Sgt1p Is a Unique Co-chaperone That Acts as a Client Adaptor to Link Hsp90 to Skp1p

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Sgt1p is a conserved, essential protein required for kinetochore assembly in both yeast and animal cells. Sgt1p has homology to both TPR and p23 domains, sequences often found in proteins that interact with and regulate the molecular chaperone, Hsp90. The presence of these domains and the recent findings that Sgt1p interacts with Hsp90 has led to the speculation that Sgt1p and Hsp90 form a co-chaperone complex. To test this possibility, we have used purified recombinant proteins to characterize the in vitro interactions between yeast Sgt1p and Hsp82p (an Hsp90 homologue in yeast). We show that Sgt1p interacts directly with Hsp82p via its p23 homology region in a nucleotide-dependent manner. However, Sgt1p binding does not alter the enzymatic activity of Hsp82p, suggesting that it is distinct from other co-chaperones. We find that Sgt1p can form a ternary chaperone complex with Hsp82p and Sti1p, a well-characterized Hsp90 co-chaperone. Sgt1p interacts with its binding partner Skp1p through its TPR domains and links Skp1p to the core Hsp82p-Sti1p co-chaperone complex. The multidomain nature of Sgt1p and its ability to bridge the interaction between Skp1p and Hsp82p argue that Sgt1p acts as a "client adaptor" recruiting specific clients to Hsp82p co-chaperone complexes.

Hsp90 is a highly conserved molecular chaperone that has been linked to maintaining the activity of a number of cellular proteins involved in signal transduction and cell division. This role has been linked to the normal cycle of protein activation and inactivation associated with the highly dynamic pathways that control cell division (1). In addition, Hsp90 has been proposed to play a more general role in "buffering" the proteome against the genetic changes associated with the rapid accumulation of mutations found in cancers or in slow accumulation of changes that contribute to gene evolution (2). How Hsp90 is targeted to its substrates, or clients, remains a major unresolved question.

A large group of Hsp90-associated proteins have been proposed to assist Hsp90 in client recognition or in the transition of client to its final active state. One class of Hsp90-associated proteins includes "co-chaperones," proteins that interact with Hsp90 and frequently are found to modulate its ATPase activity. Co-chaperones typically interact with Hsp90 through two conserved domains: (i) a tetrapeptide repeat (TPR) domain or an Hsp20/α-crystallin domain, also known as a p23-domain, after the founding member of the family (3, 4). SGT1 encodes a protein that has both a putative TPR and p23 homology domains and was originally identified in the budding yeast Saccharomyces cerevisiae as a high copy suppressor of skp1–4 (5), a temperature-sensitive allele of SKP1. The relevance of this genetic interaction lies in the fact that Skp1p is an Sgt1p-associated protein. The Skp1–4p mutant fails to interact with Sgt1p, and this failure compromises the assembly of the budding yeast centromere-DNA binding complex, CBF3 (6, 7). A major advance in the understanding of Sgt1p function came from evidence found in multiple systems for an interaction between Sgt1p and Hsp90 (7–11). Although the biochemical details remain unclear, it has been proposed that Sgt1p may link Hsp90 to Skp1p and the core CBF3 subunit, Ctf13p, thus allowing Ctf13p activation and the assembly of the CBF3 complex (6). Consistent with this possibility, CBF3 assembly in rabbit reticulocyte lysate is sensitive to inhibition of Hsp90 (7, 12).

Although Skp1p has also been implicated in function of the E3 ubiquitin ligase SCF and a vacuolar [H+] ATPase, the importance of Sgt1p in these complexes is less clear (13–17). So while it may be that Sgt1p and Skp1p function together in some contexts, it is likely that Sgt1p is important for a broad set of cellular functions, including regulation of protein kinase A signaling through control of adenylyl cyclase function and in disease resistance in plants (18–25). Recent work in human tumor cells further argues that essential mitotic functions of Sgt1p overlap with those of Hsp90 in these cells (26). Interpreting these genetic findings requires some basic understanding of the biochemical role of Sgt1p in the context of Hsp90 function.

Recent reports have shown that Sgt1p co-immunoprecipitates with the molecular chaperone Hsp90 in yeast, plants, and humans (7, 9–11). Alone, this observation does not distinguish between Sgt1p being an Hsp90 client or co-chaperone. Co-precipitation data shows only a small fraction of total Sgt1p in a complex with Hsp90 and does not address whether additional factors are required to mediate the Sgt1p–Hsp90 interaction (7, 11). Furthermore, work with known co-chaperones has sug-

* This work was funded by an American Cancer Society Grant RSG-02-035-01 (to K. B. K.) and by Grant P20 MD000262 from the Research Infrastructure in Minority Institutions Program, NCMHD, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: TPR, tetrapeptide repeat; CBF3, centromere binding factor-3; E3, ubiquitin-protein isopeptide ligase; GST, glutathione S-transferase; AMP-PNP, adenosine 5′-(β,γ-imino)triphosphate; ATPγS, adenosine 5′-O-(thiotriphosphate).
gested that they may bind with much higher affinity than seen for Sgt1p and Hsp90 in extracts; the yeast co-chaperones Sti1p and Cpr6p have dissociation constants that suggest co-chaperone-Hsp90 complexes should predominate in vivo (27). One possible interpretation is that Sgt1p forms a transient interaction with Hsp90, one more reminiscent of the essential Hsp90-targeting subunit, Cdc37p (28). To explore the relationship between Sgt1p and Hsp90 in more detail, we have used purified recombinant proteins to reconstitute Hsp90-Sgt1p complexes in vitro. We have found that the p23 homology domain of Sgt1p is required for the direct interaction between Sgt1p and Hsp90. Sgt1p binds preferentially to non-ATP bound forms of Hsp90 and does not alter the intrinsic ATPase activity of the chaperone. Interestingly, Sgt1p can form at least two distinct ternary complexes; in the first, Hsp90 interacts directly with Sgt1p and Sti1p and in the second, Sgt1p interacts with Skp1p through its TPR domain and Hsp90 through its p23 homology domain. Importantly, we demonstrate that Sgt1p can recruit Skp1p to the core co-chaperone complex containing Hps90 and Sti1p. Together, these findings argue that Sgt1p functions as a client adaptor, linking Hsp90 to a specific set of clients.

