*D*) The indicated forms of Sky1p were immunoprecipitated from yeast extracts before (lanes 2 and 3) or after (lanes 5 and 6) induction of their expression by galactose and then were tested for kinase activity by using ASF/SF2 as the substrate. Cells containing the plasmid vector alone (pYES) served as a control.

sion does not cause similar toxicity (Fig. 4*A*) suggests that the presence of Sky1p in the nucleus, rather than its absence in the cytoplasm, is responsible for growth inhibition. It remains to be determined whether the toxic effects generated by inactive versus active Sky1p reflect the same, overlapping, or distinct mechanisms.

DISCUSSION

A Single Conserved SR Protein Kinase in Budding Yeast? We have described a number of structural and functional lines of evidence demonstrating that *S. cerevisiae* expresses a member of the SRPK family. In striking contrast to mammalian cells, which express many distinct kinases that phosphorylate RS domains, including multiple forms of both SRPKs and Clk/Sty kinases, Sky1p was sufficient to phosphorylate metazoan RS domains and hence may be the only such kinase in *S. cerevisiae*. In searches of the yeast genome, *SKY1* was the only ORF that significantly resembled a gene for an SRPK based on domain structure and sequence similarity. More directly, experiments with strains deleted for the single-copy *SKY1* gene revealed that Sky1p is necessary and sufficient for generating the mAb104 phosphoepitope. In the simplest case, these results indicate that Sky1p is the only SRPK member expressed in budding yeast. However, we cannot exclude the possibility that *S. cerevisiae* expresses additional SRPKs or functional homologues of Clk/Sty that are unable to interact with metazoan RS domains *in vivo* or that the substrate specificities of these proposed additional kinases may differ from that of their mammalian counterparts.

Whether budding yeast contain a Clk/Sty has remained an enigma. Searches of the yeast database reveal several kinases with some sequence similarity to mammalian Clk/Sty, but none stand out clearly as members of this family (41). Furthermore, none of these Clk/Sty candidates in yeast contain the Clk/Sty RS-like domain, which is critical for kinase-substrate binding (17), suggesting that a yeast Clk/Sty may be unable to interact with metazoan SR proteins. Because Clk/Sty kinases might be expected to function redundantly with Sky1p, a search for mutants that are synthetically lethal with *sky1* may lead to the identification of Clk/Sty kinases in yeast. Indeed, Npl3p appears to be phosphorylated by Sky1p and at least one additional yeast kinase that is not an SRPK (C.W.S. and C.G., unpublished work). Alternatively, Sky1p may provide a nonessential, auxiliary function or play an essential role under other growth conditions.

Multiple SR-Like Proteins in Budding Yeast. Here, we have reported a number of yeast SR-like proteins, defined by using two empirical criteria: precipitation in 20 mM magnesium chloride and efficient SRPK1-catalyzed phosphorylation *in vitro*. Given that only a subset of metazoan RS-domain proteins are precipitated in magnesium (1), we may have failed to detect additional SRPK substrates that remained soluble. A search of RS/SR dipeptides in yeast ORFs also suggests other possible substrates for Sky1p. Such proteins including Mud2p, an orthologue of human splicing factor U2AF^{65} (42), Hrb1p and Gbp2p, closely related proteins that contain RNA recognition motif RNA binding domains (43), and Nrd1p, an orthologue of the mammalian rA8 RS domain-containing protein that can bind to the C-terminal repeats of RNA polymerase II (44) and may coordinate transcription and splicing (45). Coupled with our identification of a yeast SRPK, our discovery of yeast SR-like proteins further points to the existence of an SR protein system in budding yeast.

