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Fission yeast Prp4p kinase regulates pre-mRNA splicing by phosphorylating a non-SR-splicing factor

Wiebke Schwelnus, Kathrin Richert, Florian Opitz, Thomas Groß, Yasuaki Habara[1], Tokio Tani[1] & Norbert F. Käufer[+]

Institute of Genetics, Technical University of Braunschweig, 38106 Braunschweig, Germany and [1]Department of Biology, Faculty of Science, Kyushu University, Fukuoka 812-81, Japan

We provide evidence that Prp4p kinase activity is required for pre-mRNA splicing *in vivo* and show that loss of activity impairs G_1–S and G_2–M progression in the cell cycle. Prp4p interacts genetically with the non-SR (serine/arginine) splicing factors Prp1p and Prp5p. Bacterially produced Prp1p is phosphorylated by Prp4p *in vitro*. Prp4p and Prp1p also interact in the yeast two-hybrid system. *In vivo* labelling studies using a strain with a mutant allele of the *prp4* gene in the genetic background indicate a change in phosphorylation of the Prp1p protein. These results are consistent with the notion that Prp4p kinase is involved in the control of the formation of active spliceosomes, targeting non-SR splicing factors.

INTRODUCTION

The recognition and removal of introns in eukaryotes is mediated by a highly complex and dynamic machinery called the spliceosome (Will and Lührmann, 1997). The basic RNA and protein components of this machinery have been well conserved throughout evolution; however, we found distinct differences when we compared the splicing factors of fission yeast *Schizosaccharomyces pombe* with those of *Homo sapiens* and the budding yeast *Saccharomyces cerevisiae* (Käufer and Potashkin, 2000).

The modification of some of the proteins by phosphorylation appears to play an important role in the splicing process. It has been shown that kinase activity in mammals is essential for pre-mRNA splicing *in vitro* (Tazi *et al.*, 1993; Mermoud *et al.*, 1994). In mammals, several protein kinases have been identified that specifically phosphorylate the RS domain of the SR-splicing

factors *in vitro* (Gui *et al.*, 1994; Colwill *et al.*, 1996; Rossi *et al.*, 1996). SR proteins consisting of one or two RNA-binding domains (RBDs) followed by an RS domain are involved in constitutive and differential pre-mRNA splicing (Manley and Tacke, 1996). The homologue of human SR protein kinase 1 (SRPK1) in fission yeast, Dsk1p, also phosphorylates SR proteins including Srp1p and Srp2p of fission yeast, the first typical SR proteins discovered in a unicellular organism (Tang *et al.*, 1998, 2000; Groß *et al.*, 1998; Lützelberger *et al.*, 1999).

In fission yeast and mammals, we identified the protein kinase Prp4p (Groß *et al.*, 1997). Budding yeast does not contain a kinase with sequence similarity to Prp4p (Käufer and Potashkin, 2000). Prp4p kinase is essential for growth, and the temperature sensitive allele *prp4-73* shows a defect in pre-mRNA splicing. A strain containing this allele stops growing at the restrictive temperature, which correlates with the accumulation of pre-mRNA and the disappearance of mRNA (Rosenberg *et al.*, 1991; Alahari *et al.*, 1993; Groß *et al.*, 1997). An extragenic suppressor, Spp42p, of this defect encodes a homologue of the mammalian and budding yeast splicing factor p220/Prp8p (Schmidt *et al.*, 1999). Prp8p is a unique and highly conserved protein associated with U5 snRNP and is thought to be a major player in the switch from inactive to active spliceosome (Murray and Jarrell, 1999; Käufer and Potashkin, 2000).

