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Identification of a Novel HSP70-binding Cochaperone Critical to HSP90-mediated Activation of Small Serine/Threonine Kinase*[S]

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We previously reported the identification of small serine/threonine kinase (SSTK) that is expressed in postmeiotic germ cells, associates with HSP90, and is indispensable for male fertility. Sperm from SSTK-null mice cannot fertilize eggs *in vitro* and are incapable of fusing with eggs that lack zona pellucida. Here, using the yeast two-hybrid screen, we have discovered a novel SSTK-interacting protein (SIP) that is expressed exclusively in testis. The gene encoding SIP is restricted to mammals and encodes a 125-amino acid polypeptide with a predicted tetratricopeptide repeat domain. SIP is co-localized with SSTK in the cytoplasm of spermatids as they undergo restructuring and chromatin condensation, but unlike SSTK, is not retained in the mature sperm. SIP binds to SSTK with high affinity ($K_d \sim 10$ nM), and the proteins associate with each other when co-expressed in cells. *In vitro*, SIP inhibited SSTK kinase activity, whereas the presence of SIP in cells resulted in enzymatic activation of SSTK without affecting Akt or MAPK activity. SIP was found to be associated with cellular HSP70, and analyses with purified proteins revealed that SIP directly bound HSP70. Importantly, SSTK recruited SIP onto HSP90, and treatment of cells with the specific HSP90 inhibitor, 17-allylamino-17-demethoxygeldanamycin, completely abolished SSTK catalytic activity. Hence, these findings demonstrate that HSP90 is essential for functional maturation of the kinase and identify SIP as a cochaperone that is critical to the HSP90-mediated activation of SSTK.

Protein phosphorylation plays important roles during spermatogenesis (1, 2). A number of protein kinases are expressed in testis, and several of them are essential for male fertility (3). For example, disruption of the *Camk4* which is involved in postmeiotic chromatin condensation, results in impaired spermiogenesis and male sterility (4). Targeted deletion of the casein kinase 2α' catalytic subunit leads to oligozoospermia and extensive degeneration of germ cells at all stages of spermatogenesis (5), and Cdk2 is essential for completing mitotic division in germ cells (6). Three members of the testis-specific serine threonine kinase (TSSK)[2] family, namely TSSK1, TSSK2, and the small serine/threonine kinase (SSTK, also known as TSSK6), are expressed postmeiotically and are essential for male fertility (7–13).

SSTK is one of the smallest protein kinases, consists only of N- and C-lobes of a kinase catalytic domain, and forms stable associations with heat shock protein (HSP) 70 and 90 (7). Targeted deletion of *Sstk* in mice resulted in male infertility without any other visible somatic defects. SSTK-null sperm could not fertilize eggs *in vitro* and are incapable of fusing with eggs that lack zona pellucida (9). SSTK expression first appears in elongating spermatids as they undergo cellular remodeling and chromatin condensation, and the kinase is retained in mature sperm. However, the *in vivo* substrate(s) for SSTK, the mechanism of activation, and the specific function that SSTK performs in spermatids and/or sperm are not known.

The HSPs are a highly conserved group of proteins that are important for a number of diverse biological processes including germ cell development (14, 15). They function in nascent protein folding and prevention of aggregates, aid in refolding of denatured proteins, facilitate the degradation of misfolded proteins, regulate the assembly of protein complexes, help proteins transverse cellular membranes, and possess cytoprotective functions (16). Unlike the other major classes of HSPs, HSP90 appears to preferentially interact with a specific subset of proteins and is involved in stabilization and activation of a number of signaling molecules including protein kinases, transcription factors, and hormone receptors (17, 18). HSP90 has ATPase activity, which is required for mediating the necessary conformational changes that occur during maturation of client proteins (18, 19). The HSP90 chaperone complex also includes other HSPs, such as HSP70, and cochaperones that assist in stabilization and/or activation of client proteins (16, 18). These cochaperones provide functional specificity for the HSP90 client proteins and aid in the ordered assembly of the client-HSP90 machinery (20). Most cochaperones either contain a

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2 and references.

The nucleotide sequences reported in this paper have been submitted to the GenBank™ DataBank with accession numbers AY048672 and AY048673.

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[2] The abbreviations used are: TSSK, testis-specific serine threonine kinase; SSTK, small serine/threonine kinase; SIP, SSTK-interacting protein; 17-AAG, 17-allylamino-17-demethoxygeldanamycin; TPR, tetratricopeptide repeat; HSP, heat shock protein; H2A, histone 2A.

J-domain, such as in the HSP40 cochaperones of HSP70, or have tetratricopeptide repeat (TPRs) domains as found in cochaperones that interact with HSP70 or HSP90 (21). HSP70 itself has been considered a cochaperone for HSP90 and functions in the recruitment of substrates (22). The TPR-less Cdc37 assists in the HSP90 maturation of many kinases including Akt, ErbB2, Src, Raf1, and CDK4 (20, 23, 24), and to date, represents the only reported protein kinase-specific HSP cochaperone.

To identify potential regulators of SSTK activity, we performed a yeast two-hybrid screen and discovered a novel male germ cell-specific protein that localizes and binds with SSTK. This SSTK-interacting protein (SIP) is limited to mammals and possesses a predicted single TPR domain. SIP directly binds to HSP70, is found associated with HSP70 and HSP90 in cells, and facilitates HSP90-dependent enzymatic activation of SSTK. Accordingly, we have designated SIP as a cochaperone that assists in the conformational maturation of SSTK. These findings are the first demonstration of a germ cell-specific cochaperone and protein kinase that requires the HSP90 machinery for catalytic activation.

