Structurally Unique Yeast and Mammalian Serine-Arginine Protein Kinases Catalyze Evolutionarily Conserved Phosphorylation Reactions*

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Randall Lukasiewicz1, Adolfo Velazquez-Dones3, Nhat Huynh5, Jonathan Hagopian5, Xiang-Dong Fu5, Joseph Adams3, and Gourisankar Ghosh1,2

From the Departments of 1Chemistry & Biochemistry, 4Cellular & Molecular Medicine, and 5Pharmacology, University of California, San Diego, La Jolla, California 92093-0375

The mammalian serine-arginine (SR) protein, ASF/SF2, contains multiple contiguous RS dipeptides at the C terminus, and ~12 of these serines are processively phosphorylated by the SR protein kinase 1 (SRPK1). We have recently shown that a docking motif in ASF/SF2 specifically interacts with a groove in SRPK1, and this interaction is necessary for processive phosphorylation. We previously showed that SRPK1 and its yeast ortholog Sky1p maintain their active conformations using diverse structural strategies. Here we tested if the mechanism of ASF/SF2 phosphorylation by SRPK is evolutionarily conserved. We show that Sky1p forms a stable complex with its heterologous mammalian substrateASF/SF2 and processively phosphorylates the same sites as SRPK1. We further show that Sky1p utilizes the same docking groove to bind yeast SR-like protein Gbp2p and phosphorylates all three serines present in a contiguous RS dipeptide stretch. However, the mechanism of Gbp2p phosphorylation appears to be non-processive. Thus, there are physical attributes of SR and SR-like substrates that dictate the mechanism of phosphorylation, whereas the ability to processively phosphorylate substrates is inherent to SR protein kinases.

Pre-mRNA splicing and mRNA export are complex processes, which involve a large number of protein and RNA factors (1–4). SR proteins, a class of non-small nuclear ribonucleoprotein splicing factors, participate in every step in the spliceosome assembly and catalysis (5). SR proteins have unique domain architecture. The N-terminal domain contains one or two RNA recognition motifs (RRMs)3 and is responsible for RNA binding, and the C-terminal domain, known as the RS domain, is rich in long stretches of serine-arginine/arginine-serine (SR/RS) dipeptides (6). The RS domains of SR proteins are extensively phosphorylated. Although many kinases have been demonstrated to target SR proteins, members of the SR protein kinase (SRPK) family have been established as the predominant kinase (6–10). Mammalian SR protein kinase 1 (SRPK1) and the yeast enzyme, Sky1p, are the two most studied SRPKs. Members of the SRPK family display strict substrate specificity, preferring to phosphorylate only serine residues flanked by arginines. One of the well studied mammalian SR proteins, ASF/SF2, contains 20 serines within its 50-residue-long RS domain (see Fig. 1a). SRPK1 phosphorylates ~10–12 of these serines in the N-terminal RS1 region of the RS domain (11). This multsite phosphorylation occurs in a processive manner where the kinase remains bound to the substrate until all sites are phosphorylated (12). Phosphorylation of RS1 is essential for the nuclear import of ASF/SF2.

In yeast, Sky1p regulates the phosphorylation and nuclear import of SR-like proteins Npl3p and Gbp2p (13–15). However, unlike the mammalian SR proteins, the yeast proteins do not have a classic RS domain with long stretches of arginine/serine repeats (6, 16) (Fig. 1a). In the case of Npl3p, eight isolated RS dipeptides are present within the RGG domain, and the C-terminal most RS dipeptide is phosphorylated by Sky1p (15). This modification is essential for the nuclear import of Npl3p, facilitated by an importin, Mtr10p (17). Defects in phosphorylation cause accumulation of Npl3p in the cytoplasm with enhanced mRNA binding activity (14, 15). Recent experiments reveal that the Gbp2p RS-repeat motif, which contains three successive RS-dipeptides, is critical for the nuclear import of Gbp2p (Fig. 1a) (13).