We show in this report that Prp4p kinase activity is required for splicing *in vivo* whereas loss of activity leads not only, as expected, to the accumulation of pre-mRNA, but also to a cell cycle arrest phenotype, suggesting that kinase activity is essential for the transition of G_1 to S and of G_2 to M phase. Furthermore, *prp4-73* is synthetically lethal with *prp1-4* and *prp5-1*,

[+]Corresponding author. Tel: +49 531 391 5774; Fax: +49 531 391 5765; E-mail: n.kaeufer@tu-bs.de
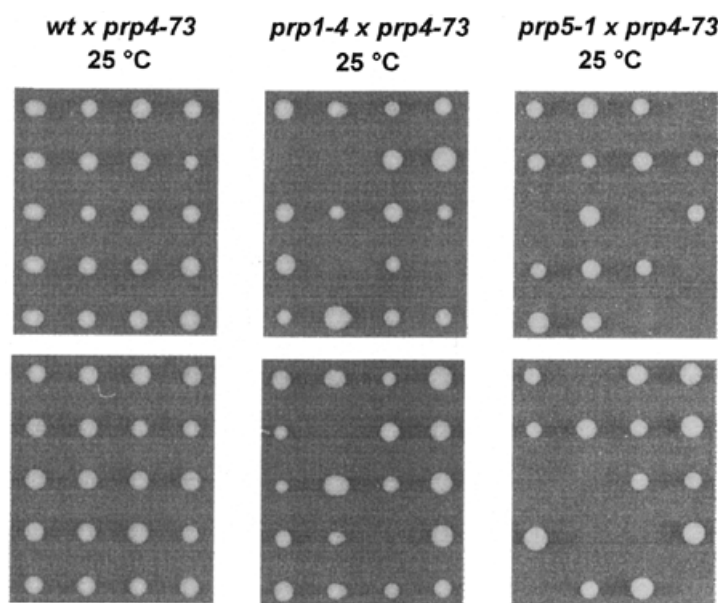
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Fig. 1. Synthetic lethality of *prp4-73* with non-SR splicing components. Tetrad analyses of crosses of the indicated mutant strains, at 25°C. Four growing colonies, parental ditype, PD; two growing colonies, nonparental ditype, NPD; three growing colonies, tetratype,TT.

indicating a functional relationship of Prp4p kinase with two additional highly conserved splicing factors. Bacterially produced Prp1p is phosphorylated by Prp4p *in vitro*. Prp1p and Prp4p also interact in the yeast two-hybrid system. We also provide evidence that the mutant allele *prp4-73* causes a change in the phosphorylation of Prp1p *in vivo*. These data are consistent with our suggestion that Prp4p may play the role of a signalling kinase, controlling the formation of active spliceosomes by activating non-SR components.

RESULTS AND DISCUSSION

Genetic interactions of Prp4p kinase with splicing factors

All fourteen temperature sensitive strains *prp1-prp14* (pre-mRNA processing) accumulate pre-mRNA at the restrictive temperature (Potashkin *et al.*, 1989; Rosenberg *et al.*, 1991; Urushiyama *et al.*, 1996). In addition, the strains containing the temperature-sensitive alleles *prp2-1*, *prp5-1*, *prp6-1* and *prp8-1/cdc28–1* arrest at the restrictive temperature with a 2C DNA content (Lundgren *et al.*, 1996; Potashkin *et al.*, 1998; Beales *et al.*, 2000).

In order to screen for genetic interactions with *prp4-73*, we crossed *prp4-73* with each of the *prp* strains and performed tetrad analyses. Spores were grown at the permissive temperature of 25°C. This analysis revealed that the double mutants *prp1-4 prp4-73* and *prp5-1 prp4-73* are not viable at the permissive temperature of 25°C (Figure 1). Whenever the *prp4-73* allele segregates with one of the indicated mutant alleles the spores do not grow out. This phenotype, called synthetic lethality, indicates a functional interaction between Prp4p protein kinase and these components.