EXPERIMENTAL PROCEDURES

Reagents—[^{32}P]ATP and enhanced chemiluminescence kit were purchased from PerkinElmer Life Sciences. MAP4K4 was purchased from Invitrogen, and histone 2A and GSK-3 fusion protein were obtained from Upstate Biotech Millipore (Lake Placid, NY) and Cell Signaling Technology (Danvers, MA), respectively. Purified HSP70 and antibodies against HSP90, HSP70, and Myc were purchased from Assay Designs (Ann Arbor, MI). Myc (9E-10), HA and GFP antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). 17-Allylamino-17-demethoxygeldanamycin (17-AAG) was purchased from Sigma. Frozen surgical samples of normal human testicular tissue and human sperm were obtained from the Ontario Tumor Bank (Ontario, Canada). All reagents were of analytical grade.

Yeast Two-hybrid Cloning of SIP—Cloning of SIP was performed with the Matchmaker GAL4 yeast two-hybrid system 3 (Clontech) by nutritional complementation. Bait construct was generated by subcloning of the complete coding region of SSTK into pGBKT7 in-frame with the GAL4 DNA-binding domain. The construct was verified by sequencing, and expression of SSTK-GAL4 DB fusion protein in yeast was confirmed by Western blotting. *Saccharomyces cerevisiae* strain AH109 was transformed to Trp prototrophy with pGBKT7-SSTK bait construct by the polyethylene glycol/lithium acetate method as recommended by the supplier. *S. cerevisiae* strain Y187 pretransformed to Leu prototrophy with pGADT7 that carried testicular cDNA library (about 3.5 million independent clones from the adult human testis) served as the prey strain. Haploid bait and prey yeast strains were mated in 2× YPDA medium (Clontech), and resulting diploids were selected under medium stringency conditions on synthetic dropout −His/−Leu/−Trp agar plates followed by selection under high stringency conditions on −Ade/−His/−Leu/−Trp dropout plates and assayed for the activation of the *MEL1* gene that encodes secreted α-galactosidase. Protein-protein interaction in yeast was confirmed by switching bait and prey plasmids and remating strains. Strain AH109 transformed with pGBKT7 vector served as negative control. Prey plasmids were isolated from MEL1-positive Ade/His/Leu/Trp prototrophs as described (25) and rescued by transformation into *Escherichia coli* DH5α. Plasmid DNA was isolated from *E. coli* clones with the plasmid mini kit (Qiagen, Valencia, CA). cDNA inserts were subjected to restriction analysis and sequenced.

Preparation of Lysates from Mouse Tissues—Mice were handled and sacrificed in accordance with the guidelines of the Animal Care and Use Committee (Center for Biologics and Evaluation Research, Food and Drug Administration). Proteins from mouse tissues and sperm were extracted in lysis buffer containing SDS and subjected to SDS-PAGE as described earlier (7).

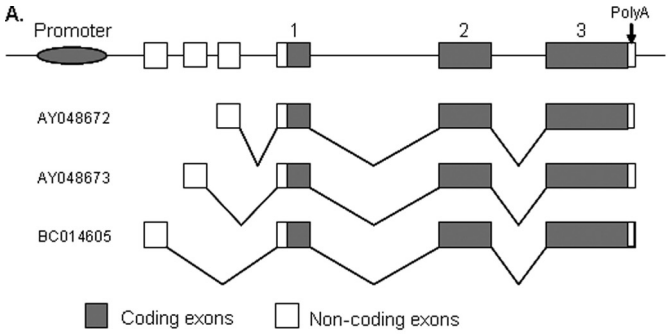
SIP and SSTK Antibodies, Immunoprecipitation, and Immunostaining—Mouse monoclonal antibodies against peptides corresponding to residues 1–51 and 218–273 of mouse SIP and SSTK, respectively, were generated and purified with protein G agarose. Rabbit antibodies against residues 80–124 of mouse SIP were produced and purified using peptide antigen immobilized on AminoLink gel from Thermo Scientific. Western blotting, immunoprecipitation, and immunohistochemistry were performed as described previously (7).

Protein Expression, Purification, and SIP-GST Binding Assay—SSTKs and EGF receptor were expressed by baculoviral infection in Sf9 insect cells and purified as described previously (26), and HA-tagged SIP-glutathione *S*-transferase (SIP-GST) was expressed in *E. coli* BL21 and purified as described in Fan *et al.* (27). SIP-GST binding was assessed by incubating 300 nM HSP70 or lysate from 5×10^6 cells (1 ml) with 5 μg of SIP-GST bound to 5 μl of glutathione-agarose for 1 h at 4 °C. Pellets were washed four times and analyzed by Western blotting. Transient expression of cDNA constructs in 293T cells was carried out as described earlier (7). When required, the cells were treated with 1 μM 17-AAG overnight and prior to cell harvest.