The domain arrangement in SRPKs is unique in that these kinases contain a large insert, bifurcating the kinase core domain (Fig. 1a). Although the insert region, known as the spacer domain, bears little sequence homology among SRPKs across species, its precise location with respect to the core kinase domain suggests that it may play a similar function in most SRPKs. Indeed, in both yeast and human kinases, the spacer domain plays a role in the subcellular localization of these kinases (18). Both SRPK1 and Sky1p also have non-homologous extensions at their N termini. Sky1p has a 40-residue-long extension at its C terminus, whereas the C terminus of SRPK1 has no such extension (19). The x-ray structure and subsequent deletion analysis of Sky1p reveal that its non-con-
erved C-terminal tail makes contact with the activation loop of the kinase. Deletion of only 10 residues from the C terminus of Sky1p drastically reduces the catalytic activity of Sky1p (19). On the other hand, the entire spacer domain and N-terminal 137 amino acids of Sky1p can be removed without compromising its in vitro activity (19). In SRPK1, both ends of the previously designated spacer domain fold into helical structures and interact with the kinase core. Deletion analyses of SRPK1 have revealed that, unlike Sky1p, removal of the designated spacer domain in its entirety results in reduced stability of SRPK1 (20).

Despite the critical differences in the primary and tertiary structures between SRPK1 and Sky1p, they can phosphorylate each other’s substrate in vivo and in vitro (21, 22). SRPK1 also can functionally substitute for Sky1p in yeast suggesting that the basic mechanism of phosphorylation is evolutionarily conserved from yeast to humans. Perhaps it is not too surprising as the yeast substrate is phosphorylated only at a single site and that recognition of the phosphorylation site peptide of Npl3p is not different between these kinases. However, it is unclear if the yeast enzyme can phosphorylate the mammalian substrates at multiple sites in a processive manner. If so, this would imply the development of an evolutionarily conserved mechanism of phosphorylation among SRPKs.

Our results show that, despite significant differences in the mechanism of how Sky1p and SRPK1 maintain their active states, these two kinases display an evolutionarily conserved mode of substrate binding and catalysis. We demonstrate here that catalysis and substrate recognition are conserved among SR protein kinases. However, only the SR protein substrates such as ASF/SF2 undergo processive phosphorylation but not the SR-like protein Gbp2p.

EXPERIMENTAL PROCEDURES

Cloning and Expression of Recombinant Proteins—Expression and purification of His-SRPK1ΔNS1, His-Sky1pΔN(137)ΔS, mutants of Sky1p, SRPK1 K109M (trap), and Npl3p have been described previously. The cloning of ASF/SF2 in Escherichia coli expression vector has been described earlier (11). The protein was then transformed into E. coli BL21(DE3) cells (Invitrogen) and grown in LB containing 150 μg/ml ampicillin. The protein was then bound to ProBond Nickel resin (Invitrogen) via batch binding for 1 h at room temperature. The resin was washed extensively followed by refolding of His-ASF/SF2 in the column with stepwise removal of urea. His-ASF/SF2 was then washed with low pH buffer (pH 4.5, 20 mM Tris, 0.1 M MOPS, 10% glycerol, 300 mM NaCl) and then eluted with a buffer of pH 2.1.

The RGG domain Gbp2p encompassing residues 1–123 was prepared by introducing a stop codon at amino acid 124 in a vector expressing full-length Gbp2p as a GST fusion protein. Gbp2p mutants were generated by stepwise single step PCR. Gbp2p was expressed in E. coli BL21(DE3) cells after growing to an A600, and inducing for 5 h at 37 °C with 0.1 mM isopropyl β-D-galactopyranoside. The cells were pelleted, suspended in 100 ml of lysis buffer (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture (Sigma)), and lysed by sonication. The lysate was centrifuged for 40 min at 13,000 rpm, and the supernatant was loaded onto a GST column. The GST column was then washed with 50 ml of lysis buffer and then eluted with lysis buffer containing 10 mM glutathione. The protein was then dialyzed against 3 liters of dialysis buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 5% glycerol, 1 mM DTT). Mutants of Gbp2p were purified similarly.

