Dominant negative mutants of the yeast splicing factor Prp2 map to a putative cleft region in the helicase domain of DExD/H-box proteins

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ABSTRACT

The Prp2 protein of *Saccharomyces cerevisiae* is an RNA-dependent ATPase required before the first transesterification reaction in pre-mRNA splicing. Prp2 binds to the spliceosome in the absence of ATP and is released following ATP hydrolysis. We determined what regions in Prp2 are essential for release from the spliceosome by analyzing dominant negative mutants in vivo and in vitro. We made mutations in conserved motif II (DExH) and motif VI (QRxGR) of the helicase (H) domain. Mutations that inactivated *PRP2* had a dominant negative phenotype when overexpressed in vivo. To test whether mutations outside of the H domain could confer a dominant negative phenotype, we mutagenized a *GAL1-PRP2* construct and screened for mutants unable to grow on galactose-containing media. Five dominant negative mutants were characterized; three mapped within the H domain and two mapped downstream of motif VI, indicating that an extended helicase domain is required for release of Prp2 from the spliceosome. Most mutants stalled in the spliceosome in vitro. However, not all mutants that were dominant negative in vivo were dominant negative in vitro, indicating that multiple mechanisms may cause a dominant negative phenotype. Structural modeling of the H domain of Prp2 suggests that mutants map to a cleft region found in helicases of known structure.

Keywords: DExH proteins; pre-mRNA splicing; RNA-dependent ATPase; structural modeling; yeast *PRP* genes

INTRODUCTION

Pre-mRNA splicing is a multistep process in which introns are removed from pre-mRNAs and exons are joined. Reactions occur in the spliceosome, a dynamic RNA–protein assembly that contains U1, U2, U4/U6, and U5 small ribonucleoprotein particles (snRNPs) as well as a number of non-snRNP proteins complexed with the pre-mRNA (Sharp, 1994; Burge et al., 1999). Following spliceosome assembly, splicing proceeds via two sequential transesterification reactions (Moore et al., 1993; Burge et al., 1999). ATP hydrolysis is required for pre-mRNA splicing, not for the transesterification reactions themselves, but perhaps for the many RNA rearrangements that are required throughout spliceosome

assembly (Ares & Weiser, 1995; Nilsen, 1998; Staley & Guthrie, 1998).

There are a number of RNA-dependent ATPases in the splicing pathway whose role may be to catalyze the RNA rearrangements that occur at many steps in splicing (Ares & Weiser, 1995; Nilsen, 1998; Staley & Guthrie, 1998). The RNA-dependent ATPases identified in the yeast *Saccharomyces cerevisiae* include Prp2 (Kim et al., 1992), Prp5 (O'Day et al., 1996), Prp16 (Schwer & Guthrie, 1991), Prp22 (Company et al., 1991), Prp28 (Strauss & Guthrie, 1991), Prp43 (Arenas & Abelson, 1997), and Brr2/Prp44 (Noble & Guthrie, 1996; Kim & Rossi, 1999) [also known as Rss1 (Lin & Rossi, 1996), Slt22 (Xu et al., 1996), Snu246 (Lauber et al., 1996)]. Prp16, Prp22, Brr2/Prp44, and its human counterpart the U5–200-kDa protein, have RNA helicase activity in vitro (Laggerbauer et al., 1998; Raghunathan & Guthrie, 1998; Schwer & Gross, 1998; Wagner et al., 1998; Wang et al., 1998; Kim & Rossi, 1999). The in vivo substrates of these enzymes are not known, although Brr2/Prp44 and the U5–200-kDa protein may unwind U4/U6 duplexes (Laggerbauer et al., 1998;

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Ragunathan & Guthrie, 1998; Kim & Rossi, 1999) and Prp28 plays a role in unwinding the U1:5' splice site interaction (Staley & Guthrie, 1999). The RNA-dependent ATPases are part of a larger DExD/H-box family of helicases or helicase-like proteins (Fuller-Pace, 1994; Will & Luhrmann, 1997; Hamm & Lamond, 1998; Staley & Guthrie, 1998). They have a similar domain structure, with a high degree of amino acid conservation in the ATPase, or helicase (H), domain. The ATPase domain can be subdivided into seven conserved motifs that are involved in ATP binding, Mg^{2+} binding, and nonspecific RNA binding (reviewed in Hamm & Lamond, 1998; Staley & Guthrie, 1998).

Prp2, an RNA-dependent ATPase, is required prior to the first transesterification reaction in pre-mRNA splicing (King & Beggs, 1990; Kim & Lin, 1993, 1996; Plumpton et al., 1994). Prp2 binds to the pre-mRNA-containing spliceosome in the absence of ATP and is released from the spliceosome following ATP hydrolysis. In this study we investigated what regions in Prp2 are essential for release of Prp2 from the spliceosome. When a release-mutant Prp2 protein is overexpressed, splicing and cell growth are inhibited (Plumpton et al., 1994), which is a dominant negative phenotype. We made mutations in motif II (the DExH box) and motif VI (QRxGR) of the conserved helicase domain by site-directed mutagenesis. Mutants that could not substitute for wild-type Prp2 also had dominant negative phenotypes in vivo. We wanted to determine what regions of Prp2 might be required for release from the

spliceosome in addition to conserved motifs in the H domain. We isolated dominant negative mutants by random mutagenesis of a *GAL1-PRP2* construct, selecting for mutants that could not grow on galactose-containing medium. All mutants mapped within or just C-terminal to the H domain. Some mutants bound to the spliceosome in the presence and absence of ATP and inhibited splicing by wild-type Prp2 in vitro as had been previously demonstrated for a mutant in motif III (SAT) of Prp2 (Plumpton et al., 1994). Other mutants showed different degrees of spliceosome binding and spliceosome inhibition in vitro, which indicates that not all dominant negative H domain mutants act by stalling in the spliceosome. Structural modeling of the H domain of Prp2 based on helicases of known structure suggests that the mutations map to a cleft region that juxtaposes residues essential for H domain activity.