Prp1p, a tetratricopeptide protein containing 19 repeats of a 34 amino acid motif (TPR) with an M_r of ~100 kDa, has been

identified and characterized by Urushiyama *et al.* (1997). Prp1 shows a polymorphic phenotype at the restrictive temperature. The investigation of several alleles, such as *prp1-1*, *prp1-4* and *zer1*, indicates that Prp1p is involved in pre-mRNA splicing, poly(A)$^+$ transport and cell cycle progression (Urushiyama *et al.*, 1997). The budding yeast homologue, Prp6p, was found to associate with snRNP U4/U6–U5 (Galisson and Legrain, 1993). Recently, the human homologue of Prp1p/Prp6p has been identified, tightly associated with the U5 snRNP (Makarov *et al.*, 2000). It is interesting to note here that the fission yeast Prp1p and human Prp1p share 66% similar amino acids, whereas fission yeast Prp1p and budding yeast Prp6p share 46% similar amino acids (Käufer and Potashkin, 2000).

Prp5p is a protein containing seven WD motifs. Mass spectrometry of proteins isolated from *in vitro* assembled spliceosomes using HeLa cell extracts identified the Prp5p homologue as a spliceosomal protein (Neubauer *et al.*, 1998). There are some hints that Prp5p might also be a U5 snRNP protein. The protein is highly conserved between mammals, fission yeast and budding yeast (Käufer and Potashkin, 2000).