EXPERIMENTAL PROCEDURES

Plasmids and Cloning—Construction of His-Sgt1p for baculoviral expression has been previously described (6) as has His-Skp1p (29). Yeast HSP82, STI1, and CPR6 were PCR-amplified with the addition of appropriate cloning sites from yeast genomic DNA and cloned into a modified pFASTBACTM vector (Invitrogen), pFBNHis10HA (6), or pMIT-77 (pFASTBAC-GST followed by PreScission Protease cleavage site; details provided upon request) to generate His-Hsp82p, GST-Hsp82p, GST-Sti1p, or GST-Cpr6p. Inserts were confirmed by DNA sequencing. p23ET, a plasmid for bacterial expression of His-tagged yeast Sba1, was a kind gift of Avrom Caplan. Site-directed mutagenesis using PCR was performed to generate mutant versions of Sgt1p and Hsp82p. Coding sequences were confirmed by DNA sequencing. Details of cloning techniques or primers used are available upon request.

Protein Expression and Purification—His-Sba1p was expressed and purified from bacterial lysates as previously described (30). All other proteins used in this paper were expressed and purified using the baculoviral expression system. High FiveTM insect cells (Invitrogen) were infected with the appropriate virus for 48 h, chilled on ice 15 min, then washed in phosphate-buffered saline. Cells were lysed on ice 15 min using 1 Triton buffer (10 mM HEPES, pH 8.0, 150 mM NaCl, 50 mM β-glycerophosphate, 0.1 mM EDTA, 1% Triton X-100, 1 mM dithiothreitol, 10% glycerol) plus protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM N-tosyl-L-phenylalanine chloromethyl ketone, and 10 μg/ml leupeptin, pepstatin, and chymostatin) then centrifuged at 4 °C for 15 min at 21,000 × g. The soluble fraction was removed, aliquoted, flash frozen, and stored at −80 °C for subsequent purification.

To purify GST- or His-tagged fusion proteins, 100 μl of glutathione-Sepharose 4B beads (GE Healthcare, Piscataway, NJ) or 100 μl of nickel-nitrioltrycic acid beads (Qiagen, Valencia CA), as appropriate, were added to soluble baculoviral extract containing the desired fusion protein diluted 1:10 in 1% Triton buffer and rocked at 4 °C for 2 h. Generally, extract containing 40 μg of fusion protein was used, although the protocol could be scaled up or down without altering purity or percent yield. After rocking, beads were washed three times in 500 μl of 1% Triton buffer, followed by three washes in 500 μl of SHB-Tris (25 mM Tris, pH 8.0, 150 mM KCl, 0.05% Triton X-100, 10% glycerol, 1 mM dithiothreitol, with protease inhibitors as described for 1% Triton buffer). GST fusion protein to be used as bait for binding assays was stored as purified at 4 °C for up to 1 week. To produce cleaved Sti1p or Cpr6p, GST fusion protein-bound beads were further washed three times in PreScission protease buffer followed by addition of 2 units of PreScission protease and overnight cleavage at 4 °C as described by the manufacturer (GE Healthcare). Eluted protein was aliquoted and flash frozen.

His fusion proteins bound to nickel-nitrioltrycic acid beads were washed three times in five bead volumes of SHB-Tris plus 50 mM imidazole, and protein was then eluted three times in one bead volume of SHB-Tris plus 250 mM imidazole. Eluted protein was combined, aliquoted, and flash frozen. Contaminating chaperones were released from Sti1p and Sgt1p protein fusions by including a final wash step with SHB-Tris buffer containing 5 mM ATP for 30 min at 4 °C, followed by three washes in SHB-Tris buffer with no nucleotide. Although this step reduced co-purifying proteins, it had no effect on the binding assays. Because the ATPase assays described below require large amounts of highly concentrated protein, the above protein purification protocol was scaled up, and eluted proteins were concentrated using Microcon YM-30 centrifugal filter devices (Millipore, Billerica, MA).

Protein Binding Assays—For the quantitative binding assays shown in Fig. 1 (A and B) proteins to be assayed were quantified using Bradford reagent (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions. 0.025, 0.050, or 0.100 nmol of Sgt1p, Sti1p, or Cpr6p with GST or GST-Hsp82p bound to glutathione beads in 200 μl of SHB-Tris and processed as described below. For the other binding reactions shown, 10 μl of GST, GST-Hsp82p, or GST-Skt1p (~2 μg of protein), bound to glutathione-Sepharose 4B beads (Amerham Biosciences) and purified as described above, was added to 200 μl of SHB-Tris followed by addition of ~2 μg of additional protein(s) to be assayed. Binding reactions were rocked at room temperature 1 h and then centrifuged at 1000 × g. Depleted supernatants were removed, and proteins were concentrated after adding 20% w/v trichloroacetic acid. Beads were washed three times in 200 μl of cold SHB-Tris. Bound and unbound fractions were resolved by SDS-PAGE and Coomassie-stained. For GST-Skp1p binding experiments, PreScission Protease was used (as described above) to cleave the fusion protein prior to SDS-PAGE because GST-Skp1p and His-Sgt1p co-migrate.

