Identification of the Spermatogenic Zip Protein Spz1 as a Putative Protein Phosphatase-1 (PP1) Regulatory Protein That Specifically Binds the PP1cγ2 Splice Variant in Mouse Testis*

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The spermatogenic zip protein (Spz1) was originally isolated from a mouse testis library and identified as a novel member of the basic helix-loop-helix family of transcription factors. Here we identify Spz1 as a specific binding partner of the γ2 catalytic subunit of protein phosphatase-1. Male mice homozygous for a null mutation in the protein phosphatase-1γ (PP1cγ) gene are infertile and display a distinct impairment in spermiogenesis despite the continued presence of closely related PP1c isoforms. Yeast two-hybrid screening using the PP1cγ2 splice variant has identified Spz1 as an interacting protein and possible mediator of the sterile PP1cγ mutant phenotype. Spz1 was shown to interact specifically with PP1cγ2 but did not show an interaction with PP1c or with a truncated version of PP1cγ2 lacking 18 amino acids from the C terminus. Interaction between full-length Spz1 and PP1cγ2 was verified by co-immunoprecipitation and co-localization experiments in COS-1 cells as well as gel-shift and sedimentation assays using whole testis lysates. Immunohistochemistry on wild type testis sections reveals a stage-specific expression pattern for Spz1 during spermatogenesis that appeared grossly abnormal in the testes of PP1cγ2 mutant mice. Protein phosphatase assays using recombinant PP1c indicate that increasing concentrations of Spz1 are able to inhibit PP1cγ2 activity while having little effect on the activity of PP1cα. Furthermore, an interaction between PP1cγ2 and Spz1 was shown to prevent binding of the latter to the consensus E-box promoter sequence. We propose that the interaction between Spz1 and PP1cγ2 may be required for proper regulation of spermatogenesis and fertility in males.

Mammalian spermatogenesis takes place in the seminiferous tubules of the testis and may be divided into three distinct stages: the proliferative, meiotic, and spermiogenic phases. Throughout the process developing germ cells are organized into a stratified epithelium by Sertoli cells, somatic cells that span the width of the tubules and help regulate differentiation through multiple signaling pathways (1). Male mice homozygous for a null mutation in the protein phosphatase-1γ (PP1cγ) gene have been found to display a severe impairment in spermiogenesis, the final phase of spermatogenesis in which haploid spermatids elongate and undergo characteristic structural changes before being released into the lumen as mature sperm (2). Although homozygous mutant males are completely infertile, females and heterozygous males appear normal, suggesting that the observed phenotype is not the result of a decrease in PP1c gene dosage. Histochemical analysis of PP1cγ mutant tubules reveals that the majority of germ cells present are at the round spermatid stage of development, although smaller numbers of abnormal cells at later stages are occasionally observed. Mutant tubules were also found to display higher levels of apoptosis (3) and aneuploidy (4) in germ cells, indicating the possibility of a secondary defect in meiosis. Preliminary analyses suggest that the observed lack of condensing and elongating spermatids may be due to premature shedding into the lumen, a hypothesis that is supported by the presence of empty regions in the epithelium where germ cells appear to have come away from Sertoli cell contacts (5).

Reversible protein phosphorylation on serine and threonine residues is a universal mechanism by which eukaryotic cells regulate internal processes and modulate responses to environmental and physiological stimuli. Type 1 protein phosphatases are holoenzymes composed of a single catalytic subunit (PP1c) in complex with one or more regulatory subunits. Catalytic subunits of PP1 have been identified in every eukaryotic organism studied and are highly conserved across phyla (6). Diversity of function has been achieved largely through association with different regulatory subunits, which vary greatly in structure and serve to mediate substrate specificity and localization of the enzyme within different cell types (7). Many PP1c regulatory proteins are known to interact through a degenerate RVXF motif, although it is not required in every case (8). In mice, four isoforms of PP1c have been identified, PP1cα, PP1cβ, PP1cγ1, and PP1cγ2, the latter two being derived through alternate splicing of a single gene (9). The isoforms are >98% identical excluding their unique C termini. Given that the PP1cγ2 splice variant is the most abundant PP1cγ isoform expressed in the testis (10), its absence seems the most likely cause of the sterile phenotype observed in PP1cγ mutant males. Both PP1cα and PP1cβ are expressed in mutant testes, suggesting that these closely related isoforms are unable to compensate for the loss of PP1cγ. We have hypothesized the presence of one or more regulatory subunits that interact specifically with PP1cγ2, allowing it to mediate a function required for proper spermiogenesis.