Prp4p kinase and Prp1p mutations arrest the cell cycle in G_1 and G_2

Next, we investigated *in vivo* the consequences of a loss-of-function mutation in the kinase domain of Prp4p when over-expressed in a *prp4* background. For this purpose a strain containing the mutant allele *prp4-K189I* was constructed. We have replaced lysine in kinase subdomain II at position 189 with isoleucine (Figure 2). This lysine is involved in positioning ATP near the active site, to mediate proper catalysis, and has been found in many kinases invariable for function *in vivo* and *in vitro*. The *prp4-73* allele harbours a mutation in kinase subdomain IV. Instead of a cysteine, a tyrosine residue is found
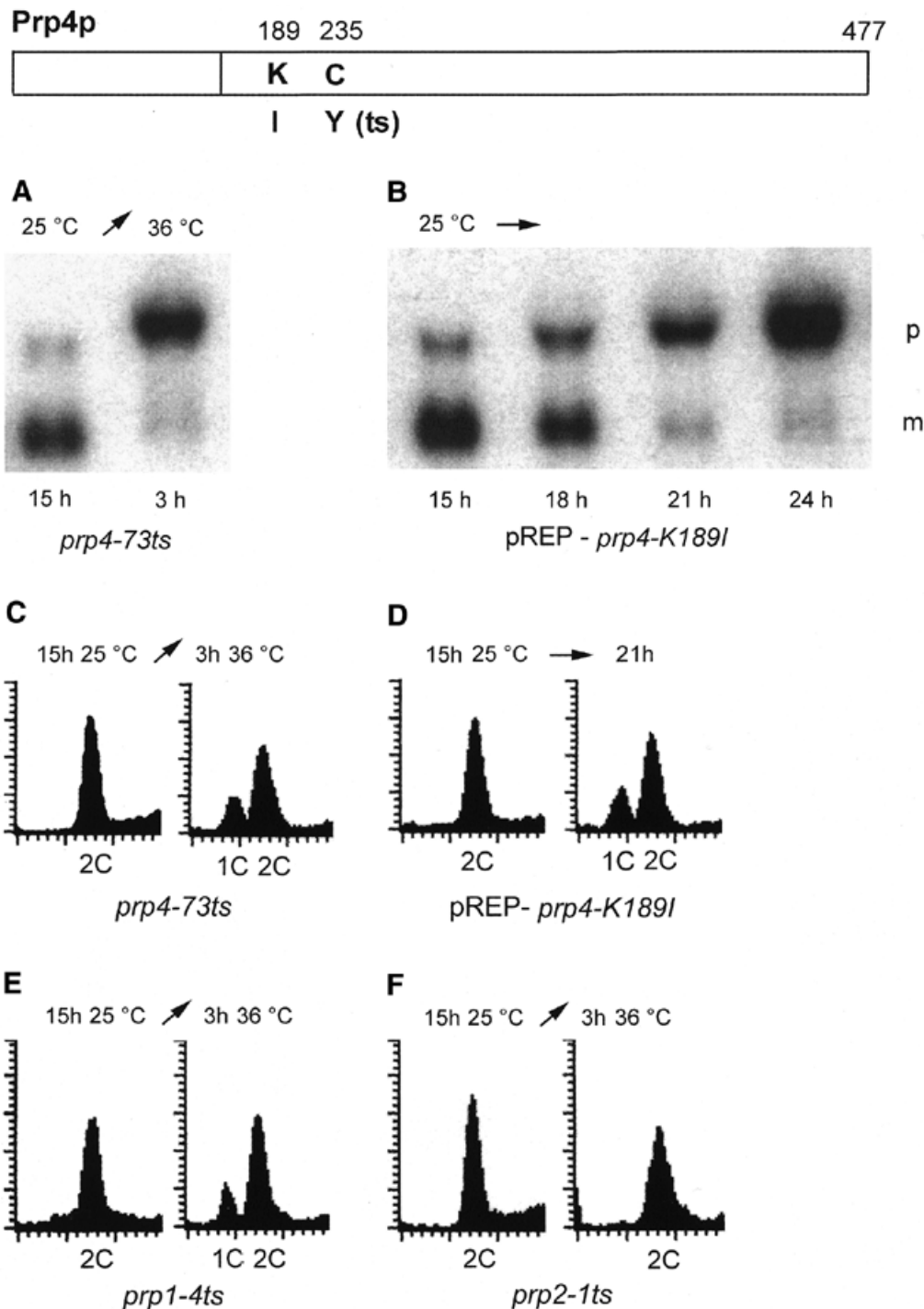
Fig. 2. Response of cells overexpressing mutant Prp4p K189I. Northern analysis: (**A**) *prp4-73ts* strain 15 h after growth at the permissive temperature (25°C) and after a shift to the restrictive temperature (36°C) for 3 h; (**B**) the strain overexpressing the mutant allele after the indicated incubation time at 25°C in medium inducing the expression of pREP *prp4-K189I*. p, pre-mRNA; m, mRNA. The radiolabelled probe used is the complete *tfIId* gene, which encodes the TATA-binding protein, containing three introns. Each lane contains 15 μg total RNA. FACS analysis: cells were stained with propidium iodide and analysed by flow cytometry. (**C**) *prp4-73ts* strain after 15 h at 25°C and 3 h at 36°C; (**D**) strain expressing pREP *prp4-K189I* after the indicated time period at 25°C; (**E**) *prp1-4ts* strain after 15 h at 25°C and 3 h at 36°C; (**F**) *prp2-1ts* strain after 15 h at 25°C and 3 h at 36°C. The peaks labelled 1C and 2C represent cells with DNA contents characteristic of those in the G_1 and G_2 phase of the cell cycle, respectively.

at position 235. This subdomain is also involved in ATP binding (Taylor and Radzio-Andzelm, 1994). A strain containing *prp4-73* ceases growing at the restrictive temperature within 3 h, which correlates with the accumulation of pre-mRNA and the

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disappearance of mRNA (Figure 2A). The mutant allele *prp4-K189I*, driven by the thiamine-repressible *nmt1* promoter, was transformed in a *prp4* background. The transformed strain grows on plates supplemented with thiamine, but does not grow on plates without thiamine (results not shown). For further analysis, we inoculated the strain pREP-*prp4-K189I* in medium without thiamine. Cells overexpressing *prp4-K189I* accumulate pre-mRNA at 25°C (Figure 2B). The accumulation of pre-mRNA also coincides with ceasing growth, which comes to a halt after 21 h (results not shown). In this approach we used the constitutively expressed *tfIId* gene as a probe; however, all other intron-containing genes tested also accumulate pre-mRNA.