To quantify bound Sgt1p, Sti1p, and Cpr6 bound to GST-Hsp82p (Fig. 1B), the intensity of the band (or doublet of bands for Sgt1p) was determined from the Coomassie-stained gel using ImageQuaNT (GE Healthcare), converted to micrograms of bound using the molecular weight ladder as an internal control, then converted to nanomoles of bound using the molecular weight of the protein.
Nucleotide Dependence of Binding—Binding reactions of Sgt1p to GST-Hsp82p with or without various nucleotides were conducted as above with the following modifications. SHB-Tris binding buffer was modified via addition of 6 mM MgOAc and 5 mM ADP, ATP, AMP-PNP (Sigma), or ATPγS (Roche Applied Science) as indicated. Geldanamycin (a kind gift of the Drug Synthesis and Chemistry Branch, Developmental Therapeutics, Division of Cancer Treatment and Diagnosis, NCI, National Institutes of Health) was diluted from a 10 mg/ml (17.8 mM) stock in Me2SO to a final concentration of 60 μM. This concentration has previously been shown to completely inhibit the ATPase activity of purified Hsp90 (31, 32). GST-Hsp82 and indicated nucleotide or geldanamycin were mixed on ice and then preincubated at 30 °C for 30 min followed by the addition of potential binding partner to be assayed, and assays were completed as described above. Reactions were prepared on ice, then GST-Hsp82, and indicated amounts of nucleotide or geldanamycin were preincubated at 30 °C for 30 min, potential binding partner to be assayed was added, and assays were completed as described above. For Sba1p binding (Fig. 1D), binding was performed as described (33), but no cross-linking reagent was used.

ATPase Assays—Pyruvate kinase/lactate dehydrogenase-coupled ATPase assays were conducted essentially as described (32, 34), except that buffer SHB-Tris (25 mM Tris, pH 8.0, 150 mM KCl, 0.05% Triton X-100, 10% glycerol) was modified with 13 mM MgOAc and 5 mM ATP. Reactions were carried out at 37 °C in 200 μl of reaction volume with 0.4 nmol of Hsp82p and/or 1.2 nmol of co-chaperone (Sgt1p, Sti1p, Cpr6p, and Sba1p) unless otherwise noted. Addition of 60 μM geldanamycin completely inhibited the ATPase activity of our His-Hsp82p preparation (data not shown), ruling out the possibility of contaminating ATPases.

RESULTS

Sgt1p Directly Interacts with Yeast Hsp90—To characterize Sgt1p-Hsp90 complexes in more detail, we expressed and purified yeast Sgt1p and Hsp82p using baculoviral expression in insect cells (see “Experimental Procedures”). To serve as positive controls and to allow comparison of Sgt1p to known Hsp90 co-chaperones, the well characterized co-chaperones Cpr6p, Sti1p, and Sba1p were also purified as either GST fusions, or multihistidine fusions (supplemental Fig. S1). Sti1p is the yeast homolog of human HOP (35) and is thought to mediate transfer of client proteins from Hsp70p to Hsp90 (36, 37). Cpr6p is a yeast cyclophilin-like molecule (38), and Sba1p is the homolog of human p23 (30). Both Cpr6p and Sba1p are believed to act at a late step of Hsp90 client activation (39).

Previous efforts have co-purified only small amounts of Sgt1p associated with Hsp90 under conditions where the presence of other potential bridging proteins could not be ruled out (7, 11). To test if the interaction between Sgt1p and Hsp90 is direct, we purified recombinant, His-tagged versions of yeast Sgt1p (His-Sgt1p) and a GST fusion of the yeast Hsp90 protein, Hsp82p (GST-Hsp82). When added at equimolar ratios, His-Sgt1p specifically interacted with GST-Hsp82, but not GST, bound to glutathione-Sepharose beads (Fig. 1A). His-Sgt1p purified from insect cells migrates as a doublet due to protein phosphorylation (see arrows, Fig. 1A); interestingly, both forms of His-Sgt1p bind equally well to GST-Hsp82 (compare with load gel, supplemental Fig. S1). To assess the relative affinity of Sgt1p for GST-Hsp82p, we compared its ability to bind with two known co-chaperones, Sti1p and Cpr6p (Fig. 1A). We assayed the binding of Sgt1p, Sti1p, and Cpr6p when added at 1:4, 1:2, or 1:1 molar ratios to GST-Hsp82p. Taking into account the two forms of Sgt1p, quantitative analysis of digitized gel images suggest Sgt1p binds to GST-Hsp82p with similar affinity to other known co-chaperones (Fig. 1B).