To date all known binding partners of PP1cγ2, including the glutamate receptor mGluR7b (11), the 78-kDa glucose-regulated protein (12), and a mammalian homologue of yeast Sds22

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3-AT, 3-aminotriazole; RT, reverse transcriptase; PBS, phosphate-buffered saline; GFP, green fluorescent protein.
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Experimental Procedures

Construction of Yeast Two-Hybrid Vectors—Codon sequences for mouse PP1cY, PP1cY, and a truncated form of PP1cY lacking 18 amino acids from the C terminus were amplified by PCR from pGEM7Z vectors containing respective full-length cDNA (18). PCR was performed using the following primer sets: for PP1cY, 5'-GGACGGCATGGCGGATAT-3' (primer 1) and 5'-ATGTTGAATTCACCACAAGG-3' (primer 2); for PP1c, primer 1 and 5'-GTCACCGGAGATCTGAGA-3' (primer 3); for PP1cO, 5'-GCACGCCCATTCCGGCGACAAGTCAGGGCGG-3' (primer 4); and for PP1cY, was insert of 1 by 100 bp for truncated PP1cY, primer 1 and 5'-CTAATGACGAAATTCGGCGCGTCG-3' (primer 6). The resulting fragments were digested with NcoI and EcoRI and ligated separately into pGBK7 bait vectors (Clontech). To clone full-length Spz1, testis RNA was isolated from a 14-week-old CD-1 male mouse using Trizol (Invitrogen), and reverse transcriptase-PCR was performed using the primer set 5'-GCTCCATGCTCATACTCGGCAATGAG-3' (primer 7) and 5'-GCTCCTGGCAGTTCTCAGCAAGAA-3' (primer 8). The Spz1 fragment was digested with NdeI and EcoRI and ligated into the multiple cloning site of pGADT7 (Clontech). Two hybrid vectors were sequenced before use in experiments to ensure the respective cDNAs had been inserted into the correct open reading frame for expression.

Yeast Two-hybrid Screening and Assays—pGBK7 bait vectors containing PP1c constructs were transformed separately into yeast Saccharomyces cerevisiae AH109 (MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4, gal80, lys2::GAL1-LAC10TATA-HIS3, GAL3-LAC10TATA-AD2, ura3::MEL1, ura3::MEL1TATA-lacZ) and transformed into synthetic dropout media lacking tryptophan (Clontech). Yeast strains expressing PP1c were proven negative for self-activation of reporter genes after replica plating on media lacking adenine and histidine. Two-hybrid screening was performed on a mouse testis library constructed in pCT2s from pooled BALB/c males aged 8–12 weeks (Clontech). The library was amplified in Escherichia coli strain BNI122 according to manufacturer's instructions (Bio-Rad). Plasmid DNA was affinity-purified using a maxi-prep kit from Qiagen. Library plasmid DNA (500 μg) was transformed into yeast AH109 expressing the PP1cY bait protein by the LiAc method as described in the Clontech manual.Transformed cells were plated on minimal selective media, and colonies were picked and replica-plated after 5–7 days of incubation at 30°C. Activation of the lacZ reporter gene was initially assessed by means of a colony-lift assay using an X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) substrate. Relative strength of lacZ expression was later quantified using a chemiluminescent assay with Galacton-Star as described in the Clontech manual. Positive interactions for each of the two-hybrid baits were assessed as described previously.

Antibodies—An antibody recognizing both PP1cY and PP1cY was generated in rabbits against the short peptide KPAEKKPNA TMPTPT common to the C-terminal region of each splice variant. The peptide was synthesized commercially with a cysteine residue at the N terminus to keyhole limpet hemocyanin was used for the peroxidase-conjugated secondary antibodies. For production of antibodies, 100 μg of purified immunogen was injected subcutaneously into New Zealand White rabbits as a 400-μl emulsion with Freund's complete adjuvant (22). Booster shots were administered using incomplete adjuvant after 14 and 28 days with Western blot on mouse testis protein. The antibody was affinity-purified against a SulfoLink® column (Pierce) containing the peptide originally used as an epitope. Antibody specifically recognizing PP1cY was a generous gift from the Dr. Srivivasan Vijayaraghavan (Kent State University). Antibodies to Spz1 were a gift from the laboratory of Dr. Hung Li (Institute of Molecular Biology, Academia Sinica, Taipei). Goat anti-PP1cY, rat anti-GATA1, and horseradish peroxidase-coupled secondary antibodies to rat, goat, and rabbit IgG were purchased from Santa Cruz Biotechnology. Chicken antibodies recognizing Myc and HA epitopes were from Akrabio. whereas Cy3-conjugated secondary antibodies were from Jackson Laboratories.