Protein Kinase Assays and K_d Determination—Kinase reactions were performed as described earlier (7). Briefly, the reaction was carried out in the kinase buffer that contained 25 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 2 mM EGTA, 60 μM ATP, 10 μCi of [γ-^{32}P]ATP, and protein/peptide substrate at room temperature for 30 min. Histone 2A, GSK-3-GST, Gab1 627Y peptide (27), or myelin basic protein was used as substrate for SSTK, Akt, EGF receptor, and MAP4K4, respectively. In some assays, reactions were terminated by acidification and transferred to P30 filtermats, and phosphorylation was quantified with a 1450 MicroBeta TriLux liquid scintillation counter (PerkinElmer Life Sciences, Turku, Finland). Alternatively, reactions were terminated with Laemmli sample buffer, resolved in SDS-PAGE gels, and developed by autoradiography. The dissociation constant for the SSTK and SIP interaction (K_d) was estimated by fitting the experimental titration data to the equation for a 1:1 binary complex

$$A = A_0 - \frac{(A_0 - A_\infty)[\mathrm{SIP}]}{K_d + [\mathrm{SIP}]} \qquad \text{(Eq. 1)}$$

where A is SSTK activity in the presence of a defined amount of SIP, and A_0 and A_∞ are the activities in the absence of SIP or in the presence of saturating SIP, respectively.



B.

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1   MERHTSHPNRKVPAKEEANAVPLCRAKPSPSYINLQASSPPATFLNIQTT  Human
1   ----------------------------------------------------  Chimpanzee
1   --Q-----------------------------------------------V---  Macaca
1   --Q-A----E-------S-------------F----------------R-  Dog
1   --QLP---TNR-R----G-------------F---------V-L-K-LP-  Cow
1   --Q---N-TKE E----D-V-C---------------N------S-TE--  Rat
1   --Q---N-TKE K----DSVACF-P--S-----D-P-NFT--N--T---  Mouse

51  KLPS VDHKPKECLGLLECMYANLQLQTQLAQQQMAVLEHLQASVTQLAP  Human
51  ---- --------------I----------T-------------------  Chimpanzee
51  ---- ----------------------------I-------------  Macaca
51  ----G-E-----------------------------I--N----M-----  Dog
51  ----GIN-----------------------------I--N----K-----  Cow
50  M---GAGE-----------S-----------R---I--N--T-LS--GS  Rat
49  --S-GAGQ---G---V----------------R-I-I--N----LS-PGS  Mouse