RESULTS

Site-directed mutagenesis of conserved H domain motifs in *PRP2*

We took a genetic approach to identify potential *PRP2* spliceosome-release mutants by screening for a dominant negative phenotype upon overexpression of the mutant Prp2 protein. First, site-directed changes were made in motif II, the DExH box, and motif VI, QRxGRxGR, of the helicase domain (Fig. 1). Previous studies on other RNA-dependent ATPases have dem-

FIGURE 1. Illustration of the restriction sites used for mapping mutants, domain structure of Prp2, and alignment with Prp16, Prp22, Prp43, and the Hepatitis C virus (HCV) NS3 proteins. N: the unique N-terminal domain; H: the highly conserved helicase domain; C: the C-terminal domain conserved among the DEAH family members Prp2, Prp16, Prp22, and Prp43. The approximate location of restriction enzyme sites used in plasmid mapping experiments is shown. Conserved motifs in the H domain of Prp2, Prp16, Prp22, Prp43, and HCV NS3 proteins are indicated by Roman numerals. The location of the site-directed and random H domain mutants made in this study are indicated by the numbers above the sequence and in bold within the sequence, with the amino acid changes indicated underneath.

onstrated the importance of these motifs in ATP hydrolysis and RNA helicase activity (Schmid & Linder, 1991; Pause & Sonenberg, 1992). We changed histidine (H) at amino acid 349 of *PRP2* to aspartic acid (D), glutamic acid (E), lysine (K), asparagine (N), or tyrosine (Y). These changes were chosen because the substitutions represent different subgroups of amino acids. We also made a double mutant, which included the mutation of histidine 349 to aspartic acid in motif II and a change in the glutamine (Q) 548 in motif VI to histidine. The double changes make the Prp2 protein more like the DEAD family, such as eIF-4a (Schmid & Linder, 1991; Pause & Sonenberg, 1992).

Some motif II mutants cannot substitute for Prp2 in vivo

The effect of the H349X mutations on *PRP2* function was tested using a plasmid shuffle assay (Sikorski & Boeke, 1991) in which the chromosomal copy of *PRP2* was disrupted by the *TRP1* gene and *PRP2* function was provided on the *URA3*-containing plasmid YCp51-*PRP2* in strain YTY1 (see Materials and methods). Mutations were introduced by site-directed mutagenesis of the plasmid pRS415-*PRP2* and were transformed into YTY1. If the mutant retains Prp2 activity, the trans-

formants can survive in the absence of the YCp51-*PRP2* (*URA3*) plasmid and can grow on 5-fluoroorotic acid (5-FOA). Cells containing a glutamic acid, aspartic acid, or lysine at position 349 could not grow in the presence of 5-FOA, indicating that these changes inactivated the Prp2 protein (Table 1). The double mutation H349D/Q548H also inactivated the protein. A change to either asparagine or tyrosine at position 349 in Prp2 allowed cells to grow in the presence of 5-FOA, and the growth rates of both mutants were similar to wild-type (Table 1; data not shown).

Three of the mutant *PRP2* genes that could not substitute for *PRP2* in the plasmid shuffle assay (H349D, H349K, and H349D/Q548H) were subcloned into the galactose-inducible pYES2 2 μ vector and the YCp-GAL CEN vector to test whether they would have a dominant negative phenotype when overexpressed in vivo. Mutants H349K, H349D, and the double mutant H349D/Q548H all grow on glucose, the suppressed condition, but not on galactose, the induced condition (Table 1; Fig. 2). The extent of growth inhibition was not as great when the H349D mutant was expressed from the low copy vector YCp-GAL at 33 °C or 37 °C, suggesting that the H349D mutant may be partially temperature-sensitive. The H349D/Q548H and H349K mutants inhibited growth equally when expressed from

TABLE 1. Phenotype of H domain mutants in vivo and in vitro.

Prp2	Rescue of Ts	Rescue of null	Overexpression				Splicing complementation	Spliceosome binding	Splicing competition
			2μ	CEN					
				26 °C	33 °C	37 °C			
Wild-type	+	+	+	+	+	+	+	+	+
H349D	–	–	–	–	±	+	–	+	+
H349E	–	–	ND	–	ND	ND	ND	ND	ND
H349K	–	–	–	–	–	–	–	–	–
H349N	+	+	ND	+	+	+	ND	ND	ND
H349Y	ND	+	ND	ND	–	–	ND	ND	ND
H349D/Q548H	–	–	–	–	–	–	–	+	+
G251D/S378L	ND	ND	–	ND	–	–	ND	ND	ND
P399S/G444E	–	ND	–	ND	–	–	–	±	±
G551N	+	–	–	ND	–	–	–	+	+
E573K	–	ND	–	ND	–	–	–	+	+
E573K/588ter	ND	ND	+	ND	–	–	–	–	–
T583I	–	ND	–	ND	–	–	–	±	±

Phenotype designation for in vivo experiments: +: wild-type growth; ±: some growth; –: no growth; ND: not determined. Rescue of the Ts phenotype was measured by transformation of *GAL-PRP2* plasmids into the *prp2-1Ts* strain D12 (for site-directed mutants) or 3.2A10C (for random mutants) and then assessing growth on SC-glucose at 26 °C, the permissive temperature, or 37 °C, the nonpermissive temperature. Rescue of null was measured in a plasmid-shuffle assay by the ability to grow on 5-FOA (see Materials and methods). The overexpression phenotype was assayed by transformation of either the pYES2 (2 μ) or YCp (CEN) *PRP2* plasmids into the wild-type strain BJ168 and then assessing growth on galactose by streaking and dilution plating. Phenotype designation for in vitro experiments: splicing complementation measured the ability of the mutant Prp2 protein to complement splicing in the absence of wild-type Prp2; +: spliced products are produced; –: no spliced products are produced. Spliceosome binding was assayed by immunoprecipitation of spliceosomes using anti-Prp2 antibodies in the presence and absence of ATP; +: strong binding, ±: intermediate binding, –: weak binding. Only wild-type Prp2 showed different binding characteristics $\pm$ ATP. Splicing competition was measured by the ability of the mutant Prp2 to inhibit splicing by wild-type Prp2; +: strong inhibition of splicing; ±: intermediate inhibition of splicing; –: weak inhibition. In most cases, in vitro results were quantified using a Molecular Dynamics PhosphorImager.