GST Fusion Protein Production—Coding regions for PP1cY and PP1cO were amplified from pGEM7Z vectors by PCR using the forward primer 5'-GCGCGGGCATCCGGCGATACC-3' (primer 9) and primer 2 or 5, respectively. Resulting cDNA fragments were digested with BamHI and EcoRI and ligated into pGEX-6P-2 (Amersham Biosciences). Full-length Spz1 cDNA was also cloned into pGEX-6P-2 after release from a pdNAS1.1 vector by restriction with Nhel and EcoRI. Each of the GST expression vectors was transformed separately into E. coli BL21 (DE3) (Stratagene). Recombinant bacteria were grown in LB media to an A600 = 0.8, and GST fusion protein production was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside followed by further incubation for 1.5 h at 37°C. Cells were pelleted by centrifugation, frozen at −70°C for 5 min, and thawed in a 1:100 culture volume of phosphate-buffered saline (PBS) containing 1 mM phenylmethylsulfonyl fluoride, 10 mM β-mercaptoethanol, and 0.2 mg/ml lysozyme. After incubation at 4°C for 20 min, Triton X-100 was added to 1%, and bacteria were lysed by sonication for 1 min at 0°C (Branson model 250/450 Sonifier, 80% pulse, output 4). The lysate was centrifuged at 10,000 × g for 5 min at 4°C, and GST fusion proteins were affinity-purified using glutathione-Sepharose 4B beads (Amersham Biosciences) as per the manufacturer's instructions. Protein samples were separated by homogenization in protein extraction buffer (100 mM NaCl, 1 mM EDTA pH 8.0, 10 mM Tris-HCl, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, with 1 μg/ml each aprotinin, leupeptin, and pepstatin A) using 500 μl of buffer per 50 μg of tissue. After centrifugation at 10,000 × g for 10 min at 4°C, the resulting supernatants were dialyzed against 200 volumes of buffer for 3 h. Protein concentrations were quantified using a Bradford dye assay as per the manufacturer's instructions (Bio-Rad).

sedimentation assays and Western Blotting—Testis protein extracts were prepared by homogenization of whole testis samples with a Dounce homogenizer in protein extraction buffer (100 mM NaCl, 1 mM EDTA pH 8.0, 10 mM Tris-HCl, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, with 1 μg/ml each aprotinin, leupeptin, and pepstatin A) using 500 μl of buffer per 50 μg of tissue. After centrifugation at 10,000 × g for 5 min at 4°C, the resulting supernatants were dialyzed against 200 volumes of buffer for 3 h. Protein concentrations were quantified using a Bradford dye assay as per the manufacturer's instructions (Bio-Rad). Varying amounts of GST-Spz1 were bound to glutathione-Sepharose 4B beads (Amersham Biosciences) as per the manufacturer’s instructions. Bovine serum albumin (BSA) was added to a concentration of 1.5 mg/ml to 1.25 mg/ml, and antibodies to PP1cO and Spz1 were used at 1:1000. Bound protein complexes were visualized using an enhanced chemiluminescence (ECL) detection system (Amersham Biosciences). Initial binding of GST-Spz1 to glutathione-Sepharose was verified via Coomassie Blue staining after elution and electrophoresis.

Co-immunoprecipitation and Co-localization Experiments—PerCP-2.8 and PerCP-Cy5.5 were expressed in a COS-1 kidney cell line derived from African green monkey using Myc- and HA-tagged pCDNA3.1 expression vectors, respectively. Cells were cultured on plates in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum according to standard procedures (25) and passaged by treatment with 0.02% EDTA and 0.25% trypsin for 5 min. For construction of expression vectors the full-length sequence of PP1cY was amplified by PCR using the primers 5'-GGACGGCGGTACGGATACC-3' (primer 10) and primer 2. PerCP-2.8 coding sequence was amplified by RT-PCR from total testis RNA using the primers 5'-GCCCGCGTACGGATACC-3' (primer 9) and primer 2. pCDNA3.1 expression vectors were transfected into COS-1 cells by electroporation using a Bio-Rad Gene-Pulser according to the method of Misumi et al. (26). Transformed cells were selected in 1 mg/ml G418 and purified by limiting dilution.
selected in 0.8 mg/ml G418 (Invitrogen) for 1 week. Two 60-mm culture dishes of the transformed line expressing PP1cy2 were grown to 90% confluency and used in a transient transfection with HA-pcDNA3.1-PP1cy2 as described (26) before being concentrated onto a single dish. After 3 days, cells were collected and lysed in 1 ml of immunoprecipitation buffer (50 mM Tris-HCL, pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 1 µg/ml each leupeptin, aprotinin, and pepstatin A) for 15 min at 4 °C. Lysates were centrifuged at 10,500 × g for 10 min at 4 °C, and the supernatant was split into two 500-µl samples. PP1cy2 and PP1cy1 were precipitated separately using 5 µg of chicken anti-Myc or anti-HA antibodies at 4 °C with gentle rotation for 1.5 h. Antibody and bound proteins were pulled down with a 50-µl slurry of anti-chicken IgY immobilized on agarose beads according to manufacturer’s instructions. Protein complexes were washed before the addition of SDS-PAGE sample buffer and separated on 12.5% acrylamide gels. Western blotting was performed as described above. For control experiments, immunoprecipitations were performed using 5 µg of anti-GATA1 or anti-PP1cy2 (Santa Cruz Biotechnology) together with 10 µl of 50% protein A-agarose beads (Transduction Laboratories).