In Vitro Kinase Assay—Kinase assays were performed in the following conditions: 50 mM Tris, pH 7.5, 10 mM MgCl2, 1 mg/ml bovine serum albumin, 1 mM DTT, 200 μM cold ATP, and 5–10 μCi of [γ-32P]ATP at 25 °C. Reactions were quenched using 2× SDS-PAGE loading dye, and samples were boiled for 1 min at 95 °C before loading onto a 10% SDS gel. Bands of radio-labeled product were cut out and quantified via a Beckman scintillation counter and normalized to background as performed previously (11). Extrapolated curves for Km of Npl3p and kobs were calculated using KaleidaGraph (Synergy Software, Reading, PA).

Trap Kinase Assay—Trap assays consisted of three separate in vitro kinase time-course reactions using equimolar amounts of kinase and substrate as performed previously (11). The positive control reaction labeled “no trap” containing no SRPK1 K109M inhibitor (trap) in which the kinase and substrate were incubated at 25 °C for 5 min before initiation with ATP. The negative control reaction labeled “trap-start” contained 50 μM trap incubated with the substrate at 25 °C for 5 min before the addition of active kinase and subsequent initiation with ATP. The last reaction labeled “start + trap” contained incubated kinase and substrate initiated with a mixture of ATP and trap to final concentrations of 200 μM and 50 μM, respectively. The mechanism of phosphorylation was determined by comparison of the phospho-content of the “start + trap” reaction to that of the positive and negative controls, indicating processive and distributive modes, respectively.

Mass Spectrometry Analysis—MALDI-TOF analyses were carried as previously described (11). Preparation of phosphorylated protein samples were carried out in 50 mM Tris, pH 7.5, 10 mM MgCl2, 1 mM DTT, 8 μM His-Sky1pΔNS, 8 μM His-ASF at 25 °C. Reactions were initiated with the addition of 3 mM ATP in a total volume of 20 μl. Reactions were quenched with three volumes of stop buffer (50 mM Tris, pH 7.5, 200 mM NaCl, 1 mM DTT, 8 μM urea).

GST Pulldown Assays—Pulldown experiments were carried out in two steps. In the first step crude extract containing GST fusion proteins (Npl3p, Gbp2, or ASF/SF2) in binding buffer (20 mM Tris, pH 7.5, 200 mM NaCl, 1% Triton X-100, 2 mM DTT, protease inhibitor mixture from Sigma, 1 mM phenylmethylsulfonyl fluoride) were bound to buffer-equilibrated glu-
tathione-Sepharose beads (10 μl), centrifuged, and washed (10 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2 mM DTT). We estimated roughly 10 μg of GST fusion protein to remain bound to beads. In the second step, His-tagged kinase (2–20 μg) was added to the bead bound to GST (control) or GST-Npl3p (or Gbp2 or ASF/SF2) in 150 μl of binding buffer. Samples were then washed four times with 750 μl of wash buffer. 10 μl of 4× SDS-PAGE loading dye was added to each sample; then each sample was boiled at 95 °C for 2 min and then loaded onto a 10% SDS gel. Proteins were visualized via Western blot or Coomassie stain.

**Western Blotting**—Protein samples were run on a 10% SDS-PAGE and transferred to an Immobilon polyvinylidene difluoride membrane (Millipore). Membranes were then probed with either anti-His antibodies or anti-GST antibodies (obtained from Qiagen and Santa Cruz Biotechnology, respectively).

**RESULTS**

Sky1p Can Phosphorylate Heterologous ASF/SF2 Substrate at Multiple Sites—It has been previously shown that SRPK1 can functionally complement SKY1 in vivo (23). The replacement of SKY1 with SRPK1 implies that the mammalian kinase can phosphorylate Npl3p and other Sky1p substrates at a sufficient level for viability. The peptide-bound structures of Sky1p and SRPK1 shown above (Fig. 1, b and c) suggest a similar mechanism of substrate recognition by these two kinases (24). However, the physiological substrates for these two kinases are different in terms of the number of potential phosphorylation sites. This prompted us to understand how each of these kinases phosphorylates the heterologous substrates.