We further analysed *prp4-73* at the restrictive temperature and the strain overexpressing *prp4-K189I* by flow cytometry. In both strains we observed the same arrest phenotype. In correlation with ceasing growth and the accumulation of pre-mRNA, the cultures show a significant cell population with a 1C DNA content, and another with a 2C DNA content (Figure 2C and D). Whereas this FACS phenotype is seen when the mutations are in the C-terminal kinase domain, most likely causing a decrease of kinase activity, we found previously that expression of mutations in the N-terminal domain of Prp4p leads to the impairment of mitosis (Groß *et al.*, 1997).

We also analysed the *prp1-4* allele, which is synthetically lethal with *prp4-73*, by flow cytometry and found that some cells arrest in G_1, and others in G_2 at the restrictive temperature (Figure 2E). Remarkably, all the other *prp* mutants display a 2C DNA content at the restrictive temperature in this analysis. As an example and control we used *prp2-1* (Figure 2F). These results indicate that Prp4p, as well as Prp1p activity, is essential for G_1–S and for G_2–M transitions.

Prp4p and Prp1p interact in the two-hybrid system

We investigated the interaction of Prp4p and Prp1p in the yeast two-hybrid system. In this approach we fused full-length Prp4p cDNA to the Gal4p DNA-binding domain, generating the plasmid pGB-Prp4, and fused the *prp1* coding region to the activation domain in a plasmid called pGAD-Prp1, and vice versa (Figure 3). Several colonies containing both plasmids were analysed for the stimulation of the reporter gene β-galactosidase using a luminiscence-photometer. In both cases the interaction between Prp4p and Prp1p appears to be specific (Figure 3), although Prp1p fused with the DNA-binding domain generated a background activation of the reporter gene by itself (Figure 3B) and the activity measured is relatively low when compared with the activity induced by the interaction of p53 and SV40 large T-antigen, which were used as a positive control.

Prp4p kinase phosphorylates Prp1p *in vitro*

We constructed a Prp1p protein containing a His_6 tag at the N-terminus. The recombinant protein complements the *prp1-4* allele when expressed in *S. pombe* (results not shown). We produced His_6-Prp1p in bacteria and isolated it from the bacterial extract using the His-tag system. Then, HA-tagged Prp4p kinase was immunoprecipitated from a *S. pombe* extract using anti-HA antibodies. HA-tagged Prp4 kinase also complements the *prp4-73* allele. We performed *in vitro* kinase assays with the immunoprecipitate, using $[\gamma\text{-}^{32}P]ATP$. Adding increasing
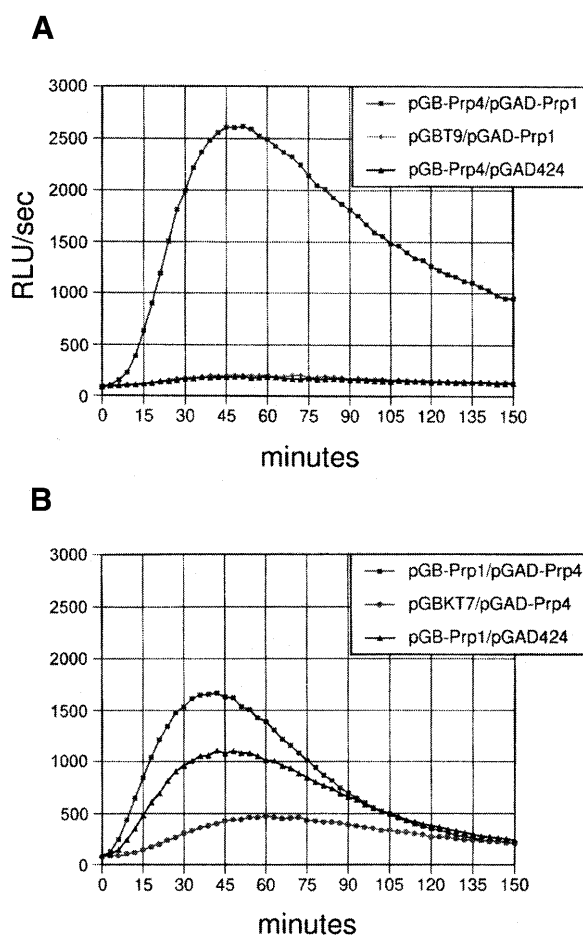
Fig. 3. Two-hybrid interaction between Prp4p and Prp1p. The yeast cells (Y190) containing the plasmids in the indicated combinations were cultured in SD media lacking leucine and uracil to mid log phase. Then, samples were mixed with an equal volume of Gal-Screen™ (Tropix, Inc.). The β-galactosidase activity in each mixture was then measured using a Microplate Luminometer LB96V (EG & G. Berthold).