Multiple modes of interactions link Hsp90 to its co-chaperone and clients. Some interactions are sensitive to conformational changes in Hsp90 mediated by nucleotide binding and hydrolysis. For example, it has been reported that Sba1p/p23 interacts preferentially with the ATP-bound form of Hsp90; Sti1p/Hop1 interacts preferentially with the ADP-bound form of Hsp90; and Cpr6p binds to Hsp90 independent of nucleotide (27, 30, 40, 41). To test the effect of nucleotide on the interaction between Sgt1p and Hsp90, we incubated purified His-Sgt1p with GST-Hsp82p in the absence of nucleotide, in the presence of ADP, ATP as well as the non-hydrolyzable ATP analogues, ATP-γ-S and AMP-PNP (see “Experimental Procedures”). His-Sgt1p bound to GST-Hsp82p in the absence of nucleotide, suggesting that binding does not require Hsp82p to be bound to nucleotide. Consistent with this possibility, geldanamycin, a highly specific Hsp90 inhibitor that displaces ATP from the active site (42–44), has no effect on the interaction of His-Sgt1p with GST-Hsp82p (Fig. 1C). Although incubation with ADP resulted in similar amounts of His-Sgt1p bound to GST-Hsp82p compared with the no nucleotide condition, ATP, and to a greater extent ATP-γ-S and AMP-PNP had an inhibitory effect on the binding of His-Sgt1p to GST-Hsp82p. As anticipated from the participation of Sti1p in the HSP90 “intermediate” complex and Cpr6p in the HSP90 “mature complex” (27), the binding of Cpr6p to GST-Hsp82p increased when GST-Hsp82 was ATP-bound, whereas Sti1p failed to interact with GST-Hsp82p in the presence of non-hydrolyzable ATP analogues (Fig. 1C). Although a weaker interaction, Sba1p bound to Hsp82p best in the presence of non-hydrolyzable nucleotide analogues and was further enhanced with the addition of sodium molybdate under conditions previously described (Fig. 1D) (33). Skp1p did not bind directly to GST-Hsp82p under any of the nucleotide conditions tested (Fig. 1B). These controls argue that the preparation of GST-Hsp82p bound to glutathione Sepharose is behaving as predicted, allowing us to conclude that, like Sti1p, Sgt1p has a lower affinity for Hsp90 in its ATP-bound conformation.

Sgt1p Does Not Alter the Enzymatic Activity of Hsp82p—The binding of Sti1p and Sgt1p to the ATP-bound form of Hsp82p raised the possibility that these two proteins have a similar effect on the enzymatic activity of Hsp82p. Previous reports have shown that Sti1p/HOP blocks access of ATP to Hsp90 (27). We therefore asked if Sgt1p could alter the hydrolysis rate of Hsp82p. For these experiments, we used a His-tagged version of yeast Hsp82p (see “Experimental Procedures”).

3 B. A. Macher and K. B. Kaplan, unpublished observations.
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**A.**

A titration of Sgt1p, Sti1p, or Cpr6p was added to 0.100 nmol of glutathione beads with 337 nmol of purified Sgt1p, Sti1p, or Cpr6p was added to glutathione beads with 100 nmol of bound GST control (GST) or GST-Hsp82p (Hsp82p), and a binding reaction was carried out as described under "Experimental Procedures." Arrows and labels mark the approximate position of the indicated protein. Only the bead-bound fractions are presented after SDS-PAGE and Coomassie staining. A, a titration of Sgt1p, Sti1p, or Cpr6p was added to 0.100 nmol of GST-Hsp82p, and binding reactions were performed as in A. Bound fraction image was digitized and quantified as under "Experimental Procedures"; nanomoles of protein bound to GST-Hsp82p are graphed for each amount of protein added. C, glutathione beads with GST-Hsp82p were preincubated with nucleotide as shown, and then Sgt1p, Cpr6p, Sti1p, Sba1p, or Skp1p was added separately to test binding. Bead-bound fractions (GST-Hsp82p bound) were analyzed as in A. The depleted fraction is also included (unbound) to show that excess protein was present in the binding reaction. Only the region of the gel relevant for each chaperone is presented. As in A, we observed no nonspecific binding of co-chaperones to GST (not shown). D, Sba1p binding was repeated in the presence of the indicated nucleotide, sodium molybdate, and in buffer as previously published (see "Experimental Procedures" and Ref. 64).

**B.**

**C.**

**D.**

**FIGURE 1.** Nucleotide dependence of Sgt1p binding to Hsp82p. A, 0.100 nmol of purified Sgt1p, Sti1p, or Cpr6p was added to glutathione beads with 100 nmol of bound GST control (GST) or GST-Hsp82p (Hsp82p), and a binding reaction was carried out as described under "Experimental Procedures." Arrows and labels mark the approximate position of the indicated protein. Only the bead-bound fractions are presented after SDS-PAGE and Coomassie staining. B, a titration of Sgt1p, Sti1p, or Cpr6p was added to 0.100 nmol of GST-Hsp82p, and binding reactions were performed as in A. Bound fraction image was digitized and quantified as under "Experimental Procedures"; nanomoles of protein bound to GST-Hsp82p are graphed for each amount of protein added. C, glutathione beads with GST-Hsp82p were preincubated with nucleotide as shown, and then Sgt1p, Cpr6p, Sti1p, Sba1p, or Skp1p was added separately to test binding. Bead-bound fractions (GST-Hsp82p bound) were analyzed as in A. The depleted fraction is also included (unbound) to show that excess protein was present in the binding reaction. Only the region of the gel relevant for each chaperone is presented. As in A, we observed no nonspecific binding of co-chaperones to GST (not shown). D, Sba1p binding was repeated in the presence of the indicated nucleotide, sodium molybdate, and in buffer as previously published (see "Experimental Procedures" and Ref. 64).