FIGURE 2. *PRP2* mutants that cannot substitute for wild-type *PRP2* have dominant negative phenotypes. Yeast strain BJ168 was transformed with the indicated pYES2-*PRP2* plasmids. Isolated colonies grown on SC-glucose were streaked onto SC-glucose or SC-galactose plates. Plates were photographed after 3 days (glucose) or 6 days (galactose) of growth at 30 °C.

either vector at all temperatures (Table 1). Therefore, the *PRP2* mutations we identified in motifs II or VI that cannot substitute for wild-type Prp2 function have dominant negative phenotypes in vivo.

Dominant negative mutants isolated after random mutagenesis of *PRP2* map within or near the H domain

Studies on *PRP2* and other members of the DExD/DExH protein families have shown that it is possible to create dominant negative mutants by mutation of con-

served motifs in the H domain (Schmid & Linder, 1991; Pause & Sonenberg, 1992; Plumpton et al., 1994; Ohno & Shimura, 1996; Arenas & Abelson, 1997; Hotz & Schwer, 1998; Schwer & Gross, 1998; Wagner et al., 1998; this study). We wanted to determine if dominant negative mutants in *PRP2* isolated by random mutagenesis would also map to the H domain or would map to other regions of *PRP2*. We mutagenized the *PRP2* gene using hydroxylamine and placed it behind the inducible *GAL1* promoter in the plasmid pYES2 (see Materials and methods). Wild-type yeast was transformed and single colonies growing on glucose were

screened for the ability to grow on galactose. There were 66 galactose-sensitive colonies isolated after three trials of mutagenesis, and 10 dominant negative mutants were characterized further.

The mutants were mapped by plasmid swap with wild-type *PRP2* to determine the location of the mutations (see Fig. 1 and Materials and methods). Regions of wild-type *PRP2* were replaced with fragments from the dominant negative clones to see which fragments conferred a Gal⁻ phenotype to the wild-type construct. All mutants mapped upstream of the *PvuII* site in *PRP2*, within the first 660 amino acids. Sequencing of the mutants showed that all mapped within or close to the H domain (Fig. 1). Five of the mutants contained a double mutation P399S/G444E; residue 399 is between motifs III and IV and residue 444 is in motif IV. The mutant G251D/S378L has mutations in motif I, the Walker A motif, and motif III, SAT. The mutant G551N has a mutation in motif VI, QRAGR. The residues mutated in these three mutants are conserved in Prp16 and Prp22 (Fig. 1). Two of the mutants, E573K (isolated twice) and T583I, are 20 and 30 amino acids, respectively, downstream of motif VI in a region conserved among the DExH family members Prp16, Prp22, and Prp43, but not in the larger DExD/H protein family.

To examine whether the dominant negative mutants retained Prp2 activity in vivo, plasmids harboring the mutant *PRP2* genes were transformed into the temperature-sensitive *prp2-1* strains 3.2A10C or D12 and then transformants were tested for growth on SC-glucose at 26 °C and 37 °C (Table 1). There is a low level of expression from the *GAL1* promoter in the pYES2 vector when cells are grown on glucose. Transformation of wild-type *PRP2* permits growth at 37 °C, the nonpermissive temperature. None of the dominant negative mutants except G551N were able to rescue, indicating that these mutant Prp2 proteins are not active in splicing in vivo. It was unexpected that the motif VI mutant G551N, which has a strong dominant negative phenotype both in vivo and in vitro, rescued the temperature-sensitive phenotype of 3.2A10C (Table 1). Nevertheless, the G551N mutant was not able to substitute for wild-type *PRP2* at 26 °C, 30 °C, 33 °C, or 37 °C as measured in a plasmid shuffle assay (Table 1; data not shown).

Mutant Prp2 proteins are stably expressed after galactose induction

To confirm that the mutant proteins were overexpressed after galactose induction, western blots were performed on extracts derived from cells grown in raffinose, the noninducing condition, or galactose, the inducing condition. In the western assay, little Prp2 protein was detectable without galactose induction (data not shown; see Fig. 3A, lane 1). Extracts prepared from cells grown in galactose for 6 h show a 200–1,000-fold increase in

expression of the mutant Prp2 proteins. All were stably expressed, indicating that the mutations in Prp2 apparently did not affect protein stability (Fig. 3A, lanes 3–8; data not shown). One of the E573K mutants acquired a termination codon at residue 588. This mutant produced a stable, truncated product as measured by western assay (data not shown), but the mutant was no longer dominant negative in vivo (Fig. 2) and in vitro (Table 1; data not shown).

Extracts from some of the dominant negative mutants inhibit wild-type Prp2 activity in vitro

To examine whether the phenotype of the *PRP2* dominant negative mutants was due to an inability to release from the spliceosome, splicing inhibition and spliceosome binding assays were performed. Glass-bead extracts, which by themselves cannot carry out a complete splicing reaction, were prepared from strain D12 (*prp2-1^{TS}*) carrying wild-type or mutant *PRP2* grown either in suppressing (glucose) or inducing (galactose) conditions. The Prp2 protein encoded by the *prp2-1* gene was inactivated during glass-bead-extract preparation (see below). In the complementation assay (Fig. 3B, lanes 1–5), glass-bead extracts were incubated with spliceosomes, which were assembled in heat-inactivated *prp2Δ* splicing extracts with ³²P-labeled actin pre-mRNA and ATP. Extract prepared from cells expressing wild-type Prp2 efficiently complement heat-inactivated *prp2Δ* splicing extract (Fig. 3B, lane 2). The pYES2 vector or H-domain mutants could not complement the heat-inactivated splicing extract (Fig. 3B, lanes 1, 3–5), although the mutant proteins are expressed (Fig. 3A, lanes 3–5). In the competition assay (Fig. 3B, lanes 6–10), purified Prp2 protein was added to the splicing reactions shown in Figure 3B, lanes 1 to 5, and incubated under splicing conditions. Glass-bead extracts from pYES2 alone or from the mutant H349K did not interfere with complementation of the *prp2Δ* extract by wild-type Prp2 (Fig. 3B, lanes 6 and 8). The H349D mutant and the double mutant H349D/Q548H, however, inhibited splicing by wild-type Prp2 (Fig. 3B, lanes 9 and 10). These results indicated that the three dominant negative mutants lost splicing activity; however, only H349D and the double mutant inhibited the action of wild-type Prp2 protein in vitro.