For co-localization experiments PP1cy2 cDNA was released from a pGEX-6P-2 vector through digestion with BamHI and EcoRI and cloned into the corresponding sites on pDeRed2-C1 (Clontech). Spz1 was amplified by PCR from a pACT2 clone containing the full coding sequence using the primers 5'-CCAGGAAAGGCGAGCAGCCCGTGGAGAATTT-3' (primer 12) and 5'-GACGTCGACCTTGGCCGCAGACCCTG-3' (primer 13). The cDNA was released from a pGEX-6P-2 vector by digestion of yeast two-hybrid baits with NcoI and SalI. Resulting cDNA was cloned into pGEX-6P-2 lacking 18 amino acids from the unique C terminus as described in Iwamura et al. (29). Tissue was sectioned to an 8-µm thickness onto slides, dehydrated, and rehydrated (29). Heat antigen retrieval was performed using a microwave with the slides immersed in 0.01 M Tris-HCL, pH 9.0, buffer. Slides were microwaved for 2 separate 8-min periods on a high setting, with a 3-min cooling period between. After microwave treatment, slides were washed for 10 min in phosphate-buffered saline with 0.4% Photoflo (Eastman Kodak Co.) for 10 min in PBS with 0.005% Triton X-100 and 10 min in PBS with 10% antibody dilution buffer (10% goat serum, 3% bovine serum albumin, 0.05% Triton X-100). Incubation with Spz1 or PP1cy2 antibody (1:250) was performed overnight at 4 °C after which slides were washed and blocked as before. Treatment with secondary antibodies was for 4 h at 4 °C using 1:4000 Cy3-conjugated goat anti-rabbit IgG. Secondary antibodies were removed in PBS with 0.4% Photoflo for 20 min before counterstaining with 4,6-diamidino-2-phenylindole dihydrochloride at 0.1 µg/ml in water for 10 min. Slides were given the final 2 final 10-min washes in distilled water and mounted (34). Sections were examined with an Olympus BX50 microscope equipped with rhodamine and 4,6-diamidino-2-phenylindole dihydrochloride filters. Images were captured using Cool Snap software and a CCD camera. Images were merged using Image Pro4.1 software.

Animals—Mice were bred using standard animal husbandry. Individual males from the PP1cy colony were genotyped using a PCR-based method previously described (2). In our laboratory the PP1cy mutant allele has been propagated in a CD-1 background (Charles River Laboratories). Rabbits were maintained using standard animal husbandry procedures. All procedures performed involving laboratory animals were approved by the Canadian Council on Animal Care.

RESULTS

Spz1 Interacts with PP1cy2 in a Yeast Two-hybrid Assay—To identify putative regulatory subunits of PP1cy2, a yeast two-hybrid screen was performed using the PP1cy2 isoform as bait to screen a mouse testis expression library. Two-hybrid screening identified a clone containing a portion of the coding sequence for the spermatogenic zip protein Spz1. The entire Spz1 cDNA was subsequently amplified by reverse transcriptase-PCR from total mouse testis RNA and cloned into a similar two-hybrid vector to confirm an interaction with the full-length protein. To determine whether Spz1 was able to interact with multiple PP1c isoforms, the full-length two-hybrid clone was also tested for interactions with PP1c1, PP1cy1, and a truncated version of PP1cy2 lacking 18 amino acids from the unique C terminus (Fig. 1A). Expression of the respective PP1c fusion proteins in yeast was confirmed by Western blotting (data not shown) before testing. A construct encoding a portion of the human lamin C gene was used as a negative control. Positive interactions were assessed through the ability of transformed cells to grow on minimal media, activating expression of Ade2 and His3 reporter genes. Expression of a lacZ reporter gene was initially assessed via colony lift assay. Using these criteria Spz1 was found to interact strongly with PP1cy2 and not PP1c1. A two-hybrid construct expressing amino acids 148–486 of the mouse homologue of staufen was used as a positive control for PP1c1 activity. Staufen is a RNA-binding protein containing an RVXF motif that was originally identified in Drosophila and has recently been recognized as a regulatory subunit of PP1 in rat brain (30). Interestingly, Staufen is also expressed in male germ cells during spermatogenesis in a stage-specific manner (31) and was isolated by us in the same two-hybrid screen as Spz1.

Two-hybrid assays were originally conducted in media containing 1 mM 3-aminio 1,2,3-triazole (3-AT), a competitive inhibitor of the HIS3 gene product. In the presence of 1 mM 3-AT Spz1 was shown to interact not only with PP1cy2 but also weakly with PP1cy1 and the C-terminal truncated version of PP1cy2. The latter two interactions were only barely able to support yeast growth on minimal media, with the resulting colonies turning a brownish color indicative of cell death. When concentrations of 3-AT were raised to 5 mM, no growth due to interaction of Spz1 with these baits was observed. The relative
Fig. 1. Spz1 interacts specifically with PP1cy2 in a yeast two-hybrid assay. A, summary of yeast two-hybrid interactions between Spz1, Staufen, and a series of PP1c bait constructs. Two-hybrid interactions were determined in either 1 or 5 mM 3-AT as described under “Experimental Procedures” and categorized as strong (+++), weak (+), or negative (−). PP1c-y2ΔC is a deletion construct lacking 18 amino acids from the C terminus. A two-hybrid bait construct encoding a portion of human lamin C was used as a negative control. B, histogram illustrating relative strength of two-hybrid interactions as determined by a chemiluminescent assay for lacZ reporter gene expression. Assays were performed using a Galacton-Star substrate as per manufacturer’s instructions (Clontech). Data points used for the histogram represent an average of three independent experiments. RLU, relative light units.