amounts of bacterially produced His_6-Prp1p to the assay leads to the phosphorylation of a protein with a M_r of ~100 kDa (Figure 4B). The protein is recognized on a western blot by anti-His antibodies (Figure 4A). The phosphorylated protein at ~62 kDa is recognized by anti-HA antibodies, suggesting that Prp4p protein kinase strongly autophosporylates *in vitro* (Figure 4B and C). In contrast, the mutant protein HA-Prp4K189I, which was immunoprecipitated and used in the kinase assay, does not autophosphorylate, nor do we detect phosphorylation activity towards recombinant His_6-Prp1p (Figure 4B). We also fused glutathione *S*-transferase (GST) to Prp4p, produced the recombinant GST–Prp4p molecules in bacteria and isolated them using glutathione–Sepharose. The fusion protein, GST–Prp4p, and Prp4p after the removal of GST show kinase activity towards His_6-Prp1p (Figure 4D). These results provide clear evidence that Prp4p kinase is capable of phosphorylating Prp1p *in vitro*, and unambiguously show that the mutation of lysine 189 to isoleucine in Prp4p leads to the abolition of kinase activity *in vitro*.
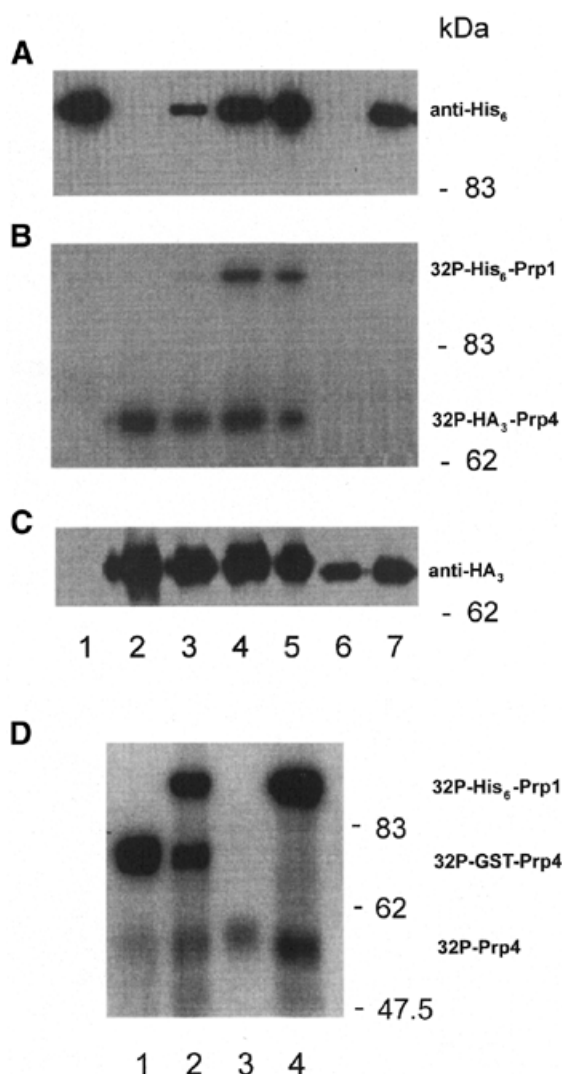
Fig. 4. Phosphorylation of bacterially produced His$_6$-Prp1p by immuno-precipitated HA-Prp4p and bacterially produced GST–Prp4p. Radiolabelled ATP was used in the kinase assay. SDS–PAGE electrophoresis. (**A**) Western blot analysis using anti-His antibodies as probe. (**B**) Autoradiography. (**C**) Western blot analysis using anti-HA antibodies as probe: lane 1, His$_6$-Prp1p; lane 2, HA-Prp4p; lane 3, His$_6$-Prp1p (0.05 µg) + HA-Prp4p; lane 4, His$_6$-Prp1p (0.3 µg) + HA-Prp4p; lane 5, His$_6$-Prp1p (0.6 µg) + HA-Prp4p; lane 6, HA-Prp4p K189I; lane 7, His$_6$-Prp1p (0.3 µg) + HA-Prp4p K189I. (**D**) Autoradiography: lane 1, GST–Prp4p (0,6 µg); lane 2, His$_6$-Prp1p (2 µg) + GST–Prp4p (0,6 µg); lane 3, PreScission Protease™ + GST–Prp4p (0,6 µg); lane 4, PreScission Protease™ + GST–Prp4p (0,6 µg) + His$_6$-Prp1p (2 µg).

