Sgt1 has been identified as a subunit of both core kinetochore and SCF (Skp1-Cul1-F-box) ubiquitin ligase complexes and is also implicated in plant disease resistance. Sgt1 has two putative HSP90 binding domains, a tetratricopeptide repeat and a p23-like CHORD and Sgt1 (CS) domain. Using NMR spectroscopy, we show that only the CS domain of human Sgt1 physically interacts with HSP90. The tetratricopeptide repeat domain does not bind to either HSP90 or HSP70. Determination of the three-dimensional structure showed that the Sgt1-CS domain shares the same β-sandwich fold as p23 but lacks the last highly conserved β-strand in p23. Analysis of the structures of Sgt1-CS and p23 revealed a similar charge distribution on one of two opposing surfaces that suggests that it is the binding region for HSP90 in Sgt1. Although ATP is absolutely required for p23 binding to HSP90, Sgt1 binds to HSP90 also in the absence of the non-hydrolyzable analog ATPγS. Our findings suggest the CS domain is a binding module for HSP90 distinct from p23-like domains, which implies that Sgt1 and related proteins function in recruiting heat shock protein activities to multiprotein assemblies.

Heat shock protein 90 (HSP90) is a molecular chaperone important for protein folding. HSP90 is different from other chaperones because most of its substrates are related to signal transduction (1). Recent studies also suggest that HSP90 plays a role in protein quality control where it facilitates the poly-ubiquitination and degradation of substrates through interaction with the co-chaperone C terminus of HSC70-interacting protein (CHIP) (2). Thus, HSP90 can be involved in protein regulation in quite different ways depending on the cellular context.

Very recently, it has been reported that Sgt1 interacts with HSP90 (3–5). Sgt1 was originally identified as a suppressor of the G2 allele of Skp1 and was found to be important for both the G1/S and G2/M transitions in the cell cycle (6). The G2/M transition involves activation of the kinetochore. Sgt1 was shown to be required for the activation of the kinetochore core complex CBP3 and to physically interact with Skp1, one component of CBP3 (6). Sgt1 also physically interacts with Skp1-Cul1-F-box (SCF) E3 ubiquitin ligase complexes through interaction with Skp1. Moreover, a yeast Sgt1 mutant was defective in Sic1 degradation through ubiquitination (6).

Sequence analysis of Sgt1 proteins from yeast, human, barley, rice, and Arabidopsis shows three conserved domains (tetratricopeptide repeat (TPR), CHORD-containing proteins and Sgt1 (CS), and Sgt1-specific (SGS)) and two variable regions (VR1 and VR2) (7). TPR domains are known as heat shock protein binding domains. The SGS domain was shown to interact with S100 calcium-binding proteins (8). The CS domain has high sequence homology with p23 and is also known as a p23-like domain (9). p23 has been shown to physically and functionally interact with HSP90, apparently serving in a role as co-chaperone. Recently, HSP90 was shown to be an essential factor required for kinetochore assembly (10) and two-hybrid screens revealed an interaction between Sgt1 and HSP90 (3).

Because Sgt1 has a standard heat shock protein binding TPR domain (3, 7), it is logical to assume that this TPR domain is the primary mediator of HSP90 interaction. However, given the homology with p23, it has been also postulated (11, 12) that the CS domain may also be involved. In fact, the CS domain has been proposed to play a direct role in binding HSP90 (3). To more fully characterize the interaction of HSP90 and Sgt1, we report here a series of biophysical and structural experiments designed to map the HSP90 binding site on Sgt1. These results are then analyzed for their implications regarding the cellular functions of Sgt1 and related proteins.

**EXPERIMENTAL PROCEDURES**

Expression Plasmids—For Sgt1 and its isolated domains, the appropriate fragments of human Sgt1 DNA were amplified from the BKK51 plasmid (6) by PCR with Pfu polymerase. PCR products and vector (pET28a from Novagen) were digested with restriction endonucleases (NdeI and BamHI, both from Promega), purified from an agarose gel using QiAquick gel extraction kit (Qiagen), and ligated by T4 DNA ligase (Promega) to produce pET28-Sgt1, pET28-Sgt1-(1–138) (TPR domain), pET28-Sgt1-(139–236) (CS domain), and pET28-Sgt1-(227–333) (SGS domain). These TPR and CS domains contain additional VR1
Sgt1-CS Domain Binds HSP90

RESULTS
HSP90 Binds to Sgt1—Recent evidence suggesting that Sgt1 interacts with HSP90 (3, 11, 12) led us to investigate whether or not there is direct interaction between human Sgt1 and HSP90 using a combination of pull-down assays and NMR spectroscopy. The pull-down assays in Fig. 1 show clear evidence for interaction between HSP90 and Sgt1. The well established ATP-dependent interaction between HSP90 and its co-chaperone p23 was used as a control to confirm the validity of our methods (13, 16). Using the non-hydrolyzable analog ATPγS, we see the expected ATP dependence of the binding of p23 to HSP90 (Fig. 1, left two lanes). Remarkably, the interaction of Sgt1 with HSP90 did not require the presence of ATPγS as did p23. There is a notable difference in the amounts of HSP90 bound to the Sgt1 resin in the absence and presence of ATPγS, which implies that the binding of HSP90 may be stimulated by the presence of ATP. However, regardless of the relative amounts of HSP90 bound, the key finding from the pull-down assays is that Sgt1 can bind HSP90 in the absence of ATP.