Fig. 2. Spz1 co-immunoprecipitates with PP1cy2 in mammalian cells. A, PP1cy2 and Spz1 were expressed in COS-1 cells with Myc and HA epitope tags, respectively. PP1cy2 was naturally expressed in COS-1 cells as judged by RT-PCR. Antibodies to epitope-tagged PP1cy2 and Spz1 were produced with Myc and HA epitope tags, respectively. PP1cy2 was immobilized on glutathione-Sepharose and incubated with testis lysates under similar conditions (32). As mentioned previously, the PP1cy2 antibody used in these experiments recognizes both splice variants. B, GST-Spz1 was immobilized on glutathione-Sepharose and incubated with testis lysates under similar conditions (32). The beads were washed, and bound proteins were eluted in high salt buffer before separation by SDS-PAGE and Western blotting with antibodies to PP1cy2 and PP1cy.

strength of the respective two-hybrid associations involving Spz1 were quantified in 1 or 5 mM 3-AT by a chemiluminescent assay using a Galacton-Star substrate to test for lacZ expression (Fig. 1B). Results of the assay indicate that the interaction between Spz1 and PP1cy2 was just as strong as those observed for the general PP1c regulatory protein Staufen.

Examination of the Spz1 amino acid sequence reveals the presence of a variation on the characteristic RVXF binding motif found in many known PP1c regulatory subunits (8). Specifically, the sequence KN’KIRF’K appears from amino acids 116–122. Mutagenesis experiments are currently under way to determine whether the interaction of Spz1 with PP1cy2 is dependent on this sequence. Preliminary analyses using deletion mutants of Spz1 lacking the KN’KIRF’K sequence did not show an interaction with PP1cy2 in a two-hybrid assay (data not shown).

Spz1 Associates with PP1cy2 in Mammalian Cells—To verify an interaction between Spz1 and PP1cy2 in mammalian cells, full-length coding sequences were cloned separately into pcDNA3.1 expression vectors and transformed sequentially into a COS-1 monkey kidney cell line for co-immunoprecipitation experiments. PP1cy2 and Spz1 were produced with Myc or HA epitope tags, respectively. Antibodies specific to Myc or HA were able to pull down the opposite protein after immunoprecipitation and Western blotting (Fig. 2A). Specific antibodies to PP1cy2 and Spz1 were used only during the final blotting step, as both were generated in rabbits and resulted in IgG bands obscuring the signal when used together in a single experiment. Although the antibody to PP1cy2 was capable of recognizing both splice variants, RT-PCR using COS-1 RNA showed that the PP1cy1 variant was not expressed (32). PP1cy2 was naturally expressed in COS-1 cells as judged by RT-PCR but did not co-immunoprecipitate with Spz1. The monkey PP1cy2 cDNA sequence is not available, but the human and mouse sequences are identical.

Specificity and relative strength of the interaction between Spz1 and PP1cy2 was further illustrated using a GST sedimentation assay. After expression and purification from E. coli, varying amounts of full-length GST-Spz1 were covalently

— C. Hrabchak and S. Varmuza, unpublished data.
Transfection of fluorescently labeled $Spz1$ and PP1$c2$ fusion constructs into COS-1 cells reveals a variable expression pattern for each protein, possibly related to different stages of the cell cycle. Using confocal imaging of live cells, GFP-$Spz1$ could be observed in either or both the nucleus and cytoplasm (Fig. 3, A–C). Cytoplasmic expression was diffuse and appeared evenly distributed, whereas nuclear expression was typically more intense and largely excluded from the nucleoli (arrowheads). Similarly, a PP1$c2$ construct fused to DsRed could also be observed in either or both the nucleus and cytoplasm (Fig. 3, D–F). In contrast with $Spz1$, PP1$c2$ expression in the nucleus was often concentrated in nucleoli. Cytoplasmic expression appeared relatively uniform. The distribution of PP1$c2$ in COS-1 cells was similar to that reported for the PP1$c2$ splice variant in HeLa cells, which followed a complex series of subcellular localizations throughout the cell cycle and also appeared concentrated in nucleoli (33, 34). When both $Spz1$ and PP1$c2$ were expressed within a single cell, the two proteins were invariably co-localized in the nucleus, with weak overlapping expression in the cytoplasm occasionally observed. Nuclear expression appeared uniform, and neither protein appeared concentrated in nucleoli (Fig. 3, G–I).