Similar splicing inhibition experiments were performed with the *prp2* mutants isolated by random mutagenesis, except that the mutants were transformed into BJ168 or B4-7-2D; both strains carry the wild-type *PRP2* gene on the chromosome. Glass-bead extracts prepared from galactose-induced strains were added to heat-inactivated *prp2Δ* extract. The extract prepared from yeast transformed with pYES2 vector complemented the *prp2Δ* extract, indicating the level of Prp2 activity endogenous to the glass-bead extracts (Fig. 3C, lane 7). Extracts prepared from dominant negative

FIGURE 3. A: Mutant Prp2 proteins are expressed and stable in glass-bead extracts. Five microliters of the indicated glass-bead extracts were separated by 10% SDS-PAGE and transferred to PVDF membrane. The membrane was incubated with rabbit anti-Prp2 followed by alkaline-phosphatase-conjugated goat anti-rabbit IgG. The band corresponding to Prp2 is indicated. A cross-reacting band present in glass-bead extracts is indicated by *. This band has not been characterized, but it is not present in purified Prp2 preparations (see Fig. 5C). Lanes 1–5: extracts were prepared from the strain D12 (*prp2-1^{Ts}*) transformed with pYES2-PRP2 plasmids containing the indicated mutation. Lanes 6–8: extracts were prepared from the strain BJ168 transformed with the indicated plasmids. **B:** In vitro complementation and inhibition assays of H domain mutants. Extracts from H domain mutants cannot complement heat-treated *prp2-1* splicing extract. Glass-bead extracts were prepared from the strain D12 (*prp2-1^{Ts}*) transformed with the indicated pYES2 plasmids and added to splicing mixtures containing heat-treated *prp2-1* extract (*prp2Δ*), ³²P-labeled actin pre-mRNA, and ATP, with or without purified Prp2 protein. Following incubation at 23 °C for 30 min, RNA was extracted and separated on a 7.5% polyacrylamide/7.5 M urea gel. The positions of the pre-mRNA, mRNA, lariat intron-exon 2 (IVS*-E2), and released intron lariat (IVS*) are indicated. Lanes 1–5: addition of the indicated glass-bead extracts; lanes 6–10: addition of the indicated glass-bead extracts and purified Prp2 protein. **C:** H domain mutants isolated by random mutagenesis inhibit splicing by endogenous Prp2 protein in vitro. Glass-bead extracts were added to splicing reactions containing heat-inactivated *prp2* extract, ³²P-labeled actin pre-mRNA, and ATP. RNA was isolated and analyzed as before. Lane 1: Buffer D was added in place of glass-bead extracts; lanes 2–7: glass-bead extracts were prepared from the wild-type strain BJ168 (lanes 2–5, 7) or B4-7-2D (lane 6) transformed with the indicated pYES2 plasmids. The positions of the pre-mRNA, mRNA, intron lariat-exon 2 (IVS*-E2), and released intron lariat (IVS*) are indicated.

strains inhibited this Prp2 activity, as seen by the lack of complementation of the *prp2Δ* spliceosome (Fig. 3C, lanes 3–6). There was a low level of splicing products in the reaction containing extracts from the P399S/G444E strain (Fig. 3C, lane 5), which was due to incomplete inhibition of the endogenous Prp2 protein by the mutant protein. These results suggest that the mutant proteins interfere with the function of the endogenous wild-type Prp2 protein in vitro as well as in vivo.

The inhibition of splicing by the mutant Prp2 proteins was further investigated by immunoprecipitation of the

spliceosome using N-terminal-specific anti-Prp2 antibodies (Fig. 4A). Aliquots of the reactions shown in Figure 3B, lanes 1–5, were immunoprecipitated and analyzed on denaturing gels (Fig. 4B, lanes 6–10). Very little ³²P-labeled pre-mRNA was coimmunoprecipitated with wild-type Prp2, which carried out splicing and exited from the spliceosome (Fig. 4B, lane 7). Pre-mRNA was coimmunoprecipitated with the double mutant and H349D Prp2 proteins, indicating that these proteins inhibited splicing by remaining associated with the spliceosome in the presence of ATP (Fig. 4B, lanes 9 and

FIGURE 4. Immunoprecipitation of splicing reactions shows that H domain mutants that are dominant negative in vivo have different phenotypes in vitro. **A:** Flow chart of the spliceosome-binding assay. Splicing reactions were assembled in heat-treated *prp2-1* extract with ^{32}P -labeled actin pre-mRNA and ATP for 30 min at 23°C. Glucose was added (5 mM final concentration) and incubation continued for 5 min to deplete ATP. For immunoprecipitation in the presence of ATP, no glucose was added. Glass-bead extracts were added and incubation continued for 30 min at 23°C. Reaction mixtures were added to anti-Prp2 antibodies immobilized on Sepharose CL4B beads in IPP-150. After mixing at 4°C for 1 h, beads were washed and then RNA was extracted and separated on a denaturing polyacrylamide gel. RNA was purified from aliquots of the reactions prior to immunoprecipitation to assess splicing. **B:** Spliceosome binding assay using extracts from site-directed mutants. The indicated glass-bead extracts were prepared from the strain D12 (*prp2-1^{TS}*). Lanes 1–5: –ATP reaction; lanes 6–10: +ATP reaction. The band corresponding to the immunoprecipitated pre-mRNA is indicated. **C:** Immunoprecipitation of wild-type and mutant Prp2 proteins from glass-bead extracts. The indicated glass-bead extracts (9 μL wild-type, 15 μL mutants) were added to anti-Prp2 antibodies immobilized on Sepharose CL4B beads in IPP-150 as above. After washing, beads were boiled in cracking buffer and proteins were separated by SDS-PAGE, transferred to PVDF membrane, and reacted with anti-Prp2 antibodies. The total extract prior to immunoprecipitation is shown in even-numbered lanes (3 μL wild-type, 5 μL mutants) and the pellet from the immunoprecipitations is shown in odd-numbered lanes. The more rapidly migrating, less distinct band is likely a background band derived from the protein-A beads.