**Sgt1p Interacts with Hsp82p via Its p23 Homology Domain**

Although the putative TPR domain in Sgt1p might suggest that it is functionally related to the TPR-containing Sti1p, their distinct effects on Hsp82p activity led us to speculate that these two proteins interact with Hsp82p in fundamentally different ways. Sti1p, like other TPR-containing co-chaperones, interacts with the last five amino acids in Hsp90 (MEEVD) (46). To determine if Sgt1p also binds this Hsp90 domain, we expressed and purified a mutant form of GST-Hsp82p lacking the MEEVD amino acids (GST-Hsp82p εMEEVD). As expected from previous reports, the TPR-containing proteins Sti1p and Cpr6p do not bind GST-Hsp82p εMEEVD (Fig. 2A) (47). However, the same levels of His-Sgt1p bound to GST-Hsp82p εMEEVD as to GST-Hsp82p, arguing that Sgt1p does not interact with carboxyl terminus of Hsp82p and is therefore distinct from both Sti1p and Cpr6p (Fig. 2A). Note that a nonspecific cleavage product of Sgt1p that we have previously shown includes the amino terminus is consistently present in these complexes.

To identify the region in Sgt1p that is required to interact with Hsp82p, we constructed a series of truncations of Sgt1p, expressed them in insect cells, and tested their ability to bind to GST-Hsp82p. We found that neither the TPR-containing N terminus of Sgt1p (residues 1–187; Sgt1p1–187) nor the C-terminal region of Sgt1p (residues 268–395; Sgt1p268–395) were able to bind to GST-Hsp82p (Fig. 2B). Next, we asked if the central region of Sgt1p, which shows homology to the Hsp90-binding p23/α-crystallin domain (19), was sufficient to mediate GST-Hsp82p binding. Sgt1p173–315 and Sgt1p173–357 were observed to weakly, but reproducibly, bind to GST-Hsp82p as visualized on Coomassie-stained SDS-PAGE gels (see arrows, Fig. 2C). To confirm the central region of Sgt1p specifically interacts with GST-Hsp82p, we repeated the binding assay and enzymatic activity of yeast His-Hsp82p produced in insect cells using baculovirus had not been previously determined, we first established conditions where reliable kinetics could be measured. Using conditions similar to previously published work, we observed His-Hsp82p to have a hydrolysis rate of 0.285 mol/min/mol at 37 °C (Table 1), a rate comparable although slightly lower than previously reported (0.4 mol/min/mol (32)). Adding 60 μM of the ATP binding site competitor geldanamycin completely blocked ATP hydrolysis in our assay, arguing that there are no nonspecific ATPases present in the His-Hsp82p preparation. Importantly, ATP hydrolysis was almost completely inhibited when we added 3-fold molar excess of Sti1p or Sba1p; in contrast, addition of 3-fold excess of Cpr6p had no effect on ATP hydrolysis by His-Hsp82p (Table 1). These results are consistent with previously published reports and further validate the integrity of the purified system (27, 45). To determine if Sgt1p could alter the rate of ATP hydrolysis, it was added at a 3:1 ratio to Hsp82p. We observed no affect on ATP hydrolysis by His-Hsp82p (Table 1). An increase in the molar ratio of Sgt1p:His-Hsp82p also had no effect on the rate of ATP hydrolysis (>10-fold Sgt1p:His-Hsp82p; data not shown). This is consistent with the preference of Sgt1p for the non-ATP bound form of Hsp82p. We conclude that, although Sgt1p and Sti1p both bind to Hsp82p with similar nucleotide dependence, they have dramatically different effects on the enzymatic activity of Hsp82p.
detected Sgt1p using a specific polyclonal antibody in an immunoblot (6). Using this approach, we confirmed that the TPR domain alone (Sgt1p1–187) does not bind to GST-Hsp82p and that the region including amino acids 173–315 (Sgt1p173–315) is sufficient to interact with GST-Hsp82p but not with GST alone (Fig. 2, D and E). The slight differences in the amount of each Sgt1p fragment that binds may reflect subtle changes in the fold of the Hsp82p-interaction domain (compare Sgt1p173–357 with Sgt1p173–315 or Sgt1p173–395, Fig. 2D). It is interesting to note the increased interaction when the carboxyl terminus of Sgt1p is deleted; this may reflect the previously observed ability of the carboxyl terminus to inhibit amino-terminal binding of Skp1p (compare Sgt1p173–357 with Sgt1p173–395, Fig. 2D) (11). To more specifically test the interaction of the p23 homology region with Hsp82p, we attempted to create disrupting point mutants. The limited homology between p23 and Sgt1p made conserved residues difficult to identify. Despite the limited homology, we made changes in likely conserved residues (see Refs. 19 and 48 and our alignment, supplemental Fig. S2A). Unfortunately, single amino acid changes in this region gave rise to insoluble protein upon expression in insect cells or did not perturb binding (see supplemental Fig. S2, A and B). However, our deletion analysis is consistent with published spectroscopy data suggesting that the p23 domain of human Sgt1p binds Hsp90p (10), supporting our assertion that the p23 homology region of yeast Sgt1p mediates its binding to Hsp82p.

The TPR Motifs in Sgt1p Interact with Skp1p—Many co-chaperones, especially those with essential cellular functions (i.e. Cns1p and Cdc37p) have been proposed to link Hsp90 to specific clients. For example, the interactions between kinase clients and Cdc37p are transient and sensitive to the nucleotide-bound state of Hsp90 (49–51). The published reports describing the transient interaction between Sgt1p and Skp1p raise the possibility that Sgt1p links Hsp90 to Skp1p as a potential substitute for Cdc37p.