We conducted an in vitro kinase assay to determine the ability of Sky1p and SRPK1 to phosphorylate ASF/SF2 (Fig. 2a, middle lanes). We measured the phosphate content of Sky1p-phosphorylated His-ASF/SF2. We used MALDI-TOF mass spectrometry to quantify the number of phosphates added to ASF/SF2 by wt SRPK1 and Sky1p. In these studies, His-tagged ASF/SF2 (8 μg) was incubated with a stoichiometric amount of Sky1p or wt SRPK1 for 5 h in the presence or absence of ATP at room temperature and then analyzed using MALDI-TOF mass spectrometry (Fig. 2, b–d). In the spectra, we observe that the ASF/SF2 M + 1 and M + 2 peaks shift before and after ATP treatment. Both peak maxima shift to a higher mass/charge (443 for M + 1 and 857 for M + 1) after adding ATP in the case of SRPK1 wt, consistent with the incorporation of ~10 phosphates per substrate mole-

**FIGURE 1.** Structure and domain organization of SR protein kinases and their substrates. a, the domain organizations of SRPKs and their SR and SR-like substrates. The kinase and spacer domains of SRPKs are shown in blue and red, respectively. N- and C-terminal kinase extensions are represented in white. The sequences of entire RS and RGG domains are shown. Dispersed RS dipeptides in these proteins are shown in red. b and c, ribbon presentation of the crystal structures of Sky1p (b) and SRPK1 (c). Active sites are shown in red boxes.
SR Protein Kinases Share a Conserved Phosphorylation Mechanism

cule (Fig. 2c). Also a shift in peak maxima was observed (558 for M + 2 and 1064 for M + 1) upon the addition of Sky1p, corresponding to the incorporation of ~13 phosphates per substrate molecule by Sky1p (Fig. 2d). Longer incubation of the enzymes with ASF/SF2 and ATP resulted in no further changes in phosphoryl content (data not shown), indicating that an end point in the reaction was achieved. These data provide a definitive measure of phosphoryl content that is independent of total active ASF/SF2 concentration.

Sky1p Binds ASF/SF2 with High Affinity—We tested the relative binding efficiencies of ASF/SF2 for Sky1p and SRPK1 using a GST co-precipitation assay. Pulldown experiments showed that GST-ASF/SF2 efficiently retained both SRPK1 and Sky1p (Fig. 3, a and b, left panels), whereas GST-Npl3p bound Sky1p only (Fig. 3, a and b, right panels). Although SRPK1 can phosphorylate Npl3p, GST-Npl3p failed to pull down SRPK1 in conditions where it interacted with Sky1p. This suggests that binding affinity of the SRPK1/Npl3p complex is lower than the Sky1p/Npl3p complex.

The GST co-precipitation experiment is a qualitative assay and does not provide a quantitative assessment of affinity. It has been shown previously that SRPK1 stably binds ASF/SF2 with a $k_d$ of ~50 nM (12). We wanted to test if Sky1p could bind to ASF/SF2 with a similar affinity. To estimate a $k_d$, single turnover profiles were measured at different kinase concentrations with a fixed ASF/SF2 concentration (20 nM). Under single turnover conditions at low SRPK1 concentrations, it was shown previously that ASF/SF2 phosphorylation is biphasic, but at high SRPK1 concentrations, phosphorylation profiles are monophasic (12). The amplitude of the first phase is directly related to the amount of enzyme-bound ASF/SF2 so that a $k_d$ can be determined from a plot of fraction bound versus total enzyme concentration.

To estimate the affinity of the Sky1p:ASF/SF2 complex, we varied Sky1p concentrations from 500 nM to 50 nM to determine at which concentration the progress curves became noticeably biphasic (Fig. 3c). We observed that above 100 nM Sky1p, the progress curves followed a single exponential, rapidly reaching an end point. However, at 50 nM Sky1p, a biphasic trend in the phosphoryl transfer mechanism was observed. These findings suggest that, at 100 nM Sky1p or higher, most of the ASF/SF2 is bound by the enzyme, implying that the $k_d$ for Sky1p and ASF/SF2 is lower than 100 nM. These observations indicate that Sky1p has a similar affinity for ASF/SF2 as SRPK1.