10). In contrast, H349K was not stably associated with the spliceosome (Fig. 4B, lane 8). The inability of H349K to bind to the spliceosome was also observed when ATP was depleted before the addition of the glass-bead extract (Fig. 4B, lane 3). However, wild-type and the other two mutants stably bound to the spliceosome under the same conditions (Fig. 4B, lanes 2, 4, and 5).

To further investigate the range of in vitro phenotypes, the binding of partially purified dominant negative proteins to the spliceosome was analyzed. Prp2 proteins purified by Ni-chelation chromatography were incubated with *prp2Δ* spliceosomes in the presence of ATP. A western blot showed that approximately equal amounts of protein were added to each reaction (Fig. 5C). RNA was purified from an aliquot of the reaction to examine the ability of the purified proteins to complement *prp2Δ* extract for splicing (Fig. 5A); the

rest of the reaction was immunoprecipitated with anti-Prp2 antibodies (Fig. 5B). Spliced products were present in the aliquot of the wild-type reaction taken prior to immunoprecipitation (Fig. 5A, lane 2). None of the purified dominant negative proteins were able to complement heat-inactivated *prp2Δ* extract for splicing (Fig. 5A, lanes 3–6). The wild-type protein released from the spliceosome, as the amount of pre-mRNA immunoprecipitated was the same as the sample without Prp2 (Fig. 5B, compare lanes 1 and 2). Both H349D/Q548H and G551N remained bound to the spliceosome (Fig. 5B, lanes 3–4), whereas the mutants T583I and P399S/G444E remained weakly associated.

To test whether the lack of signal in the spliceosome immunoprecipitation assays was caused by the inability of the anti-Prp2 antibodies to recognize the mutant proteins, glass-bead extracts were subjected to im-

FIGURE 5. A: Partially purified dominant negative Prp2 proteins do not complement *prp2Δ* for splicing. Prp2 proteins were purified by Ni-column chromatography and equivalent amounts were added to a splicing reaction with *prp2Δ* splicing extract and ATP, followed by incubation for 30 min at 23°C. Lane 1: Buffer D was added in place of protein; lanes 2–6: the indicated proteins were added. The positions of the pre-mRNA, mRNA, intron lariat-exon 2 (IVS*-E2) and released intron lariat (IVS*) are indicated. **B:** Spliceosome binding of Prp2 proteins in the presence of ATP. An aliquot of the splicing reactions in **A** was added to anti-Prp2 antibodies as described in the legend to Figure 4. The position of the pre-mRNA is indicated. **C:** Western analysis of Prp2 proteins purified by Ni-column chromatography was performed as described in the legend to Figure 3A. The amount of protein shown on the western blot is the amount added to the splicing reaction.

munoprecipitation and western blot analysis (Fig. 4C). All of the Prp2 proteins tested were immunoprecipitable (Fig. 4C, odd-numbered lanes). The H349K protein was clearly immunoprecipitated by the anti-Prp2 antibodies, although immunoprecipitation was not as efficient as for the wild-type or other mutant Prp2 proteins (Fig. 4C, lane 7). There was a more rapidly migrating and less distinct band sometimes observed in the immunoprecipitates (Fig. 4C, lanes 1, 3, and 11). This background band was likely derived from the protein-A beads and not related to Prp2, as it was not present in any of the extracts. Therefore, the data from both the splicing competition and spliceosome-binding assays suggest an inefficient association of the H349K and P399S/G444E mutant proteins with the spliceosome in vitro.

PRP2 H domain mutants have reduced ATPase activity

ATP hydrolysis is required for Prp2 to release from the spliceosome (Plumpton et al., 1994; Kim & Lin, 1996). Histidine-tagged wild-type and mutant proteins were purified by Ni-chelation chromatography followed by

poly(U) agarose to measure their ATPase activity in vitro (Plumpton et al., 1994; Kim & Lin, 1996). As expected for mutations in the ATPase domain, all mutants tested showed greatly reduced ATPase activity in the absence and presence of RNA (Fig. 6). Proteins that did not release from the spliceosome in vitro have 2–6% of wild-type RNA-dependent ATPase activity. Two of the proteins that do not associate strongly with the spliceosome in vitro, P399S/G444E and T583I, have slightly higher relative ATPase activity (14–16%), but still very low when compared with the wild-type protein. Therefore, H domain mutants that are dominant negative in vivo have reduced ATPase activity in vitro.

DISCUSSION

We examined the interaction between Prp2 and the spliceosome by identifying and characterizing mutants that were dominant negative in vivo. Site-directed mutants were made in motifs II and VI; mutants isolated after random mutagenesis also mapped within an extended H domain (Fig. 1). Mutants that could not substitute for wild-type *PRP2* in vivo showed a dominant negative phenotype when overexpressed using the in-

ATPase activity of Prp2 H domain mutants

FIGURE 6. H domain mutants show reduced ATPase activity. Purified Prp2 wild-type or mutant proteins (20 ng) were incubated with $\gamma^{32}\text{P}$ -ATP in the presence or absence of poly(U) RNA for 30 min at 23°C. Released P_i was separated from the mixture by absorption with activated charcoal for 10 min on ice and was counted as ATP hydrolysis product. The background activity resulting from incubation in the absence of protein was subtracted from each value. Each value represents the average of two independent results.

ducible *GAL1* promoter and had diminished ATPase activity in vitro (Fig. 6). In addition, we identified a mutant, H349K, that is dominant negative in vivo but not in vitro. This study supports and extends observations made on Prp2 and other members of the DExD/H-box family members with respect to amino acids that are essential for H domain function. We present a model to explain the phenotypes of these mutants based on the homology of the H domain of Prp2 with members of the DExD/H family whose structure has been determined (Fig. 7).