| Protein(s) | Percentage of Hsp82 |
|------------|---------------------|
| Hsp82p     | 100                 |
| Hsp82p + Sgt1p | 96          |
| Hsp82p + Sti1p | 6          |
| Hsp82p + Cpr6p | 97          |
| Hsp82p + Sba1p | 9          |
| Sgt1p      | 0.016 ± 0.01      |
| Sti1p      | 0.015 ± 0.01      |
| Cpr6p      | 0.016 ± 0.01      |
| Sba1p      | 0.000 ± 0.01      |
tial client (5–7, 11). To begin to define the relationship between these proteins, we asked whether the interaction between Sgt1p and Skp1p required Hsp82p. We used GST-Skp1p bound to glutathione beads as an affinity reagent as we previously published (11). However, the similar molecular weights for GST-Skp1p and His-Sgt1p required that we proteolytically cleave Skp1p from GST, releasing formed complexes into the supernatant (see “Experimental Procedures”). Incubation of GST-Skp1p and His-Sgt1p in the absence of other proteins showed that nearly equivalent molar ratios of His-Sgt1p bound to Skp1p (see protease eluted lanes, Fig. 3A). To rule out the possibility that a low level of contaminating Hsp90 from insect cells was contributing to the Skp1p-Sgt1p interaction, we used the highly specific Hsp90 inhibitor, geldanamycin, to block any Hsp90-related chaperoning function in our preparations. The interaction between Sgt1p and Skp1p was unaffected by geldanamycin (Fig. 3B). These data clearly demonstrate that Hsp82p is not needed for Skp1p to bind Sgt1p. This conclusion is further supported by our finding that a fragment of Sgt1p that cannot bind Hsp82p nonetheless binds robustly to Skp1p (see below).

Previous work in lysates implicated the amino-terminal half of Sgt1p in the interaction with Skp1p (7, 11). To confirm these results using purified proteins, we used the Sgt1p truncations described in Fig. 2 to test their interaction with GST-Skp1p. The fragment of Sgt1p containing the TPR (Sgt1p1–187) domain bound to GST-Skp1p more robustly than full-length Sgt1p (Fig. 4B). However, Sgt1p268–395 was unable to bind to GST-Skp1p. We also performed an immunoblot and observed that Sgt1p fragments lacking the TPR motifs (Sgt1p173–315, Sgt1p173–357, and Sgt1p173–395) bind to Hsp82p, we believe that they have folded properly (see Fig. 2); we conclude that Skp1p binds to the amino-terminal region of Sgt1p.

**FIGURE 3.** Sgt1p interacts with Skp1p independent of Hsp82p. Purified GST-Sgt1p and His-Skp1p were incubated as described under “Experimental Procedures.” The fractions left associated with beads (cleaved beads), eluted by protease cleavage (protease eluted), or left in the supernatant (unbound) were analyzed by SDS-PAGE and Coomassie staining. Proteins were incubated in the absence of nucleotide (A) or in the presence (GA) or absence (−) of geldanamycin (B). Arrows indicate the approximate position of each labeled protein, and the asterisk marks the position of the Sgt1p, proteolytic fragments; Prot., the position of the protease used to cleave GST-Skp1p from the beads. We typically observed a small fraction of protease that releases from the beads during incubation, regardless of the fusion used.

**FIGURE 4.** Sgt1p requires its TPR domain to interact with Skp1p but not Hsp82p. A, an alignment of the three proposed TPR motifs in Sgt1p with yeast TPR motifs in Sti1p and Cdc23p (numbers in parentheses refer to starting amino acid position of each TPR motif); identical residues are boxed black, blocks of similar amino acids are dark gray, and the conserved residues are light gray. The asterisks indicate the positions of single amino acid changes analyzed. GST-Skp1p (B–D), GST-Hsp82p (E), or GST alone (F) bound to glutathione beads were incubated with the indicated Sgt1p proteins (numbers indicate the Sgt1p amino acids included in the protein). For B and D, GST-Skp1p was cleaved from GST using Precision Protease prior to SDS-PAGE analysis as GST-Skp1p and His-Sgt1p migrated in the same position on the gel. Fractions of Sgt1p left associated with beads (cleaved beads), eluted by protease cleavage (protease eluted), or left in the supernatant (unbound) were analyzed by SDS-PAGE and Coomassie staining. The asterisks in B indicate the approximate position of each Sgt1p truncation. C, GST-Skp1p bound to glutathione beads were incubated with the indicated fragments of Sgt1p, and the bound fraction was analyzed by immunoblot with antibodies against Sgt1p. No cleavage of GST-Skp1p was carried out in C.
Because the amino terminus of Sgt1p has been proposed to contain a TPR domain composed of three TPR motifs, we decided to test whether this domain is required to mediate the interaction with Skp1p. We chose conserved amino acids that are predicted from structural studies of other TPR motifs to disrupt each of the TPR motifs. Alanine residues at position 23, 67, or 107 were chosen for mutagenesis, because these residues match the same position in the TPR consensus for the first, second, and third TPR fold (see alignment in Fig. 4A and Ref. 3). When these mutants were produced as full-length His-tagged fusion proteins in insect cells, purified, and added to glutathione bead-bound GST-Skp1p, we observed that Sgt1pA23E and Sgt1pA107Y (or Sgt1pA107E) failed to bind Skp1p (Fig. 4D). Sgt1pA67Y, however, bound to GST-Skp1p like wild-type (Fig. 4D). This shows that the first and third TPR domains of Sgt1p mediate binding to Skp1p, and it may mean that the middle TPR domain remains available for binding of additional (or alternate) proteins. We cannot rule out, however, that Sgt1pA67Y fails to disrupt the second TPR fold and that additional mutants in this domain would block Skp1p binding. Importantly, we saw no effect on the binding of any of these Sgt1p TPR domain point mutants to GST-Hsp82p (Fig. 4E), confirming that these mutants are not severely misfolded and that the TPR domain of Sgt1p is not required for its interaction with Hsp82p. A summary of the Sgt1p domains that interact with Skp1p and Hsp82p is presented in Fig. 6.