**PP1$c2$ and $Spz1$ Expression Patterns Overlap in Wild type Mouse Testis**—To examine the expression pattern of $Spz1$, immunohistochemical analyses on formalin-fixed testis sections were performed using a polyclonal antibody kindly provided by Dr. Hung Li, Academia Sinica, Taipei. The antibody was proven effective before use via Western blot on GST-$Spz1$ and full-length $Spz1$ from recombinant yeast strains (data not shown). In wild type testis sections, $Spz1$ staining was stage-specific during spermatogenesis, with staining observed in only a subset of tubules (Fig. 4, A–D). Consistent with previous reports (16), $Spz1$ staining was commonly concentrated in the cytoplasm of later stage spermatids surrounding the lumen of the seminiferous tubules. However, small points of expression could be seen in earlier stage cells that had not been previously described. At higher magnification, this punctate $Spz1$ expression appeared associated with the edges of germ cell nuclei (Fig. 4D). Interestingly, purely nuclear expression of $Spz1$ as seen in COS-1 cells was never observed in testis sections. In some cases, cytoplasmic expression in germ cells ringing the lumen did not extend the full circumference of the tubule. The interstitial testis compartment was autofluorescent under our conditions.

Immunohistochemistry on wild type testis sections using an antibody specific to the unique C terminus of PP1$c2$ revealed expression of the protein in all stages of spermatogenesis (Fig. 4, E and F). Although the intensity of the PP1$c2$ signal often appeared to vary between tubules, the overall expression throughout spermatogenesis was uniformly high. PP1$c2$ was observed in the cytoplasm of both Sertoli cells and germ cells ranging from primary spermatocytes to elongating spermatids. Conversely, PP1$c2$ expression was relatively weak in spermatogonia and along the basement membrane of the seminiferous epithelium. It is important to note that expression of PP1$c2$ in the cytoplasm of later stage germ cells would coincide with the observed expression pattern for $Spz1$. PP1$c2$ did not show any obvious nuclear localization at any stage.

**$Spz1$ Expression Appears Abnormal in PP1$c2$ Mutant Testes**—Given that $Spz1$ interacts with PP1$c2$, we asked whether $Spz1$ expression was altered in PP1$c2$ null mice. Immunohistochemical analysis of mutant testis sections revealed $Spz1$ expression was stage-specific during spermatogenesis but appeared abnormal in comparison with wild type patterns (Fig. 5, A–C). The wild type pattern in which $Spz1$ appeared concentrated in the cytoplasm of germ cells adjacent to the lumen was
Spz1 expression appears disrupted in PP1cγ−/− mutant mice. A–C, immunohistochemistry was performed on testis sections from 14-week PP1cγ−/− mice using an antibody to Spz1 with Cy-3-labeled secondary antibodies as described under “Experimental Procedures.” Punctate expression of Spz1 was dependent on an antigen retrieval step in the immunostaining procedure and appeared only in a subset of tubules. D, a negative control immunostaining with a PP1cγ2 antibody on a PP1cγ null testis section. Panels A, B, and D appear at 400× magnification with bars measuring 100 μm. Panel C appears at 1000× magnification with a 40-μm bar.

Fig. 5. Spz1 expression appears disrupted in PP1cγ−/− mutant mice. Given that PP1cγ mutant tubules display a general lack of later stage spermatids, it is possible that the absence of Spz1 staining could simply reflect an absence of these cells. To address this question we searched for the few examples of mutant tubules containing condensing and elongate cells. Even in those cases the intense adluminal Spz1 expression pattern characteristic of wild type tubules was not observed (Fig. 5B). By comparison, punctate Spz1 expression associated with germ cell nuclei could still be observed in the mutants. Although the punctate staining often appeared more intense than in wild type sections, this may be due to a quirk of the program used for capturing the images, which automatically adjusts light levels to minimize saturation. As expected, immunohistochemistry on mutant testicular sections with a PP1cγ antibody was negative (Fig. 5D).

Inhibition of PP1cγ2 Activity by Spz1—Phosphorylase a is the classic substrate for dephosphorylation by PP1. Previous studies show that association of PP1c with targeting subunits often decreases activity of the enzyme in vitro (for review, see Refs. 1, 7, and 35). To examine the effect of association with Spz1 on PP1c activity, we expressed recombinant PP1cα and PP1cγ2 separately in E. coli for use in phosphatase assays performed in the presence of increasing concentrations of full-length Spz1 fused to GST. Given that E. coli has no native phosphatase activity toward phosphorylase a (27), crude bacterial lysates in physiological salt concentrations including 2 mM MnCl2 were used in all cases rather than affinity-purified enzyme. Phosphatase activity was calculated as a percentage of control (Fig. 6A). Under these conditions chimeric Spz1 decreased PP1cγ2 activity in a dose-dependent manner with a 60% decrease in total at 10−6 M, the highest concentration assayed. The calculated IC50 for the inhibition was calculated at ~0.5 μM. By comparison, chimeric Spz1 had relatively little effect on PP1cα activity, with just under a 10% decrease observed from control levels at the highest concentration (Fig. 6B). To ensure the reliability of the assay, phosphatase activities were also measured in the presence of neurabin I and the skeletal muscle glycogen-targeting subunit (Gm), both known regulatory subunits of PP1 that have been previously characterized in terms of their inhibitory effect (15, 36). Neurabin I recruits PP1 to the actin cytoskeleton in brain neurons and was recently shown to interact preferentially with PP1α and PP1cγ2, but not PP1cβ (32). A possible interaction with PP1cγ2 was not investigated. GST-neurabin I containing amino acids 374–516 inhibited PP1cα activity up to 80% of control levels with an IC50 of ~0.01 μM, consistent with previous data (15). PP1cγ2 was inhibited up to 80% of control levels with a similar IC50. The glycogen targeting subunit Gm has a high binding affinity for PP1c but is not as potent an inhibitor of phosphatase activity as might be expected. Gm was previously reported as decreasing PP1cα activity toward phosphorylase a up to 50% of maximum (36). In our assay a GST construct containing amino acids 1–240 of Gm was found to inhibit PP1cα and PP1cγ2, respectively, at 40 and 49% of maximum using 10−5 M. These data indicate that at high concentrations Spz1 is comparable with Gm as an inhibitor of PP1cγ2 activity while having only a small effect on PP1cα.