The extent of the helicase domain in the DExD/H family, which begins at motif I and ends after the conserved motif VI QRAGR, was previously determined based on sequence conservation in at least 75% of proteins within the SF2 superfamily (Gorbalenya & Koonin, 1993). Two of the mutants we identified by random mutagenesis, E573K and T583I, are 20 and 30 amino acids, respectively, beyond motif VI, the last of the seven conserved motifs in the H domain (Fig. 1). The region C-terminal to motif VI is conserved among the DEAH family members Prp2, Prp16, Prp22, and Prp43, but not in the greater DExD/H-box protein family. T583I had an intermediate phenotype in the spliceosome binding assay (Fig. 5B). E573K, however, strongly in-

hibited splicing in vitro, even in the presence of exogenously added wild-type Prp2 (Fig. 3C; data not shown). Both proteins showed greatly diminished ATPase activity and the degree of splicing inhibition seen correlated with ATPase activity levels (Fig. 6). The E573K mutant with the C-terminus truncated is no longer dominant negative, although the protein is stably expressed, suggesting that the C-terminus of Prp2 may be required for interaction with the spliceosome. Therefore, the domain required for complete H domain activity in Prp2 is larger than the H domain conserved in the DExD/H family.

Studies on other members of the DExD/H family have established the importance of the H domain for RNA-dependent ATPase and in some cases RNA helicase activity. In the DEAH subfamily of proteins the histidine in motif II, DEAH₂, is conserved. Substitution of histidine 349 in motif II with the acidic amino acids aspartic acid or glutamic acid or with the basic amino acid lysine resulted in mutants that cannot substitute for *PRP2* in vivo. Overexpression of these mutants resulted in a dominant negative phenotype in vivo (Table 1). We identified a mutant G251D/S378L that has a mutation in motif I, the Walker A motif, in addition to a SAT-to-LAT mutation in motif III. Plumpton et al. (1994) previously

FIGURE 7. A: Ribbon model of the H domain of Prp2 based on homology with the HCV NS3 protein (Yao et al., 1997; Cho et al., 1998; Kim et al., 1998) using the Swiss-Model program (Guex et al., 1999). The NTPase domain is shown on the left and the RNA-binding domain is shown on the right. Residues mutated in our study are numbered as in Figure 1 and indicated by yellow balls. Conserved motifs are shown in red. Motif I includes residue 251, motif II includes residue 349, motif III includes residue 378, and motif VI includes residue 551. **B:** Alignment of Prp2 and the HCV NS3 protein used for modeling of the Prp2 H domain. Residues mutated in our study are indicated in bold. Conserved motifs are underlined and numbered as in Figure 1.

used the *GAL-PRP2* system to identify a dominant negative mutant of *PRP2* that had the SAT-to-LAT mutation. Site-directed mutagenesis of motifs within the H domain of *PRP16* has demonstrated the importance of

individual residues in vivo (Hotz & Schwer, 1998). Mutation of the residue equivalent to G551N in *PRP16*, G688A, results in a protein that cannot substitute for Prp16 in a *PRP16*-deleted strain. The mutant has a

dominant negative phenotype when overexpressed in a wild-type strain, which is the same phenotype as the *PRP2* G551N mutant we identified by random mutagenesis (Hotz & Schwer, 1998; see Table 1).

The combination of DEAD in motif II and HRxGR in motif VI is found in the DEAD box family, whereas the DEAH/QRxGR combination is found in the DExH group of proteins. Extensive mutational analyses have been done on the DEAD-box protein translation factor eIF4A (Schmid & Linder, 1991; Pause & Sonenberg, 1992; Pause et al., 1993). A mutant in which the last aspartic acid of motif II was replaced with histidine (DEAH) and the first histidine in motif VI was replaced with glutamine (QRAGR) showed increased ATP binding and ATPase activity but no RNA helicase activity. This mutation is analogous to our double mutant (H349D/Q548H), which had a strong dominant negative phenotype and low ATPase activity. Therefore, the *PRP2* double mutant has the low level of ATPase activity corresponding to the motifs found in eIF4A (Pause & Sonenberg, 1992).

The helicase domain of Prp2 can be aligned with other members of the DExD/H box protein family, including the hepatitis C virus (HCV) NS3 protein (Figs. 1, 7B). The crystal structure of the HCV NS3 protein has been determined and shows a Y-shaped structure with three domains (Yao et al., 1997; Cho et al., 1998; Kim et al., 1998). In the NS3 protein, the NTPase domain includes motifs I and II that face toward the RNA binding domain, which includes motif VI. The structural model of Prp2 was developed based on its similarity with the HCV-NS3 helicase using the Swiss-Model program (Peitsch, 1995, 1996; Guex & Peitsch, 1997; Guex et al., 1999). In Figure 7A the NTPase domain is shown on the left side and the RNA-binding domain is shown on the right. Modeling of the H domain suggests that most of the residues identified in this study fall within a critical region on the interface between the two domains (Fig. 7A). Crystal structures of other helicase domains, including Pcr (Subramanya et al., 1996) and eIF4A (Benz et al., 1999) also indicate that lysine in motif I interacts with aspartic acid in motif II in the binding of ATP and coordination of Mg^{2+} . The histidine in motif II hydrogen bonds with the threonine (serine in the case of Prp2) of motif III (TAT or SAT) prior to ATP hydrolysis and then with the threonine two residues to the C-terminus after ATP hydrolysis (Cho et al., 1998). This change in hydrogen bonding pattern is thought to transmit the signal from the hydrolysis of ATP to the helicase activity. It is clear from the structure how motifs II and III residues could interact. Motif VI is also poised to interact in the cleft formed between the two domains. Placing the residues mutated in our random mutants on the model indicates that the residue P399S is adjacent to motif III in the cleft and E573K also faces into the cleft adjacent to motif VI (Fig. 7A). The T583I mutation lies at the bottom of the cleft. In one model, this area is near the

RNA-binding domain (Kim et al., 1998; Lin & Kim, 1999). It is interesting that although the mutations we identified were dispersed across the H domain, from motif I to downstream of motif VI, all except G444E map in a critical cleft region in this structural model.

Most of the mutants that had a dominant negative phenotype in vivo inhibited splicing and did not release from the spliceosome in the presence of ATP (Figs. 3, 4, and 5). Three of the mutants, however, H349K, P399S/G444E, and T583I, were dominant negative in vivo but did not bind efficiently to the spliceosome with or without ATP in vitro. P399S/G444E and T583I bound more strongly to the spliceosome than H349K but not as strongly as H349D, H349D/Q548H, or G551N. The P399S/G444E and T583I mutants had the highest ATPase activity of the mutant proteins tested, suggesting that these two mutants are simply weaker dominant negative mutants. The proline-to-serine change in P399S may not completely abolish essential interactions within the H domain, perhaps with motif III.