NMR spectroscopy was used to further characterize the nature of the interaction between Sgt1 and HSP90 (Fig. 1B). For these experiments, samples of 15N-enriched Sgt1 were titrated with increasing amounts of HSP90 and 15N–H2 HSPC NMR spectra were recorded in the absence of ATP. NMR signals are exquisitely sensitive probes of protein structure, and in these spectra, signals are observed for each amide group in the protein. The NMR approach thus provides a large number of site-specific probes of the structure to be monitored as the binding of one component to another occurs. As HSP90 was titrated into the solution of labeled Sgt1, there was a general decrease in intensities of isopropyl methyls of Val and Leu were observed using a mixture of biosynthetically directed 10% fractional 13C-labeled protein (18). Nuclear Overhauser effects (NOEs) between CH2 and aromatic protons observed in a three-dimensional 13C-resolved NOESY were used as starting points for sequence-specific assignments of aromatic side chains. These NOEs in combination with two-dimensional constant time (CT) 13C–1H HSQC and homonuclear two-dimensional 1H–1H TOCSY were used to complete the assignment of each aromatic side-chain spin system. The 10% fractionally 13C-labeled sample was used for a two-dimensional 13C–1H CT-TROSY-HSQC (19) with a 36-ms CT period to assign the 13C and attached protons of Phe residues. In addition, this spectrum also helped identify δ and ε positions of Phe and Tyr residues. All of the spectra were processed using NMRPipe (20) and analyzed using Sparky (21).

Structure Calculations—Distance restraints were obtained from three-dimensional 15N-edited and 13C-edited NOE-HSQC. Assignments and volume integration were made with the program PIPP (22). Distance-filtered NOE assignments using preliminary structures were made to expedite the process. Upper distance bounds for the structure calculations were set to 3.0, 4.5, and 6.0 Å for the three-dimensional 13C-edited NOE-HSQC and 3.0, 4.0, 5.0, and 6.0 Å for the three-dimensional 15N-edited NOE-HSQC. Backbone torsion angles were obtained from the program TALOS (20). An ensemble of 50 starting structures were generated using the program DYANA, version 1.5 (24), and refined using the SANDER module of AMBER (25) using 1535 distance restraints (3131 intraresidues, 349 sequential, 133 medium-range, and 474 long-range) and 118 torsion angle restraints. The program FINDFAM (26) was used to determine that a minimum of 20 structures was needed to represent the family accurately. The structures were selected on the basis of a combination of lowest constraint violation energies and large negative molecular energies. The final ensemble of 20 structures gave no distance violations greater than 0.2 Å and no torsion angle violations greater than 5° (Table I). Structures were analyzed graphically using MOLMOL (27). The stereochemistry of the final family of structures was analyzed using the PROCHECK NMR software (28) showing that 97.5% of all residues fall within the favorable regions of the Ramachandran plot.

Data deposition—The 1H, 13C, and 15N chemical shift assignments have been deposited in the BioMagResBank under accession code 6012. The coordinates of the final family of 20 structures and the full list of NMR restraints used in the structure calculations have been deposited in the Protein Data Bank (PDB) under accession code 1RLI.
attenuation of peak intensity in the NMR spectrum, consistent with the binding of the large 180-kDa HSP90 dimer to the 38-kDa Sgt1. However, a comparison of the two panels in Fig. 1 shows that a select number of peaks were more severely affected than the others. This critical observation indicates that the binding of HSP90 occurs at a specific site on Sgt1.

Sgt1 Is a Modular Protein—As noted above, Sgt1 is comprised of TPR, CS, and SGS domains. The fact that binding of HSP90 to Sgt1 leads to selective effects on a subset of NMR signals suggested that the HSP90 binding domain of Sgt1 is structurally independent of the other domains. These observations prompted the subcloning of each of the three Sgt1 domains (7) for in-depth biophysical analysis: TPR-(1–138); CS-(139–226); and SGS-(227–333). Successful bacterial expression of these constructs under standard conditions suggests that stably folded protein domains are produced.

To determine whether or not the Sgt1 domains are structurally independent, 15N-enriched samples of intact Sgt1 and the three domains were prepared and 15N-1H HSQC NMR spectra were acquired under identical conditions (Fig. 2). The spectra for the isolated TPR and CS domains are well dispersed, indicating that they have a well folded globular structure. The spectrum of the SGS domain is much less dispersed, which indicates that it is not a well folded globular domain. An analysis by CD spectroscopy indicates there is a limited degree of helical secondary structure in the SGS domain. The C-terminal domain of calcyclin-binding protein (amino acids 178–229), which shows high sequence homology with Sgt1, has a similar
Thus, the SGS domain probably has some nascent helical elements but does not occupy a compact well folded globular structure.