Interaction with PP1cγ2 Inhibits Binding of Spz1 to the E-box Consensus Sequence—As a putative member of the bHLH-Zip family of transcription factors, Spz1 was originally characterized for its ability to bind the consensus E-box promoter sequence CANNTG (16). The E box promoter sequence is nor-
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Fig. 7. Interaction of Spz1 with PP1cγ2 inhibits binding to the E-box consensus sequence. Electrophoretic mobility shift assays were performed using full-length GST-Spz1 and the radiolabeled oligonucleotide 5′-AAGAAAGAAGCCAGCTGGAGAGAAAAATT-3′ as described under “Experimental Procedures.” Increasing amounts of GST-PP1cγ2 were added in lanes 3–7 as indicated.

mally recognized by charged basic amino acids immediately upstream of the HLH region in members of the bHLH-Zip family (37). Given that the variant RVXF motif of Spz1 is located at the beginning of the HLH region, we examined the possibility that binding of Spz1 to PP1cγ2 may disrupt an association with the E-box. Electrophoretic mobility shift assays using GST fusion proteins indicate that increasing concentrations of chimeric PP1cγ2 were able to inhibit Spz1 interaction with a synthetic oligonucleotide containing the E-box sequence in a dose-dependent manner (Fig. 7). Interaction with radiolabeled oligonucleotide could also be competed-out with the addition of increasing amounts of cold probe (data not shown).

DISCUSSION

Higher eukaryotes typically contain multiple isoforms of the catalytic subunit of protein phosphatase-1. In different cell types these isoforms interact with combinations of PP1c regulatory subunits that are often mutually exclusive (35). The combined interaction of these regulatory subunits with a pool of PP1 AP1 catalytic isoforms creates subsets of unique PP1 holoenzymes that may be targeted to particular substrates (38). In this way, a shared pool of PP1c is able to regulate multiple cellular processes, some of which proceed concurrently in the same cell types. In mice lacking the PP1cγ gene, a mutant phenotype is observed only in the testis, which exhibits a severe defect in spermatogenesis leading to male infertility (2). Such an association would likely require the unique C terminus of PP1cγ2 for binding.

We have identified the spermatogenic zip protein Spz1 as a unique binding partner of PP1cγ2. Spz1 has previously been characterized as a novel member of the bHLH zip protein family of transcription factors that is expressed only in the testis (16). We have shown that Spz1 interacts strongly with PP1cγ2 in a yeast two-hybrid assay as well as in co-immunoprecipitation experiments using mammalian cells. Spz1 was also able to interact specifically with the native form of PP1cγ2 from whole testis lysates in sedimentation assays. Spz1 did not interact with PP1cα, and deletion of the unique C terminus of PP1cγ2 was sufficient to abolish the two-hybrid association. Examination of the Spz1 amino acid sequence reveals the presence of a variation on the RVXF motif common to many PP1 regulatory subunits, thought to interact with a hydrophobic channel on respective catalytic isoforms (8). Given that the point of interaction for the RVXF motif is common to all PP1c, other regions along the length of each protein may be required to facilitate a specific association with the γ2 splice variant. It is important to note that a slight interaction was also observed between Spz1 and PP1cγ1 when two-hybrid assays were performed in conditions of 1 M 3-AT. However, this association could barely support growth and was abolished by increasing the concentration of inhibitor. Given that a strong viral promoter was used to drive expression of the two proteins in yeast, it seems likely that the interaction was forced and may not be indicative of a real association in the testis.