100 GRGSNNSSLPALSPNPLLNHLPQFSK  Human
100 --------------------------  Chimpanzee
100 --------------------------  Macaca
100 ----KK----V--R-L-----S----  Dog
100 -KENK--------R-L--S-------  Cow
100 --E-KTC---G-CC-L--------H-  Rat
 99 --E-K-C---G-CCKL--------H-  Mouse
```

FIGURE 1. **Gene structure of *SIP* and alignment of SIP proteins from various mammalian species.** *A*, schematic representation of structure of the human *SIP* gene and alternatively spliced transcripts are given. Coding and non-coding exons are shown as *closed* and *open boxes*, respectively. Coding exons are *numbered*, and the schematic is not drawn to scale. *B*, alignments of SIP proteins from human (NM_144627), chimpanzee (XP_513883), macaque (AB168871), dog (predicted from AAEX01012740), cow (BC102609), rat (XP_342277), and mouse (AK006195) are shown. Amino acid identities are represented by *dashed lines*, and residues absent relative to human are blank. The *bold line* above residues 64–97 represents a predicted TPR domain (28).

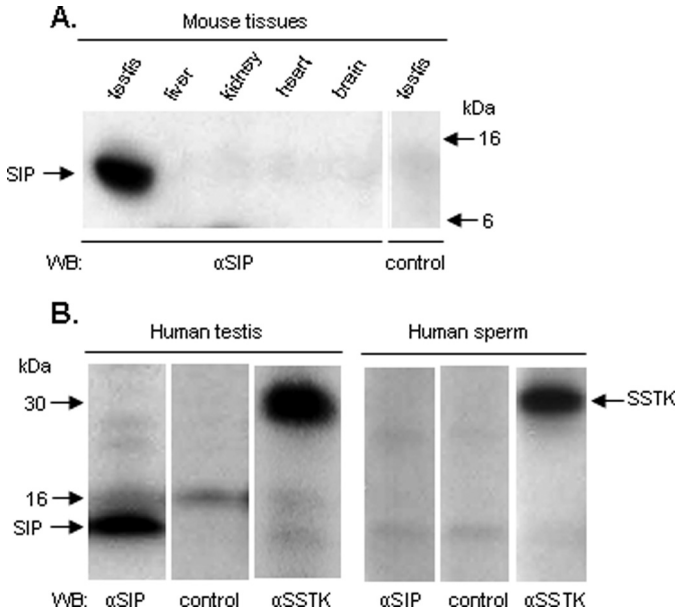

FIGURE 2. **Expression of SIP in tissues and sperm.** *A*, Western blotting (*WB*) of mouse tissue lysates was performed using a mAb against SIP or an isotype control mAb. *B*, human testis and sperm lysates were probed with SIP, SSTK, or control antibodies.

RESULTS

Identification of SIP as an Interacting Partner of SSTK—To identify proteins that bind and regulate SSTK function, a yeast two-hybrid screen of an adult human testicular cDNA library by nutritional complementation was performed using the full-length coding sequence of human SSTK as the bait. Screening of $\sim 17 \times 10^6$ clones resulted in isolation of ~200 clones that grew under medium stringency conditions on His/Leu/Trp-deficient medium and 17 clones that grew stably under the high stringency conditions, expressing the Ade+/His+/Leu+/Trp+ and Mel1+ phenotype. Of these 17 clones, 10 clones contained the complete coding sequence of a novel gene, which encodes SIP.

Human *SIP* undergoes extensive alternative splicing, and we cloned two splice variants of *SIP* that carry the same open reading frame but utilize different non-coding exons in the 5′-untranslated regions (Fig. 1*A*). The sequences were submitted to GenBank™ (accession numbers AY048672 and AY048673). Yet another splice variant (accession number BC014605) was later submitted to GenBank by a different research group (Fig. 1*A*). *SIP* from different mammalian species encode short (124–126 residues) and divergent polypeptides with gain or loss of amino acids occurring at exon-intron junctions (Fig. 1*B*). The predicted molecular mass and pI of human SIP are 13.7 kDa and 9.18, respectively, and the TPRpred software tool (28) predicted the presence of a single TPR domain (residues 64–97). The gene is situated on chromosome 1 at location 1q22 and chromosome 3 at location 3F1 in the human and murine genome, respectively. No orthologue of *SIP* was found in non-mammalian vertebrates, invertebrates, and lower organisms.

Expression of SIP and Co-localization with SSTK in Testis—To facilitate the study of SIP, monoclonal and polyclonal antibodies against residues 1–51 and 80–124 of murine SIP, respectively, were generated. The monoclonal antibody (mAb) recognizes mouse, but not human SIP, and the polyclonal antibody recognizes SIP from both species (data not shown). Western blot analysis of mouse tissues with the mAb detected the ~14-kDa SIP in testis, but not in other tissues, and no signal was detected using a control mAb (Fig. 2*A*). Similarly, the polyclonal antibody detected SIP in human testis lysate (Fig. 2*B*). Notably, SIP was not detected in sperm lysate from human (Fig. 2*B*) and mouse (data not shown). However, probing of the same human sperm lysate with an anti-SSTK mAb confirmed the presence of SSTK (Fig. 2*B*). Hence, SIP is expressed in testis and, unlike SSTK, is absent from mature sperm. These results are consistent with evidence from the human Expressed Sequence Tags (EST) database in that most of the *SIP* expressed sequence tags originated from either testis or testicular tumors, with a few from other organs.

To determine the cell types where SIP is expressed in testis and localization of SIP in relation to SSTK, immunohistochemical staining was performed with antibodies against SIP and SSTK on adjacent serial sections from adult mouse testis (Fig. 3). SIP was localized in the inner luminal layer of the seminiferous tubules in the cytoplasm of elongated spermatids (developmental steps 9 through 14) that undergo restructuring of the