Sky1p Processively Phosphorylates ASF/SF2—To further investigate potential differences in the mechanism of ASF/SF2 phosphorylation by Sky1p, we used a trap assay as previously...
reported (11). In this assay, the kinase and substrate are preincubated, and the reaction is started with the simultaneous addition of ATP and excess trapping agent designed to bind any free ASF/SF2 (Fig. 4). Here, we used a catalytically inactive form of full-length SRPK1 K109M (trap) that binds well but cannot phosphorylate ASF/SF2 (11). If the reaction is processive, no free ASF/SF2 is generated as the reaction progresses, so the trap cannot inhibit the reaction (Fig. 4a).

However, if the reaction is distributive one or more phospho-intermediates of ASF/SF2, released from the active site of Sky1p, will be readily trapped by K109M resulting in reaction inhibition. We used 100 nM ASF/SF2, 100 or 500 nM Sky1p, and 50 μM trap for the reaction (Fig. 4, b and c). We observed that, even at 100 nM Sky1p concentration, the reaction is processive. There is a noticeable difference in phosphorylation of ASF/SF2 in the control trap-start reaction between two different concentrations of Sky1p. In the trap-start reaction, ASF/SF2 is preincubated with the trap before adding active Sky1p. Perhaps the trap is unable to fully inhibit the kinase at 500 nM concentration. However, these observations clearly show that, similar to SRPK1, Sky1p is a processive enzyme.

Sky1p and SRPK1 Use a Similar Docking Groove to Bind ASF/SF2—We have previously shown that the SRPK1 docking groove is important for binding and processive phosphorylation of ASF/SF2 (24). We have also shown that Sky1p contains a homologous docking groove (25). Using mutations within the docking groove, we showed that the docking groove in Sky1p plays an important role for Npl3p phosphorylation. Next, we wanted to test if this same docking groove of Sky1p is also important for ASF/SF2 recognition.

We tested the binding between Sky1p docking groove mutants (QM and 4M) and GST-ASF/SF2 using GST co-precipitation assays. Each of these mutants contains four amino acid changes to alanine (QM: Ile-653, Lys-657, Trp-659, and Lys-668; and 4M: Asp-601, Asp-617, Glu-624, and Lys-668). Both sets of mutations in the docking groove (QM and 4M) drastically reduced the binding of ASF/SF2 compared with wt Sky1p (Fig. 5a, lower panel). In all, our results suggest that both yeast and human SRPKs use common regions to recognize ASF/SF2, and the kinase docking groove is key to their interaction. Our results also imply that the docking groove-docking motif interaction plays a major role in substrate phosphorylation.
Docking Interaction Plays a Role in Processive Phosphorylation of ASF/SF2—We next tested the effect of the Sky1p docking groove mutations (QM and 4M) on processive phosphorylation of ASF/SF2 using the start-trap assay as described earlier (Fig. 5, b and c). We used 100 nM ASF/SF2, 100 nM of the mutant kinases, and 50 μM trap for the reactions. We observed that ASF/SF2 phosphorylation by Sky1p QM is not affected by the addition of trap (Fig. 5b). Our results demonstrate that Sky1p QM processively phosphorylates ASF/SF2, whereas 4M does so only partially (Fig. 5c). These observations are consistent with our previous report that the Sky1p QM mutant did not affect phosphorylation of Npl3p, whereas the Sky1p 4M mutant did (25). However, these results appear to be inconsistent with GST co-precipitation experiments that showed that both mutants were defective in ASF/SF2 binding. It is difficult to differentiate the affinities of the mutant kinases for ASF/SF2 relative to each other using a co-precipitation assay. Although we see that both Sky1p mutants are unable to bind ASF/SF2 in a pulldown assay, it is possible that the 4M mutant has a significantly lower affinity toward ASF/SF2 than the QM mutant. It is possible that we simply used a protein concentration during the co-precipitation that is below the threshold of the stabilities of both complexes. It appears that the affinity of the ASF/SF2-Sky1p QM complex is sufficient enough for the phosphorylation to occur processively. Sky1p 4M exhibits partial processivity, which suggests that, although affinity is significantly reduced, this mutant still interacts with the substrate to an extent that partial processive activity remains. Processivity could also be affected by the ATP concentration, and the effect of ATP may be more important for relatively weak kinase-substrate complexes.