A Possible Role for Sky1p and Other SRPKs in Regulating RNA Metabolism. Definitive identification of Sky1p substrates should provide important clues to the biological functions of Sky1p. The candidate substrates listed above all appear to be RNA binding proteins and/or to function in some aspects of RNA metabolism. Specific functions have been ascribed to one Sky1p substrate, the Npl3 protein, which we showed is efficiently phosphorylated *in vitro* by both Sky1p and SRPK1. Npl3p displays many of the structural (ref. 39; C.W.S. and C.G., unpublished results) and functional (C.W.S. and C.G., unpublished results) properties of an SR protein. Importantly, Npl3p has been described as a potential carrier of mRNA from the nucleus to the cytoplasm because it shuttles between the nucleus and cytoplasm, dependent on general pol II transcription (38), and mutations in *NPL3* lead to the nuclear accumulation of bulk Poly(A)$^+$ RNA, indicative of decreased mRNA export (38). Of interest, we recently have found that deletion of *SKY1* increases the cytoplasmic localization of Npl3p (C.W.S. and C.G., unpublished work) as well as of the exogenously expressed mammalian SR protein SC35 (46), suggestive of a role in the nucleocytoplasmic shuttling of substrate RNA binding proteins.

Important clues to Sky1p function also should come from further studies of the intracellular localization of the kinase. We have shown that Sky1p localizes primarily to the cytoplasm under steady-state conditions in interphase cells, dependent on the presence of the spacer domain that is characteristic of SRPK family members. A similar localization pattern, also dependent on spacer domains, has been reported for other members of the SRPK family (ref. 30; L.F. and X.-D.F., unpublished results). *A priori*, the spacer domain could provide a cytoplasmic anchor, restricting Sky1p to the cytoplasm, or a NES, stimulating the export of Sky1p that enters the nucleus. Consistent with this latter hypothesis, a leucine-rich NES of the Rev class was described in the spacer domain of Dsk1 (40), an SRPK member in *S. pombe* (31). However, this NES sequence is not conserved within the

Sky1p spacer domain and fails to function as an NES when fused to a green fluorescent protein reporter protein (C.W.S. and C.G., unpublished results). Hence, different signals or mechanisms may dictate the cytoplasmic localization of distinct SRPK members.

The cytoplasmic localization of Sky1p may reflect a function for Sky1 in this compartment. For example, Sky1p-catalyzed phosphorylation could provide a mechanism for distinguishing cytoplasmic and nuclear pools of substrate proteins, including shuttling RNA binding proteins such as Npl3p. This hypothesis, together with the observation that a subset of mammalian SR proteins also shuttle (23), further suggests that SRPK-mediated phosphorylation may represent an evolutionarily conserved mechanism to control substrate localization or shuttling. An alternative, though not mutually exclusive, hypothesis is that the cytoplasmic sequestering of the kinase may regulate Sky1p nuclear functions by controlling its nuclear concentration. In support of this hypothesis, SRPK1 and SRPK2 induce the redistribution of splicing factors in the nuclei of transfected cells (19). Notably, the constitutive localization of Sky1p to the nucleus, induced by deletion of the spacer domain, dominantly inhibited growth. Because both active and inactive forms of the kinase were inhibitory, it will be of great interest to determine whether the two forms inhibit through the same mechanism—for example, by titrating and sequestering nuclear substrates that are crucial for growth—or via distinct mechanisms—for example, by respectively causing the hyper- and hypophosphorylation of nuclear targets.

These results beg the question of the normal nuclear functions of Sky1p. Our observation that the nuclear localization of bulk Sky1p inhibits growth, together with the observation that a high level of SRPK1 inhibits pre-mRNA splicing *in vitro* (12), suggests that nuclear SRPKs may negatively regulate RNA processing. Notably, Dsk1 enters the nucleus during mitosis, and a similar shift in localization seems to occur with Sky1p (L.F. and X.-D.F., unpublished observations). Thus, SRPKs may be involved in a cell-cycle-dependent regulatory event, perhaps down-regulating RNA processing during mitosis or in other events, such as a response to specific growth conditions or extracellular signals.

Whether the function of Sky1p in yeast will generally apply to SRPK mechanisms in other species hinges on the extent to which SRPK functions have been conserved in yeast and metazoans. One extreme argument would assert that yeast and metazoan SRPKs have evolved to fill different roles with distinct biological consequences. Alternatively, yeast and metazoan SRPKs may function similarly in homologous pathways. In either case, further genetic analysis of Sky1p in yeast will provide a powerful complement to current biochemical studies of metazoan SRPKs, together leading to a mechanistic description of how SRPKs regulate RNA processing *in vivo*.

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