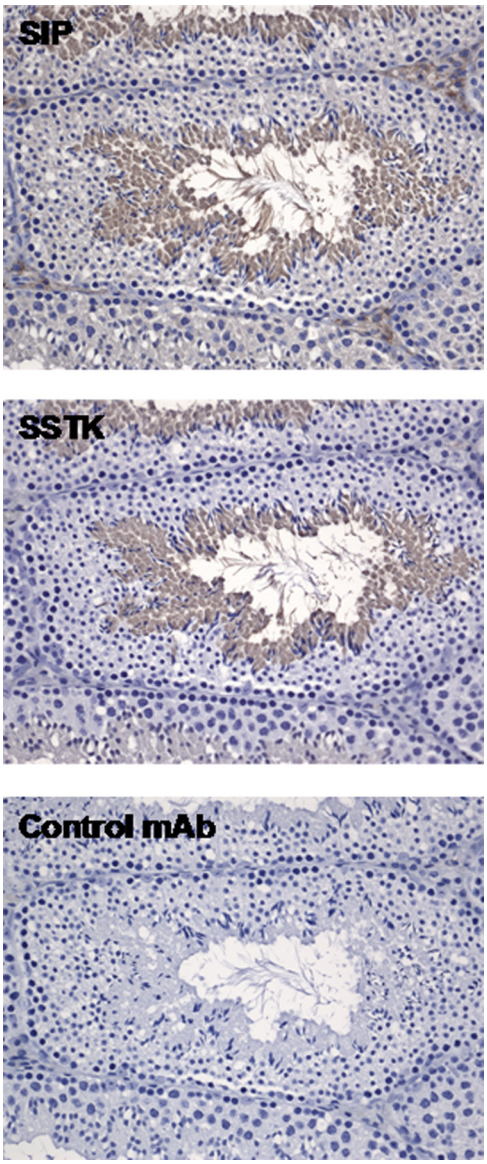

FIGURE 3. **Immunohistochemical localization of SIP and SSTK in mouse testis.** Serial sections (4 μm thick) of mouse testis were immunostained with mAbs against SIP (*upper panel*), mAbs against SSTK (*middle panel*), or isotype control mAb (*lower panel*). The sections were counterstained with hematoxylin (*blue*). *Brown staining* represents immunoreactivity with SIP and SSTK mAbs.

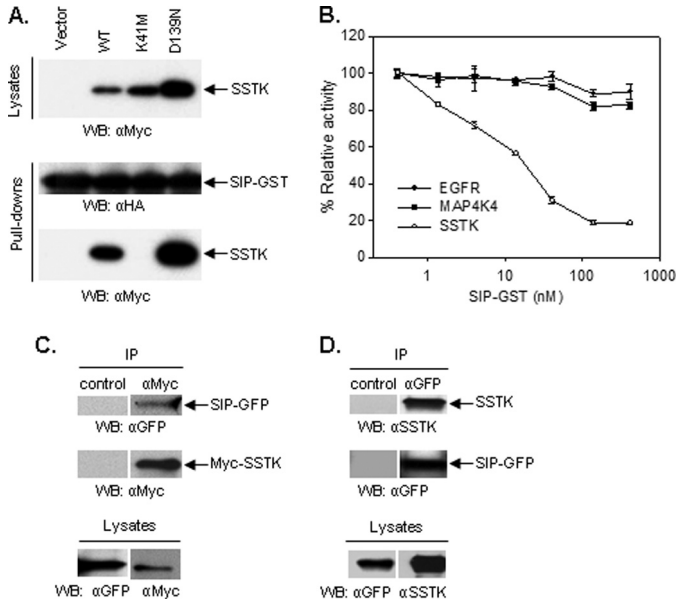

FIGURE 4. **Association of SSTK and SIP.** *A*, Sf9 cell lysates containing Myc-tagged SSTK or kinase-inactive SSTK were incubated with SIP-GST bound to glutathione agarose. Binding was analyzed by Western blotting (*WB*), and kinases and SIP-GST were detected with antibodies against Myc and HA epitopes, respectively. *B*, purified SSTK, MAP4K, or EGF receptor was preincubated in the presence of varying concentrations of purified SIP-GST at 25 °C for 10 min, and then kinase reactions were performed as described under "Experimental Procedures." Enzyme activities are shown as percentage relative to activity in the absence of SIP-GST. The dissociation constant (K_d) was calculated using a binary SIP-SSTK model and determined to be 9.7 ± 1.6 nM. Data represent mean ± S.E. ($n = 4$). *C*, SSTK and SIP-GFP cDNAs were transfected into 293T cells, and immunoprecipitations (*IP*) with Myc or isotype control mAb were performed on lysates. Western blotting analyses of immunoprecipitates and lysates were performed with GFP and Myc antibodies. *D*, immunoprecipitation with anti-GFP antibody or control rabbit antibody was performed on lysates from cells co-expressing SSTK and SIP-GFP. SSTK and GFP antibodies were used in Western blotting.

cytoplasm and chromatin condensation (*upper panel*). It should be noted that the staining of SIP was also detected in sperm in lumen of the seminiferous tubules. However, because SIP is absent from ejaculated sperm (Fig. 2*B*), we consider that this staining is associated with the residual bodies that are eventually lost from sperm. Immunostaining for SSTK revealed a similar expression pattern, and strong staining was observed in elongated spermatids and luminal sperm (*middle panel*). Similar to SIP, expression of SSTK peaked in elongated spermatids approximately at developmental steps 11–12. The specificity of the immunoreactivity with the SIP and SSTK antibodies was confirmed using control antibody (Fig. 3, *bottom panel*). These immunohistochemical studies revealed co-localization of SSTK and SIP in testis.

Association of SIP with SSTK—To confirm the interaction between SIP and SSTK, we expressed and purified a SIP-GST fusion protein in *E. coli*. Myc epitope-tagged SSTK or the kinase-inactive K41M or D139N mutants were expressed in Sf9 cells via baculovirus-mediated infection, and cell lysates were baited with SIP-GST immobilized on glutathione agarose (Fig. 4*A*). Wild type and D139N were found to bind to SIP-GST, whereas K41M SSTK did not. No SSTK binding was detected when GST replaced SIP-GST (data not shown). Next, SSTK was purified from Sf9 cells (26) and used to evaluate the effect of SIP on SSTK enzymatic activity. The addition of SIP-GST resulted in a dose-dependent inhibition of SSTK catalytic activity, although it did not completely inhibit the kinase even at saturating concentrations (Fig. 4*B*). The dissociation constant for the *SIP-SSTK* complex (K_d) calculated from the kinase inhibition data was 9.7 ± 1.6 nM. SIP-GST had no significant effect on the kinase activities of the serine/threonine mitogen-activated protein kinase MAP4K4 or on the EGF receptor tyrosine kinase (Fig. 4*B*).

The *in vivo* association between SSTK and SIP could not be confirmed by co-immunoprecipitation using testis lysate as both SSTK and SIP required SDS for their extraction and were not soluble in non-ionic detergents. Therefore, we utilized SIP- and SSTK-null 293T cells to ectopically express these proteins and further study their association and function. All attempts

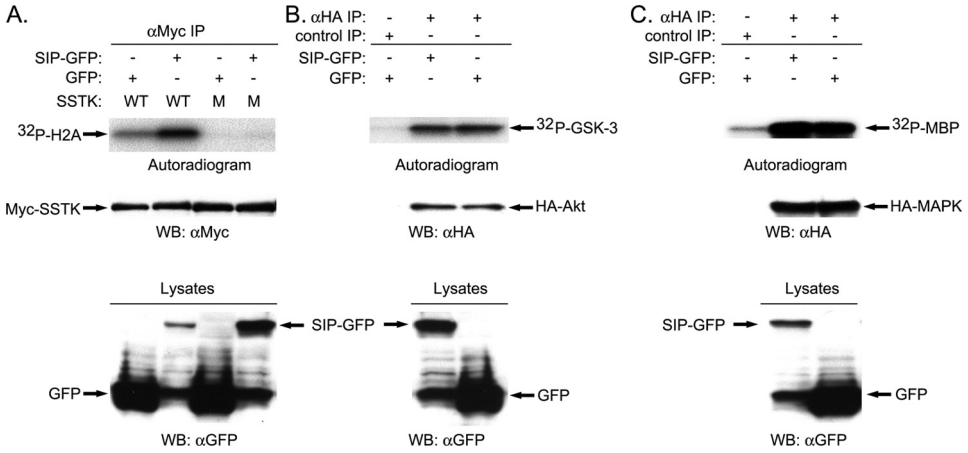