**FIGURE 5.** Sgt1p acts as an “adaptor,” linking co-chaperone complexes to Skp1p. GST-Hsp82p (A), GST-Skp1p (B), or GST-Sti1p (C and D) bound to glutathione beads were incubated with the indicated proteins and/or nucleotide. The GST-Skp1p fusion in B was cleaved with Precision Protease to allow for separation of Skp1p and Sgt1p; the position of the Precision Protease is shown; the asterisk marks the position of a small amount of uncleaved GST-Skp1p. Only the fraction of proteins bound to beads is presented; approximately equal moles of each protein were added. Arrows and labels indicate the approximate position of each protein on the SDS-PAGE gel.

The ability of Sgt1p to bind both Hsp82p and Skp1p raised the possibility that Sgt1p could form a ternary complex with Hsp82p and its binding partner Skp1p. Interestingly, Skp1p could not interact directly with GST-Hsp82p (Fig. 5A); this finding distinguishes Skp1p from Hsp90 clients that interact directly with the chaperone (Ref. 52 and “Discussion”). However, if Sgt1p was included in the binding reaction, Skp1p was found in the GST-Hsp82p-bound fraction (Fig. 5A). We conclude from this data that a ternary complex of Sgt1p-Skp1p-Hsp82p can form in vitro. We propose that Sgt1p binds simultaneously to Hsp82p via its p23 domain and to Skp1p via its TPR domains. To test whether the ternary complex was sensitive to the ATP-bound state of Hsp82p, we formed the complex using a GST-Skp1p fusion in the presence of ADP or ATPγS. Consistent with the behavior of each pair of components (see Figs. 1C and 3), we observed that Sgt1p-Skp1p complex associates independently of Hsp82p and nucleotide, whereas the association of Hsp82p with the complex is inhibited by incubation with ATPγS (Fig. 5B).

The ability of GST-Hsp82p<sup>ΔMEEVD</sup> to bind Sgt1p raised the possibility that Sgt1p could form a complex with Hsp82p bound to other TPRs containing co-chaperones via MEEVD. To test this possibility, we examined Sti1p, because, like Sgt1p, it prefers the non-ATP-bound form of Hsp82p (see Fig. 1). We expressed and purified GST-Sti1p and observed, as expected, its interaction with His-Hsp82p (Fig. 5C) (53). In contrast, when Sgt1p alone was added to bead-bound GST-Sti1p, it failed to bind, arguing that these two proteins do not directly interact (Fig. 5B). However, when Hsp82p and Sgt1p were added together, both proteins bound robustly to GST-Sti1p (Fig. 5C). These results are consistent with distinct domains on Hsp82p binding to Sti1p and Sgt1p. In support of this idea, we observed that, although Sgt1p can bind Hsp82p<sup>ΔMEEVD</sup> (Fig. 2 and Ref. 46), Sti1p could not form a ternary complex with Sgt1p-Hsp82p<sup>ΔMEEVD</sup>.<sup>4</sup>

The ability of Sgt1p to bind both Hsp82p and Skp1p raised the possibility that Sgt1p acts to recruit clients, such as Skp1p or

<sup>4</sup>M. G. Catlett and K. B. Kaplan, unpublished observations.
Sgt1p Is a Unique Hsp90 Co-chaperone

A.

| Binding | Skp1p |
|---------|-------|
| Yes     | Yes   |
| No      | Yes   |
| (Yes)   | No    |
| (Yes)   | No    |
| No      | No    |
| No      | Yes   |
| (Yes)   | No    |

B. Summary of Sgt1p binding analyses and a model of chaperone complexes. A, a graphical summary of the binding data presented in Figs. 2 and 4. The parentheses denote weak but significant binding. Colors indicate the domain boundaries of Sgt1p. B, schematic of Sgt1p domains (TPR, p23, and SGS), showing their interactions with the core chaperone complex and potential cellular clients (arrows). The configuration of Sgt1p domains is implied by alignments of amino acid homologies and previously published limited proteolysis studies (numbers refer to amino acids in Sgt1p) (5, 10, 11). The Sti1p interaction with Hsp82p was previously demonstrated (27). Other clients suggest the possibility that the TPR motifs in Sgt1p interact with other cellular proteins. Additionally, Skp1p interacting proteins that contain F-box and leucine-rich repeat domains may also be recruited to the chaperone complex (see "Discussion").

Skp1p-associated proteins (see "Discussion"), to the core chaperone machinery. We therefore asked whether Sgt1p could link Skp1p to the Hsp82p-Sti1p core chaperone complex. To test this, we incubated GST-Sti1p with each of the proteins separately and observed that only Hsp82p could interact directly with Sti1p (Fig. 5D). Incubation of Sgt1p and Skp1p with the GST-Sti1p-Hsp82p co-chaperone complex resulted in the formation of quaternary complex containing all four proteins (i.e. Skp1p, Sgt1p, Hsp82p, and GST-Sti1p, Fig. 5D; also see Fig. 6B). Together these results lead us to conclude that Sgt1p acts as an adaptor, to link Skp1p complexes to the core chaperone machinery (i.e. Hsp82p-Sti1p; see model in Fig. 6B).