To verify two-hybrid interactions, we have shown that Spz1 is able to co-immunoprecipitate with PP1cγ2 but not PP1cα in mammalian COS-1 cells. The caveat to this result is that PP1cα was not overexpressed in our experiments because it did not need to be produced from a vector. Sedimentation assays reveal that Spz1 is also able to bind native PP1cγ2 from testis lysates with similar affinity to that shown by the neurabin PP1 regulatory proteins isolated from brain (32). It is interesting to note that, whereas fluorescently tagged Spz1 and PP1cγ2 had characteristic distributions in COS-1, they were invariably co-localized in the nucleus when expressed together. Although this result provides further support for an in vivo interaction between the two proteins, it should not be viewed as a reflection of the native subcellular distribution of either Spz1 or PP1cγ2 in testis cells. This conclusion is reinforced by the fact that neither Spz1 or PP1cγ2 is normally expressed in COS-1 cells and that the cellular environment of germ cells undergoing spermatogenesis is not analogous to that of immortalized monkey kidney cells in culture. In fact, the cellular distribution of Spz1 and PP1cγ2 in the seminiferous tubules observed via immunohistochemistry is strikingly different.

In wild type testes Spz1 expression was concentrated in a subset of tubules within the cytoplasm of condensing spermatids adjacent to the lumen, consistent with previously published reports (15). This region of expression overlaps with the cytoplasmic expression of PP1cγ2, which is uniformly high in spermatocytes and spermatids but not spermatagonia. The fact that both Spz1 and PP1cγ2 are expressed at the same time and in the same place during spermatogenesis is consistent with an in vivo interaction. It should be noted that the distribution of Spz1 and PP1cγ2 would not be expected to coincide completely, since each would also have other protein partners. In addition to the observed cytoplasmic expression of Spz1 in germ cells adjacent to the lumen, points of expression were also associated with germ cell nuclei throughout the seminiferous epithelium. This punctate expression of Spz1 has not been previously described and appears to be dependent on an antigen retrieval step performed during preparation of testicular sections. Interestingly, uniform Spz1 expression was never observed in the nuclei of germ cell types, although we cannot exclude the possibility of a transient nuclear localization. Because Spz1 was originally identified as a transcription factor and shown to bind the consensus E-box promoter sequence, this seems a curious result. It is likely that Spz1 plays a multifunctional role within the testis and that the cytoplasmic distribution observed in elongating spermatids may reflect one aspect of that role. One obvious possibility for a function in elongating germ cells would...
be in the regulation of PP1cγ2. Transcription factors with dual functions (for example β-catenin) are certainly not uncommon.

In PP1cγ mutant testes cytoplasmic expression of Spz1 in germ cells adjacent the lumen is not observed as in wild type. Although condensing and elongating spermatids are largely absent from PP1cγ mutants, a lack of Spz1 expression is still evident in those few tubules where clusters of elongating spermatids are still present. Given that the punctate expression of Spz1 persists and that mRNA transcripts continue to be detected by RT-PCR, it is possible that the stability of the Spz1 in the cytoplasm may be affected by loss of PP1cγ2.

Interactions between PP1c and corresponding regulatory subunits are thought to localize the enzyme within appropriate subcellular compartments, positioning the holoenzyme in close proximity to target substrates (1). In targeting an isoform to one particular substrate, a regulatory subunit will often decrease its activity toward others (35). In phosphorytase assays using phosphorylase a, all PP1c regulatory subunits identified to date have either inhibited phosphorytase activity or had no effect. A good example is the glycogen-targeting subunit Gα, which inhibits phosphorytase activity toward phosphorylase a but increases activity toward both glycogen phosphorylase and glycogen synthase, two of the principal enzymes regulating glycogen metabolism (35, 36). In our assay, increasing concentrations of chimeric Spz1 were found to inhibit the phosphorytase activity of PP1cγ2 toward phosphorylase a by up to 60% of maximum. As is the case for the glycogen-targeting subunit, Spz1 may increase PP1cγ2 activity toward other substrates in vivo. It remains a possibility that Spz1 represents a target for PP1cγ2 within the seminiferous tubule.

If the interaction between Spz1 and PP1cγ2 is important for proper spermatogenesis, one might expect the mutant phenotypes of Spz1 and PP1cγ knockout mice to be somewhat similar. Although a knockout mouse model for Spz1 has not yet been created, Hsu et al. (39) recently overexpressed Spz1 in multiple tissues of transgenic mice, including the testis. The resulting males displayed dysfunctional spermatogenesis with increased proliferation and apoptosis of germ cells. Developing spermatids that survived meiosis were grossly abnormal and multiple tissues of transgenic mice, including the testis. The relative abundance of the PP1cγ2 splice variant in the testis has allowed for coevolution of a corresponding set of regulatory proteins and that under the direction of these proteins PP1cγ2 has evolved to play a fundamentally important role during mammalian spermatogenesis. Combinatorial control of PP1cγ2 by a subset of regulatory proteins, including Spz1, has the potential to best explain the phenotype of PP1cγ null mice. Those regulatory proteins that interact specifically with PP1cγ2 and not other PP1c isoforms would likely require the divergent C-terminal sequence for binding. This may help explain why the C-terminal sequence of PP1cγ2 is so completely unique and why it has been conserved to within a single amino acid throughout the mammalian lineage.

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Identification of the Spermatogenic Zip Protein Spz1 as a Putative Protein Phosphatase-1 (PP1) Regulatory Protein That Specifically Binds the PP1c γ2 Splice Variant in Mouse Testis

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