FIGURE 5. **Effect of SIP on SSTK kinase activity.** *A*, wild type (*WT*) or kinase-inactive K41M (*M*) Myc-SSTK cDNAs were co-transfected in 293T cells with either GFP or SIP-GFP cDNAs. Myc immunoprecipitation (*IP*) was followed by kinase assay using [^{32}P]ATP and H2A. Reaction mixtures were fractionated in SDS-PAGE gels, and autoradiography (*upper panel*) and Western blotting (*WB*) with Myc antibody (*middle panel*) were performed. Lysates were probed with GFP antibody (*lower panel*). The presence or absence of a component is represented by + and − signs, respectively. *B* and *C*, transfection, immunoprecipitation, and kinase assays were performed as in *A* except that HA-tagged Akt (*B*) or MAPK (*C*) cDNA replaced SSTK, GSK-3-GST, and myelin basic protein (*MBP*) were used as substrates for Akt and MAPK, respectively, and a control antibody immunoprecipitation was performed.

using various buffers and detergents to extract soluble SIP from 293T or other cell lines were unsuccessful. However, a fusion protein of SIP and green fluorescent protein (SIP-GFP) was found to be soluble in 1% Triton X-100 lysis buffer. SIP-GFP and Myc-SSTK were co-expressed in 293T cells, and association between SIP and SSTK was evaluated by co-immunoprecipitation analysis. Anti-GFP antibody detected SIP-GFP in the immunoprecipitate of Myc-SSTK (Fig. 4*C*, *upper panel*), and in the reverse experiment, SSTK was detected in the immunoprecipitate of SIP-GFP (Fig. 4*D*, *upper panel*). In both *C* and *D*, immunoprecipitations using control antibodies confirmed the specificity of the GFP and Myc immunoprecipitations, respectively. These results demonstrate that SIP can directly interact with the kinase in cells.

Cellular Activation of SSTK by SIP—To further study the effects of SIP on SSTK kinase activity, Myc-SSTK was expressed in 293T cells in the presence or absence of SIP-GFP. Myc-SSTK was immunoprecipitated, and *in vitro* kinase reactions were carried out with [^{32}P]ATP and histone 2A (H2A) as substrates. The kinase reaction mixtures were fractionated by SDS-PAGE, and autoradiography was performed. As expected, H2A was phosphorylated by SSTK and not by the kinase-inactive K41M mutant (*M*) (Fig. 5*A*, *upper panel*). Importantly, when SIP-GFP was co-expressed with SSTK, the level of phosphorylation of H2A by SSTK increased significantly when compared with the GFP control. The results demonstrate that SIP mediates activation of SSTK. To test whether the effect of SIP is specific to SSTK, SIP was co-expressed with the serine/threonine kinase Akt in cells. HA-Akt was immunoprecipitated using anti-HA mAb, and kinase activity was measured using GSK-3-GST fusion protein as substrate. Akt phosphorylated GSK-3-GST, but the presence of SIP did not influence this phosphorylation (Fig. 5*B*). Similar experiments were performed assessing the effect of SIP on mitogen-activated protein kinase (MAPK; extracellular signal-regulated kinase 2) activity, and like Akt, MAPK activity was not affected by SIP (Fig. 5*C*). Taken together, the results indicate that SIP specifically activates SSTK.

Association of SIP with HSP70—Our observation that SIP inhibits SSTK *in vitro* but mediates the activation of the kinase in cells indicates that other proteins are involved in the cellular activation of SSTK. It is known that SSTK associates with HSP70 (7), and therefore, we tested whether SIP itself associates with HSP70. SIP-GFP was expressed in 293T cells, and anti-GFP immunoprecipitates were analyzed for the presence of HSP70. HSP70 was detected in immunoprecipitates of lysates from cells expressing SIP-GFP but not from control cells expressing GFP (Fig. 6*A*). In the absence of SSTK, HSP70 antibody detected two bands in the immunoprecipitates, and in the presence of SSTK, the intensity of the lower band of HSP70 decreased, whereas the upper band increased significantly. HSP70 is known to undergo phosphorylation (29), and the SIP-associated HSP70 doublet bands may represent differentially phosphorylated HSP70. To determine whether SIP can directly bind HSP70, we performed binding assays using purified HSP70. Pure recombinant HSP70 was found to directly bind to SIP-GST but not to GST (Fig. 6*B*). These results demonstrate that SIP directly engages HSP70 and associates with HSP70 in cells.