In vivo phosphorylation of Prp1p

We introduced an HA-tagged *prp1+* gene into the genome of a strain containing the *prp4-73* allele. The strain grows normally at 25°C and stops dividing 3 h after the shift to the restrictive temperature. This strain was labelled *in vivo* with [^{32}P]*ortho*-phosphate for 4 h at the permissive temperature of 25°C and at the restrictive temperature of 36°C. Total protein was extracted and separated by SDS–PAGE. In the protein extract of the strain labelled at 25°C, we detect a signal in the range of M_r 100 kDa, which is much less intense in the protein extract of the cells
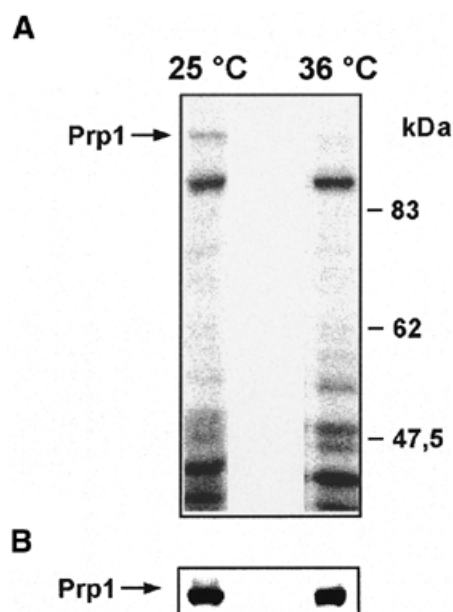

Fig. 5. *In vivo* labelling of HA-tagged Prp1p. [^{32}P]*ortho*-phosphate was incubated for 4 h with a strain containing an HA-tagged *prp1+* and the *prp4-73* allele at 25 and 36°C. Total protein extract was subjected to SDS–PAGE. (**A**) Autoradiography. (**B**) Western blot analysis using HA antibodies as probe; the arrow indicates the labelled Prp1p protein.

labelled at 36°C (Figure 5A, arrow). A western blot analysis using HA-antibodies as probe indicates that the signal identified at 25°C is phosphorylated HA-Prp1p, which is not detected at 36°C (Figure 5B, arrow). These results indicate that *in vivo* phosphorylation of Prp1p appears to be dependent on Prp4p activity. Several labelling experiments in this series revealed that the amount of phosphorylated Prp1p must be very low *in vivo*.