Sky1p Interacts with Gbp2p and Phosphorylates It at Multiple Sites—The ability of Sky1p to carry out processive phosphorylation of heterologous substrates motivated us to look for natural yeast substrates that might be processively phosphorylated at multiple sites. Yeast protein Gbp2p, contains an RGG/RS domain like Npl3p where three serines are present within a continuous stretch of RS dipeptides (RS motif) (Fig. 1a). Gbp2p also contains an RGG domain C-terminal to the RS motif. In our previous studies, we have shown that the docking groove of Sky1p interacts with the RGG domain of Npl3p, and this docking interaction is important for efficient phosphorylation of Npl3p (25). We also showed that the RGG domain of Npl3p makes electrostatic interactions with an acidic docking groove in Sky1p (25). The presence of an RS motif located in the N terminus (Arg-12 to Ser-17) with an RGG motif nearby in Gbp2p suggests that Sky1p may also mediate similar docking interactions with the RGG domain to phosphorylate the serines in the RS motif.

To test if Sky1p interacts with Gbp2p, we have expressed the RGG/SR domain of Gbp2p (residues 1–123) as a GST fusion protein (GST-Gbp2p1–123) (Fig. 6a). Gbp2p is an extremely unstable protein, and several attempts to purify this fusion protein to homogeneity failed. We tested its binding to wt Sky1p and the docking groove mutants (QM and 4M) using GST co-precipitation experiments (Fig. 6a). Pulldown assays were carried out in duplicates where two different amounts of these kinases (2 μg and 10 μg) were used. We observed Sky1p wt and QM bind similarly to Gbp2p, but the 4M mutant bound poorly. We also observed that SRPK1 could also bind Gbp2p. Together, these results suggest that Sky1p utilizes a similar binding mechanism to interact with Gbp2p and Npl3p.
To test if Sky1p phosphorylates these serines or other serines within the RGG/RS domain, we mutated all three serines in the RS repeat region at positions 13, 15, and 17 of GST-Gbp2p (1–123) to alanine. In vitro phosphorylation of the GST-Gbp2p (1–123) triple mutant by Sky1p was dramatically reduced compared with Gbp2p (1–123) wt (Fig. 6b). To test if multisite phosphorylation occurs in Gbp2p, we carried out kinase reactions with three single mutants where each of these serines is mutated to alanine (Fig. 6b). The kinase assay results demonstrated that Sky1p phosphorylates Gbp2p at multiple sites between Ser-13, Ser-15, and Ser-17. We further created a double mutant (S13A,S15A), which also undergoes phosphorylation by Sky1p (data not shown). Our experiment clearly suggests that Sky1p acts on this RS motif. An acidic residue at the P-2 position (Glu-409) in Npl3p was previously shown to be important for Npl3p phosphorylation (19). An aspartate at position 11 could serve as a P-2 residue with respect to Ser-13. We mutated this aspartate to alanine, and the mutant revealed no effect on Gbp2p phosphorylation. Finally, we investigated if Sky1p processively phosphorylates these serines using the trap assay (Fig. 6c). We did not observe phosphorylation of Gbp2p (over that of the negative control) at a concentration of 0.3 μM of the Sky1p-Gbp2p complex. In the absence of clear knowledge of apparent dissociation constant of the complex, it is difficult to know the exact mechanism of phosphorylation. The absence of even partial phosphorylation of Gbp2p suggests that multisite phosphorylation of this substrate may not occur in a processive manner.