DISCUSSION

Sgt1p is a highly conserved protein that has been implicated in a variety of cellular processes, including chromosome segregation, signal transduction, ubiquitin-mediated protein degradation, and disease resistance in plants. The co-purification of Hsp90 with Sgt1p from cell extracts suggests that Sgt1p may mediate this array of cellular functions by influencing the activity of the Hsp90 chaperone. Using biochemical reconstitution, we show that Sgt1p interacts with Hsp90 in a way that is distinct from other co-chaperones. Its preference for the non-ATP bound form of Hsp90, the second TPR domain of Sgt1p mediates binding to Hsp90 (54), whereas the TPR domains of Sgt1p are dispensable for binding to Hsp90. Furthermore, the p23/α-crystallin regions of Sba1p and Sgt1p both mediate binding to Hsp90, but Sba1p binds to Hsp90-ATP, whereas Sgt1p prefers to bind to Hsp90-ADP. The use of purified components is a powerful approach to characterize the protein domains and biochemical activities sufficient for chaperone complex interactions; however, it is important to acknowledge that the in vivo characteristics of these complexes may differ in the context of the multiple chaperone complexes that can form in cells.

Another indication that Sgt1p behaves differently than other co-chaperones comes from the genome wide screens that robustly
detect interactions between Hsp90 and known co-chaperones but inconsistently detect the interaction between Sgt1p and Hsp90 (55, 56). For example, Zhao et al. (55) did not find Sgt1p to interact by two-hybrid but did find Sgt1p in Hsc82-TAP pull-downs (notably, under conditions that lack ATP). Further, Millson and colleagues (56) failed to detect an Hsp90-Sgt1p interaction by two-hybrid using an Hsp82 E33A mutant as bait. However, this mutant form of Hsp82p delays the chaperone cycle at a late stage in client activation (32), and its failure to bind Sgt1p is consistent with our finding that Sgt1p binds to Hsp90-ATP with lower affinity. We propose that the difference between these global screens and our purified system is due to the ability of Sgt1p to act as a co-chaperone for only a small subset of Hsp90 clients (such as Ctf13p); thus, the number of Sgt1p-Hsp90 complexes per cell at any time is much lower than for other Hsp90-co-chaperone pairs. This model is supported by the low abundance of Sgt1p, estimated at only 1340 molecules per cell, whereas Cpr6p, Sba1p, Sti1p, and Hsp82p are 14-, 25-, 50-, and 333-fold more abundant, respectively (57).

Sgt1p Acts as a “Client Adaptor” to Link Hsp90 to Skp1p Complexes—Previous work using deletions, temperature-sensitive alleles and this work make a compelling case that Sgt1p interacts directly with Skp1p through its TPR domain (5–7,11). However, in contrast to the relatively weak binding between Sgt1p and Skp1p and Skp1p reported in extracts, the purified proteins we use here exhibit a very robust interaction (6, 7, 11). We suggest that a straightforward resolution to this discrepancy lies in the use of cellular extracts. We speculate that Skp1p-Sgt1p-Hsp90 complexes remain transient in vivo. Consistent with this possibility, addition of geldanamycin to extracts prior to TAP-Sgt1p purification increased the amount of associated Skp1p, potentially due to changes in the formation and turnover of Skp1p-Sgt1p-Hsp90 complexes in extracts (11).

The interaction of Sgt1p with both Skp1p and Hsp90 raises the question of what role Skp1p plays in these complexes. Our observations that Skp1p cannot bind directly to Hsp90 argues against it being formally considered a co-chaperone. Rather, our findings that Sgt1p can stably link Skp1p to a large co-chaperone complex (i.e. Sti1p, Hsp82p, Sgt1p, and Skp1p) raise the possibility that Skp1p is an Hsp90 client. Arguing against this simple interpretation is the fact that Skp1p and Sgt1p interact independently of Hsp90 activity. However, it is possible that Hsp90 chaperones the interaction of Skp1p with other cellular targets. In this light, it is interesting to recall that Skp1p interacts with a conserved F-box motif found in a number of proteins, including the core CBF3 component, Ctf13p (13, 29, 58, 59). Therefore, we suggest that Sgt1p links Hsp90 to Skp1p or Skp1p complexes containing F-box proteins or other common protein folds associated with F-boxes (e.g. leucine-rich repeats or WD40 repeats).

The potential role of Sgt1p in linking Hsp90 to clients is reminiscent of the relationship between Cdc37p and Hsp90 (28). Cdc37p is a well characterized co-chaperone that directs Hsp90 to kinases client proteins. Unlike many yeast co-chaperones, both Sgt1p and Cdc37p are essential (5, 60). Both Sgt1p and Cdc37p interact robustly with Hsp90 in vitro; like the interaction between Sgt1p and Ctf13p or Skp1p (6, 11, 12), only trace amounts of Cdc37p are found in complex with Hsp90 or kinase clients from extracts (61–63). Thus, we favor a model in which Sgt1p and Cdc37p play similar roles as co-chaperones for a distinct set of client proteins (see Fig. 6B). We propose the use of the term “client adaptor” for these obligate links between the Hsp90 chaperone machinery and their unique set of client proteins. Because Cdc37p (62) and Sgt1p (this study) bind to Hsp90 at a site that leaves the carboxyl-terminal TPR binding domain of Hsp90 free to interact with other co-chaperones, client adaptors serve to dock their clients to the core Hsp90 chaperone machine in a way that allows other co-chaperones, such as Sti1p, to bind and act upon the client-bound chaperone complex. Ultimately, testing the activity of clients in a purified, reconstituted system will be important for beginning to understand the details of Hsp90 co-chaperone complexes.

Acknowledgments—We thank Nichole Rasters for help making SGT1 point mutants, members of the Kaplan laboratory, Dr. Enoch Baldwin, and members of the UC Davis Cytoskeletal Club for helpful discussions. We thank Dr. Avrom Caplan for the Sba1p expression plasmid.

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