Recruitment of SIP to HSP90 by SSTK and a Requirement for HSP90 in SSTK Catalytic Activity—Like HSP70, we have previously demonstrated that HSP90 can be detected in anti-SSTK immunoprecipitates (7). HSP90 was detected in the anti-GFP immunoprecipitates from cells expressing SIP-GFP, but not from control cells expressing GFP (Fig. 7*A*), and co-expression of SSTK significantly increased the level of HSP90 in the SIP-GFP immunoprecipitates (Fig. 7*A*). However, in contrast to our findings using purified HSP70, analogous binding experiments performed similar to those in Fig. 6*B*, but using purified HSP90, were not able to detect any direct binding between SIP and HSP90 (data not shown). Nevertheless, association of HSP90 with SSTK and SIP in cells, and the fact that SIP facilitates the enzymatic activation of SSTK, suggested that SIP and the HSP90 chaperone machinery work together in the functional maturation of SSTK. The ATPase activity of HSP90 is required for chaperone function and the maturation of client kinases. As shown in Fig. 7*B*, treatment of cells with the highly specific HSP90 ATPase inhibitor, 17-AAG, completely abolished the catalytic activity of SSTK as assessed by the phosphorylation of H2A, and this effect was independent of the presence of SIP. Interestingly, 17-AAG treatment did not lead to degradation of SSTK or SIP but blocked association between SIP and SSTK (Fig. 7*B*). We also tested whether purified HSP90 could activate SSTK *in vitro* in the presence of HSP70 and SIP using standard methodology (30, 31). Unfortunately, the conditions of the reconstitution resulted in enzymatic inactivation of SSTK, and

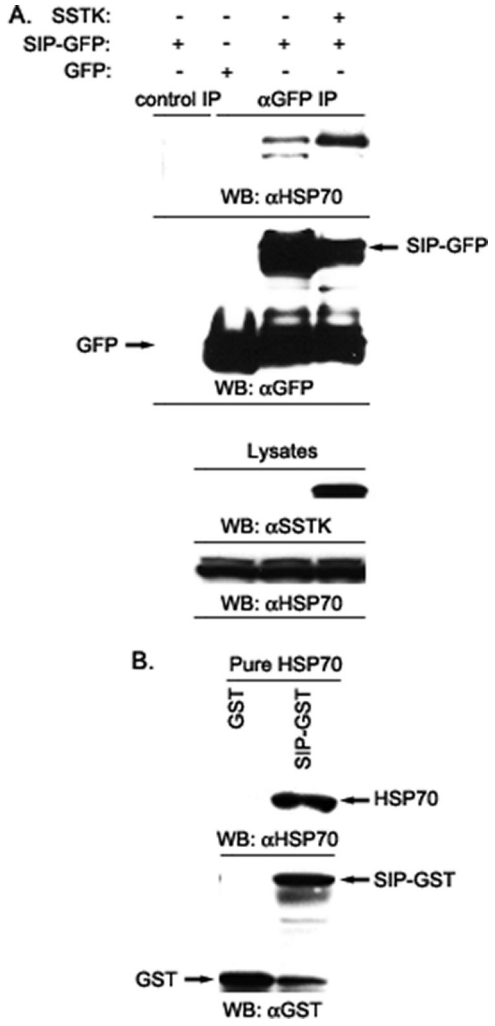

FIGURE 6. **Association of HSP70 with SIP.** *A*, SIP-GFP or GFP was expressed in 293T cells with or without SSTK, and immunoprecipitations (*IP*) were carried out using GFP or control antibody. Immunoprecipitates were probed with HSP70 and GFP antibodies. Cell lysates were probed with SSTK or HSP70 antibodies, *WB*, Western blot. *B*, 5 μg of purified GST or SIP-GST immobilized on glutathione agarose was incubated with 300 nM pure recombinant HSP70. Binding was analyzed by Western blotting using HSP70 and GST antibodies. The presence or absence of a component is represented by + and − signs, respectively.

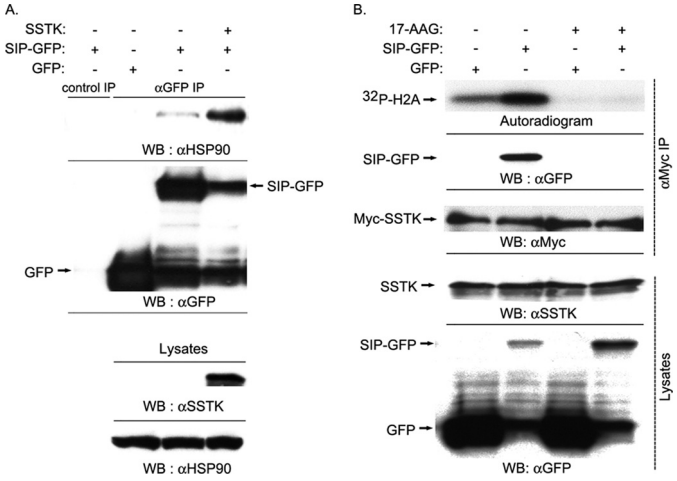

FIGURE 7. **Association of HSP90 with SIP and effect of 17-AAG on SSTK enzymatic activity.** *A*, SIP-GFP or GFP was expressed in 293T cells with or without SSTK, and immunoprecipitations (*IP*) were carried out using GFP or control antibody. Immunoprecipitates were probed with HSP90 and GFP antibodies. Cell lysates were probed with SSTK and HSP90 antibodies *WB*, Western blot. *B*, Myc-SSTK and GFP or SIP-GFP cDNAs were transfected into 293T cells and exposed to 1 μM 17-AAG for 24 h. SSTK was immunoprecipitated using Myc antibody, and *in vitro* kinase assays and analyses were performed as in Fig. 5*A*. The presence or absence of a component is represented by + and − signs, respectively.

exhaustive attempts to rescue SSTK activity were not successful (data not shown). However, using purified proteins, we were able to demonstrate that SSTK associates with HSP90 *in vitro* (supplemental Fig. S1). Together, the results demonstrate that SSTK can recruit SIP to HSP90 and that HSP90 is required for SSTK to possess kinase activity in cells.

DISCUSSION

SIP is a highly conserved protein in human and other mammals and was not identified in non-mammalian vertebrates, invertebrates, and lower organisms. Western blotting analysis demonstrated that SIP is expressed only in testis, and this finding is supported by the human EST database. Similar to SSTK, immunohistochemical localization demonstrated the presence of SIP in the inner luminal layer of the seminiferous tubules within the cytoplasm of elongated spermatids. However, unlike SSTK, SIP was not detected in ejaculated sperm, suggesting that SIP is no longer needed after kinase maturation and activation.

SSTK consists solely of a 273-amino acid residue protein kinase domain, and SIP binding to SSTK *in vitro* resulted in decreased kinase activity. Conversely, co-expression of SSTK and SIP in cells resulted in catalytic activation of the kinase and implicated other proteins in this process of cellular enzymatic activation. Molecular chaperones such as the HSPs aid in the native folding, stabilization, and signaling of client proteins. HSP90 is a constitutively and ubiquitously expressed protein that is shown to be required for the maturation of diverse client proteins, including steroid receptors, transcription factors, and a variety of serine/threonine and tyrosine kinases (18). HSP90 interacts with its client proteins in an ATP-dependent manner that is dependent on other chaperones and cochaperones (18, 32). In the case of the steroid hormone receptors, HSP70 and cochaperones act in conjunction to assemble the functional client-HSP90 machinery (33). Previously, we reported that SSTK associates with HSPs (7), and here, we demonstrate that HSP90 is absolutely required for SSTK catalytic activity. Cochaperones are proteins that are not