**DISCUSSION**

In this study, we have demonstrated that, like SRPK1, Sky1p phosphorylates ASF/SF2 at multiple sites. SRPK1 phosphorylates ASF/SF2 at an average of 10–12 sites, whereas Sky1p phosphorylates 13 sites. These similar amounts of phosphorylation by both kinases suggest that identical serines of ASF/SF2 might also be phosphorylated by these kinases. Previously, we showed that both SRPK1 and Sky1p contain a peptide docking groove near the bottom of the large lobe of the kinase formed by an insert unique to the mitogen-activated protein kinase, helix αG, and the loop connecting helices αF and αG (24). The x-ray structures of Sky1p and SRPK1 bound to an Npl3p-derived peptide revealed that the kinase docking groove recognizes the peptide in a manner observed for many other peptide-protein complexes where the peptide occupies a groove in the kinase complementing it in both shape and size. Both Sky1p and SRPK1 utilize their docking grooves to interact with their respective substrates, Npl3p and ASF/SF2. We show here that Sky1p binds ASF/SF2 with high affinity and mutations in the Sky1p docking groove affect ASF/SF2 binding. Therefore, results shown here clearly suggest a conserved general mechanism of ASF/SF2 recognition and phosphorylation by both SRPK1 and Sky1p.

Like the conserved docking grooves in SRPK1 and Sky1p, the docking motifs of their substrates also share conserved features. The docking motif of ASF/SF2 lies between the second RRM and the RS domain is rich in basic residues. Similarly, the docking motif of Npl3p, imperfectly repeated throughout the entire RGG domain, is also rich in basic residues (25). These basic residues are the driving force for an electrostatic interaction with conserved acidic residues in the kinase docking groove, leading to substrate recognition. In the case of Gbp2p, the phosphorylation sites are located in the N-terminal portion RGG/RS domain, whereas an RGG repeat region resembling the docking motif of Npl3p is found C-terminal to the phosphorylation site. Although the physical orientation of the phosphorylation site and the docking motif are reversed in Npl3p and Gbp2p, their recognition by Sky1p is similarly affected by disruption of the acidic docking groove in Sky1p. Similar behavior in GST-co-precipitation experiments suggests that Sky1p recognizes Npl3p and Gbp2p similarly.

We have demonstrated that Sky1p possesses the ability to processively phosphorylate a heterologous SR protein substrate. This begs the question if there are native yeast substrates that also undergo processive phosphorylation. The most likely recipient of processive phosphorylation is Gbp2p, which we have demonstrated to be phosphorylated multiple times by Sky1p. However, trap assay experiments suggest a mechanism that is more likely to be distributive rather than processive. We did not determine the affinity of the Sky1p-Gbp2p complex. However, it is important to note that Gbp2p, like ASF/SF2, was able to pulldown both Sky1p and SRPK1 at ~100 nM concentration. We used 300 nM kinase and substrate, which is 3-fold above the concentration used in the pulldown experiments and well above that of the $k_d$ of the Sky1p-ASF/SF2 complex. In this concentration phosphorylation was completely inhibited. This lack of even some phosphorylation compared with the control suggests that phosphorylation of Gbp2p by Sky1p is most likely distributive. We can at least suggest that the mechanism of phosphorylation of SR and SR-like substrates by SRPKs is different.

The trap assay results raise an interesting issue of what dictates processive phosphorylation. Results from previous studies and experiments herein suggest a complex mechanism. In addition to the ability of a substrate having multiple phosphorylation sites and being able to bind the kinase with high affinity other criteria might be required. Perhaps the coordinated interaction between the kinase and substrate at the docking and active sites is essential for processive phosphorylation. It is important to note that the second RRM of ASF/SF2 is also involved in the binding interactions with SRPK1 and presumably with Sky1p as well. In contrast, the RRMs of Npl3p are not involved in any binding interaction. Using this analogy, it is possible that additional contacts and modes of these contacts through the RRM domain might be required for processive phosphorylation. Thus, all RGG proteins such as the viral protein ICP27 (26–28) with multiple phosphorylation sites may undergo distributive phosphorylation. Further investigation is required to resolve this issue.

Lastly, a clear role for processive phosphorylation in biological regulation has yet to be established. We suggest that processive phosphorylation is advantageous over distributive phosphorylation, because extremely fast phosphorylation achieved through processivity allows the substrates to be involved in their biological functions in a rapid manner in the nucleus. These functions are far more important in higher eukaryotes for their survival. In yeast, the rare occurrence of
splicing may not require these SR/RGG-like proteins to function in such a manner.

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