There are a number of possibilities to explain the difference between the in vivo and in vitro phenotypes of the H349K mutant. For example, if Prp2 plays a role in proofreading prior to the first transesterification reaction, the H349K mutant could act by preferentially sending splicing complexes to a discard pathway. Such a mechanism has been established for a mutant in *PRP16*, *prp16-1*, which is a dominant negative mutant with a mutation in the H domain and reduced ATPase activity (Schwer & Guthrie, 1992; Burgess & Guthrie, 1993). The mutant was originally identified as a suppressor of a branch site mutation that allowed a pre-mRNA with a mutant branch site to be spliced in vivo due to a relaxed fidelity in splicing (Couto et al., 1987). The potential role of Prp2 in proofreading was not explored, because we did not test the ability of our mutants to splice mutant substrates in vivo or in vitro. Another possibility to explain why the H349K mutant protein does not stall in the spliceosome in vitro is that it could affect spliceosome assembly by inactivating or sequestering an essential splicing factor such as the protein Spp2p in vivo (Roy et al., 1995). We have not examined the interaction between Spp2p and the H349K mutant Prp2 protein.

This is the first study to present a tentative model of the H domain of Prp2 that explains the phenotypes of H domain mutants. The amino acids C-terminal to conserved motifs that affect H domain function in Prp2 may be a part of the cleft between domains 1 and 2. Related family members Prp16, Prp22, and Prp43 may also have extended helicase domains that fold in a similar way to known helicases. In addition, although many dominant negative mutants of Prp2 (Plumpton et al., 1994; this study) and Prp16 (Schwer & Guthrie, 1992) act by stalling in spliceosomes, alternative mechanisms of inhibition of splicing by dominant negative mutants are possible.

MATERIALS AND METHODS

Yeast strains and culture

Yeast genetic manipulations, tetrad dissections, and growth conditions were performed as described in Rose et al. (1990). Yeasts were transformed using the lithium acetate method as described in Rose et al. (1990) or Gietz and Woods (1994). Strains used were BJ168 (*ura3, leu2, trp1, prb, pep4, prc*), B4-7-2D (*Mata, his3-Δ200, lys2-801, ura3-52*), D12 (*Mata, leu2, his3, prp2-1*), YTY1 (*Mata, PRP2::TRP1, lys2-801, his3-Δ200, leu2-3,112, trp1-1, ura3-52/YCp51-PRP2*), 3.2A1D (*Mata, prp2-1, ade2, his3, lys2-801, ura3*), and 3.2A10C (*Mata, prp2-1, ade2, his3, lys2-801, ura3*).

Generation of mutants

Site-directed mutagenesis was performed as described (Kunkel et al., 1987). pRS415-*PRP2* was mutagenized using the degenerate oligo 5'-GATGAAGCTN₁AN₂TGAACGTACTC-3', where N₁ represents a mixture of 3 nt, T, A, and G, and N₂ represents a mixture of A and T. The double mutant H349D/Q548H was made using the fourfold degenerate PRP2-XRAGR oligo 5'-TCAGTTGATNATCGTGCCGGT-3' and single-stranded DNA from pRS415-*PRP2* (DEAD). The G551N mutation in pRS415-*PRP2* was made using the oligo 5'-GCAGCGTGCCAATAGGGCTGGTC-3'. Mutations were confirmed by sequencing using Sequenase (USB) or by the City of Hope DNA sequencing facility.

To isolate random release mutants, three trials of hydroxylamine mutagenesis and transformation into strain B4-7-2D were performed using plasmid pYES2-*PRP2*. Ura⁺ transformants were replica-plated on galactose-containing plates to identify transformants that failed to grow on galactose. In the first screen, 5 μg of pYES2-*PRP2* DNA was treated with hydroxylamine for 1 h, yielding mutants G551N and T583I from about 3,000 Ura⁺ transformants. In the second and third screens, 17.5 μg of DNA were treated with hydroxylamine for either 1 h (second screen) or 2 h (third screen) at 75 °C. There were 24 galactose-sensitive isolates out of 10,000 Ura⁺ transformants in the second trial and 40 galactose-sensitive colonies out of 14,000 Ura⁺ transformants in the third trial. The 24 Gal⁻ colonies from the second screen were combined as a pool (pool I) and the 40 Gal⁻ colonies from the third screen were combined as a separate pool (pool II). Plasmid DNA from each pool was prepared and used to transform *Escherichia coli* to generate pool I and pool II DNA. The two DNA pools were transformed separately into strain B4-7-2D, and Gal⁻ transformants were reisolated.

Plasmid construction

pRS415-*PRP2* was constructed by insertion of a 4.4-kb *Bam*HI fragment carrying the wild-type *PRP2* gene into pRS415. The *Hind*III-*Pvu*II fragment within the *PRP2* open reading frame in pBM-*PRP2* (King & Beggs, 1990) was replaced with the corresponding fragment from the site-directed *PRP2* mutants. The *Bam*HI fragment from wild-type or mutant pBM-*PRP2* was inserted into the 2 μ galactose-inducible vector pYES2 (Invitrogen). Some pYES2 constructs were further modified to include six contiguous histidine residues at the

N-terminus of the Prp2 protein. The oligonucleotides 5'-TAACAAGCAAGCTTATGTCAAGT(CAT)₆ATTACATCTGAAACCGGT-3' for the histidine tag and 5'-AGCTTCATCAATCAT-3' from the internal *PRP2* sequence were used to amplify a his-tagged *PRP2* fragment by PCR. The amplified PCR fragment was digested with *Hind*III and ligated to *Hind*III-cut wild-type or mutant pYES-*PRP2* plasmid.