This observation is consistent with the finding that we cannot co-immunoprecipitate Prp4p and Prp1p unless we overproduce both proteins on expression vectors (results not shown).

CONCLUSION

Collectively, the genetic and biochemical data presented above are consistent with the idea that Prp4p kinase activity is required for pre-mRNA splicing *in vivo*. The genetic and biochemical interactions between Prp4p and Prp1p, and the Prp4p kinase-dependent phosphorylation of Prp1p *in vivo*, suggest that Prp1p is a physiological substrate of Prp4p. In addition, the genetic interaction of *prp4* with several genes encoding splicing components is consistent with our proposal that Prp4p is a kinase involved in the formation of active spliceosomes, targeting non SR-splicing factor(s). It remains to be determined whether Prp4p kinase functions as a signalling kinase, for example, labelling pleiotropic factors, such as Prp1p, as splicing factors. The human homologue of Prp1p has been discussed as functioning as a bridge between snRNPU5 and snRNPU4/U6 (Makarov *et al.*, 2000). Therefore, it is conceivable that phosphorylation of Prp1p is part of a control mechanism to assemble a proper tri-snRNPU4/U6–U5 as a prerequisite for an active spliceosome. This is the first report

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showing that a kinase is involved in splicing regulation via phosphorylation of a non-SR splicing factor.

METHODS

Plasmid constructs and mutagenesis. The N-terminal HA-tagged expression vector pREP42 HA was used to insert *prp4* cDNA using *Bam*HI as cloning site as described (Craven *et al.*, 1998; Lützelberger *et al.*, 1999). To produce Prp1p in bacteria, a *Sal*1 fragment containing the ORF of *prp1* was cloned into the vector pQE31 containing an N-terminal His-tag (Qiagen). All constructs were sequenced. The His-tag was used to isolate bacterially produced His$_6$-Prp1p via Ni-NTA–agarose and to detect His$_6$-Prp1p with the monoclonal anti-His antibodies. To construct the mutant allele *prp4-K189I*, site-specific mutagenesis was performed as described previously (Groß *et al.*, 1997; Lützelberger *et al.*, 1999).

***In vitro* phosphorylation assay.** Protein extracts were made exactly as described (Groß *et al.*, 1997). For immunoprecipitation, 400 µl of protein extract were used incubated with anti-HA antibodies for 2 h at 4°C. Twenty-five microlitres of protein A–Sepharose were added and incubated for a further 2 h at room temperature. The immunoprecipitate was first washed with 10 mM Tris pH 7.5, 0.4 M NaCl, 0.5 mM dithiothreitol (DTT), followed by 10 mM Tris pH 7.5, 1 M NaCl, 0.5 mM DTT, and then with kinase buffer (20 mM HEPES, 3 mM MgCl$_2$, 0.5 mM DTT, 5% glycerol). Kinase assays were performed in a 20 µl volume containing kinase buffer, 5 µCi [γ-^{32}P]ATP, 100 µM ATP and 5 µl immunoprecipitate. Bacterially produced Prp1p was added in the range of 0.05–0.6 µg protein. The samples were incubated at 37°C for 30 min.

***In vivo* labelling.** Cultures were labelled as described by Moreno *et al.* (1991). Briefly, cells were grown in minimal medium (EMM) plus supplements at 25°C to mid log phase. Growing cells were resuspended in minimal low phosphate medium (EMMP). Five millilitres of the culture (5 × 10^6/ml) were kept at 25°C; another 5 ml of culture were shifted to 36°C. To each culture 0.5 mCi [^{32}P]*ortho*-phosphate was added and incubated for 4 h. After 4 h labelling time, the cells were diluted with 15 ml of cultures kept under the same conditions without [^{32}P]*ortho*-phosphate. Protein was extracted as described previously (Groß *et al.*, 1997).

ACKNOWLEDGEMENTS

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