clients of HSP70 or HSP90 and may be loosely defined as proteins that are involved in the function of other chaperones (21). Accordingly, our interpretation that SIP is a cochaperone that facilitates the HSP-mediated enzymatic activation of SSTK is based upon the following lines of evidence. (i) SIP binds with high affinity to the HSP90-associated SSTK and co-localizes with the kinase in the cytoplasm of elongated spermatids, and SIP-SSTK expression peaks at the same developmental steps 11–12; (ii) SIP directly engages HSP70; (iii) SIP associates with SSTK, HSP70, and HSP90 in cells; (iv) expression of SSTK results in the recruitment of SIP to HSP90; and (v) the presence of SIP results in a specific catalytic activation of SSTK that requires HSP90 function.

TPR domains are characterized by two antiparallel α-helices, are often found in tandem arrays, and are involved in protein-protein interactions (34). They are commonly present in pro-

teins that control cell cycle and transcription and in cochaperones that facilitate the maturation of clients by HSPs. TPRpred (28) predicted that SIP possesses a single TPR domain encoded within its third exon. A modeling analysis of residues 64–97 in SIP supported the idea that this region adopted a helix-turn-helix structure that strongly resembles the consensus TPR fold in consensus tetratricopeptide repeat (CTPR) (supplemental Fig. S2) (35). SIP contains conserved hydrophobic core residues at positions Tyr-70, Leu-73, Leu-90, and Pro-97 that are essential for the structural integrity of a TPR fold (35). This putative TPR domain may mediate the binding to HSP70 and/or to SSTK. The protein kinase-HSP90 cochaperone Cdc37 lacks a TPR, and thus, SIP may represent the first HSP cochaperone involved in kinase activation that contains a TPR domain.

In contrast to HSP90, SIP was not strictly required for SSTK enzymatic activity. SSTK possessed detectable kinase activity, albeit significantly lower, when expressed in the absence of SIP. Activation of protein kinases involves a conformational change from an "off" state that has minimal activity to an "on" state that is maximally active (36), and SIP appears to help HSP90 increase the proportion of SSTK in the on conformation. The precise mechanism and the nature of the molecular interactions that occur between SIP and the client SSTK and HSPs, and how SIP exactly mediates the HSP90-dependent activation of SSTK are likely to be quite complicated. The K41M mutation in the N-lobe of SSTK blocked binding of SIP but does not affect association of the kinase with HSPs (7), suggesting that SIP and HSPs bind to different regions of SSTK. One striking finding was that 17-AAG completely blocked the co-immunoprecipitation of SIP and kinase, implying that stalling of the HSP90 ATPase cycle results in a collapse of the SIP-SSTK complex on the HSP machinery. Consistent with our data, SIP may bind to HSP70 first and be recruited to HSP90-bound SSTK, where it engages SSTK and facilitates the conformational activation of the kinase. Association of SIP with HSP90 was significantly increased by co-expression of SSTK, demonstrating a recruitment of SIP to HSP90 by the kinase. However, we could find no evidence for HSP90 binding directly to SIP, and thus, this recruitment likely involves HSP70 and SSTK. It is important to note that in addition to SIP, HSP70, and HSP90, other factors may be involved and/or required for SSTK activation. Nevertheless, SIP appears to play a critical role in the ordered assembly of SSTK and the HSP70-HSP90 machinery, and this function appears to be specific. For example, HSP90 is involved in the maturation of Akt, but SIP does not influence Akt enzymatic activity.

Genetic deletion of the genes that encode the testis isoform of HSP70 (37) and the chaperone calmegin (38) resulted in male sterility. The testis HSP70-null male mice are infertile due to meiotic arrest in spermatocytes (17, 37, 39), and HSP70 may be essential in the later stages of spermatogenesis that involve SIP and SSTK. To our knowledge, SIP represents the first demonstration of a germ cell-specific cochaperone for a protein kinase that requires the HSP90 machinery for activation, and in addition to Cdc37, is only the second cochaperone identified that plays a role in protein kinase maturation. It will be interesting and important to determine whether SIP, like SSTK, is strictly required for male fertility or whether SIP functions to increase male fertility.

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