Mapping of mutants

DNA fragments from the mutants generated by random mutagenesis were inserted into wild-type pYES2-*PRP2* to determine the location of the dominant negative mutations (Fig. 1). In the first step, the 2.7-kb *Pvu*II fragment of the wild-type plasmid, which includes the promoter and first 2,180 nt of *PRP2*, was replaced with the corresponding fragment from the mutant. Each of the *Pvu*II fragments from the mutants was able to transform wild-type yeast cells to Gal⁻. In the second test the 1.7-kb *Xba*I fragment from the first step, which includes nt 1595 of *PRP2* to a site in the pYES2 vector downstream of *PRP2*, was inserted into wild-type pYES2-*PRP2*. Three of the *Xba*I chimeric DNAs (G551N, E573K, T583I) transformed cells to Gal⁻, indicating that the mutations were between the internal *Xba*I and *Pvu*II sites. Two of the *Xba*I chimeric DNAs (G251D/S378L, P399S/G444E) did not transform cells to Gal⁻, indicating that the mutations responsible for the dominant negative phenotype were located between the *Pvu*II site in the vector and the internal *Xba*I site. The swapped plasmids with the Gal⁻ phenotype were used for sequencing and biochemical experiments.

Purification of histidine-tagged proteins and ATPase assay

Histidine-tagged Prp2 proteins were purified by Ni-affinity chromatography (Plumpton et al., 1994). Glass-bead extracts were mixed with an equal volume of 2× sample buffer (20% glycerol, 20 mM Tris-HCl, pH 7.9, 1 M NaCl) with imidazole added to 1 mM final concentration. Samples were loaded twice onto a 0.4-mL Ni column (Novagen) and equilibrated in BC buffer (10% glycerol, 20 mM Tris, pH 7.9) containing 500 mM KCl (BC₅₀₀). Columns were washed with BC₅₀₀ containing 20 mM imidazole and BC buffer containing 100 mM KCl (BC₁₀₀) and 50 mM imidazole. Prp2 protein was eluted stepwise with BC₁₀₀ containing 100 mM, 150 mM, 200 mM, or 250 mM imidazole. Peak Prp2-containing fractions were combined and loaded onto a poly(U) Sepharose column equilibrated with buffer BP₅₀ (20 mM HEPES, pH 7.9, 0.5 mM EDTA, 0.01% NP-40, 10% glycerol, 50 mM KCl). Columns were washed with BP₅₀ and Prp2 protein was eluted step-wise with buffer BP containing 100 mM, 150 mM, 200 mM, 250 mM, or 300 mM KCl. Protein concentrations were determined by silver staining and Bradford assay. ATPase assays of isolated Prp2 proteins were performed as described (Kim & Lin, 1993) using 20 ng mutant Prp2 protein with or without 0.5 μg/mL poly(U) RNA.

In vitro splicing assays

Yeast whole-cell extracts were prepared as described (Lin et al., 1987). Preparation of glass-bead extracts was per-

formed as described in Kim et al. (1999). Splicing assays including substrate preparation were performed as described (Kim & Lin, 1996). Results were quantified using a Molecular Dynamics PhosphorImager.

Spliceosome binding assays and immunoprecipitation

Splicing extract was prepared from strain 3.2A1D (*prp2-1*). The splicing extract was incubated at 37°C for 30 min to inactivate Prp2 prior to addition to the splicing reaction mixture containing ³²P-labeled actin pre-mRNA and ATP as described (Kim & Lin, 1996). The reaction was incubated at 23°C for 30 min to permit spliceosome assembly. For reactions in the absence of ATP, glucose was added (5 mM final concentration) and incubation continued for 5 min to deplete ATP, and then the mixture was divided into 24-μL aliquots. For reactions in the presence of ATP, no glucose was added. 3 μL of glass-bead extract and 3 μL Buffer D were added to each tube and the reactions were incubated at 4°C for 10 min to permit binding of Prp2 to the spliceosome. When purified proteins were used, the proportion of protein and Buffer D was adjusted to add equivalent amounts of protein to each reaction. Aliquots of the reactions were removed prior to immunoprecipitation and RNA was analyzed to assess splicing. The remainder of the reactions were added with 200 μL IPP-150 (10 mM Tris-Cl, pH 8, 150 mM NaCl, 0.1% NP-40) to Protein-A-Sepharose CL4B beads (Sigma) bound with N-terminal specific anti-Prp2 antibodies and mixed for 1 h at 4°C. Following incubation, the beads were pelleted and the supernatant saved as the unbound fraction. Beads were washed three times with IPP-150, and then 200 μL splicing reaction stop buffer (50 mM NaOAc, 1 mM EDTA, 0.1% SDS, 2.5 μg/mL glycogen) was added, followed by phenol extraction of the bead mixture. RNA was precipitated and then separated on a 7.5% acrylamide, 7.5 M urea gel. Results were analyzed using a Molecular Dynamics PhosphorImager.

Immunoprecipitation of wild-type and mutant Prp2 proteins from extracts was performed by adding extract to 5 μL Protein A-anti-Prp2 beads in 50 μL IPP-150. Incubation was done as for the splicing assays. After washing in IPP-150, 2× sample buffer was added, the samples were boiled, and proteins were separated by SDS-PAGE. Western analysis was performed as described below.

Western analysis of Prp2 expression

Five microliters of glass-bead extract or purified protein was separated by SDS-PAGE and electrophoretically transferred to PVDF membrane (Millipore) in Tris-Glycine transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine) (Kim et al., 1992). In some cases, following protein transfer, the filter was stained for 1 min with 50 mL of an aqueous solution containing 0.25 g Ponceau S and 0.5 mL acetic acid and then destained for 2 min in water. The filter was photographed and then further destained prior to incubation in Blotto (5% nonfat dry milk in PBS) for 2 h at room temperature. Further manipulation was done as described (Kim et al., 1992). N-terminal-specific anti-Prp2 polyclonal antisera was used at 1:500–1:2,000 dilution as described in Kim and Lin (1996).

Modeling of the Prp2 H domain

Residues 221–621 of Prp2, which includes all of the H domain and part of the C-terminus, were aligned with the HCV NS3 protein (Kim et al., 1998; Yao et al., 1997; Cho et al., 1998) using the Pileup program (see Fig. 7B). The aligned Prp2 sequence was modeled on the known crystal structures of the HCV NS3 protein using the Swiss-Pdb viewer (Peitsch, 1995, 1996; Guex & Peitsch, 1997). The aligned structure was submitted to Swiss-Model for model building (Guex et al., 1999).

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