Comparison of the NMR spectra for the isolated domains to that of intact Sgt1 reveals peaks in identical positions (Fig. 2). Because the NMR chemical shift is extremely sensitive to structure, the similarity of peak positions constitutes unambiguous evidence that there are no differences in the structures of the domains, whether they are alone or tethered together in the intact protein. The line-widths of the peaks in the spectrum of intact Sgt1 are smaller than expected for a single domain molecule over 30 kDa, strongly implying that the linkers between the domains are flexible and that any contacts between domains are not long-lived. Hence, the domains probably function in a coordinated but not necessarily cooperative manner.

**The Interaction of Sgt1 with HSP90 Occurs via the CS Domain**

The modular nature of Sgt1 enabled NMR to be used to identify the HSP90 binding region on Sgt1. This involved titration of HSP90 into solutions of 15N-enriched Sgt1 domains as described above for the intact protein. Fig. 3 shows the 15N-1H HSQC spectra after adding 1 molar equivalent of HSP90 to the TPR, CS, and SGS domains of Sgt1. Surprisingly, there is no effect on the TPR domain, contrary to the hypothesis that the Sgt1 TPR domain might be the main binding domain to HSP90 (11, 12). In contrast, the disappearance of the majority of signals in the spectrum of the CS domain is a clear indication of interaction with HSP90. The few peaks remaining in the spectrum of the CS domain in the presence of HSP90 are attributable to very flexible residues in loops and at the N and C termini. A detailed analysis of the peaks that disappear in the spectra of intact Sgt1 upon binding of HSP90 reveals that these signals correspond very closely to those affected by binding of HSP90 to the isolated CS domain (cf. Fig. 1B). Hence, all of the observations are consistent with the interaction of Sgt1 with HSP90 being mediated by the CS domain. Experiments on Sgt1-CS were also performed in the presence of ATP, and no detectable differences were observed, supporting the conclusion from the pull-down assay that, unlike p23, the interaction of Sgt1 with HSP90 can occur in the absence of ATP.

**The Structure of Sgt1-CS Domain Is Distinct from p23**

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observation that the interaction of Sgt1 with HSP90 did not require ATPγS indicates that the Sgt-CS domain is biochemically distinct from p23. Although the sequence homology of Sgt1-CS and p23 implies that they share a common structural framework, the difference in biochemical properties implies that there are significant (although probably subtle) differences in their structures. To test this hypothesis and obtain possible insights into the reasons why Sgt1-CS and p23 have different biological functions, we determined the structure of Sgt1-CS by multidimensional heteronuclear NMR.

Details of the protocols for structure determination are provided under “Experimental Procedures,” and key structural statistics are presented in Table I. The representative NMR structural ensemble of the Sgt1-CS domain is shown in Fig. 4A. Overlays of the Sgt1-CS domain and p23 structures (Fig. 4C) reveal that the proteins are very similar. The proteins superimpose to a root mean square deviation of 1.6 Å. There are a series of conserved residues among the Sgt1-CS domain, p23, and other CS domain-containing proteins, which include Trp-144, Val-151, Leu-192, Ile-196, Ser-201, Lys-216, Trp-222, and Leu-225 (Sgt1 numbering). These residues are all found in the hydrophobic core (Fig. 4C) and are presumably important for maintaining the β-sandwich fold.

There is one significant difference between the structures of p23 and Sgt1-CS. Sgt1-CS lacks the last β-strand found in p23 (Fig. 4B). The absence of this structural element is intrinsic to the Sgt1 protein, because all of the Sgt1-CS residues corresponding to the last β-strand in p23 are present in the construct. Moreover, we have directly observed all of their NMR signals and find that these residues appear to be significantly more flexible than the rest of the domain. They have unusually narrow line-widths and are not involved in any long-range NOEs. Interestingly, this region is highly conserved among p23 proteins but appears not to be conserved in CS domains including Sgt1-CS, Siah-1-interacting protein (SIP)-CS, and other “p23-like” domains (9). It is intriguing to consider how the structural difference at the C-terminal edge of the β-sandwich may be the critical factor enabling CS domains to bind to HSP90 in the absence of ATP.

An analysis of the distribution of electrostatic charge at the surface of Sgt1-CS and p23 was also highly informative. The left and right panels of Fig. 4D show that these proteins share a strikingly similar charge distribution on one surface, which we call surface I. The high degree of similarity at this surface strongly suggests a similar function and prompted a further comparative analysis of these proteins.

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Fig. 3. Sgt1 interacts with HSP90 via its CS domain. $^{15}$N-$^1$H HSQC NMR spectra of the isolated TPR (left), CS (center), and SGS (right) domains in the presence of HSP90. The samples and experimental conditions were the same as those used for Fig. 2 with the exception of the presence of 1 molar equivalent of HSP90. Note that only the spectrum of Sgt1-CS is different from the corresponding spectra for the isolated domains shown in Fig. 2 and peaks that disappear are circled.

TABLE I  
Statistics for the Sgt1 CS domain structural ensemble

| Constraint violations, mean ± S.D. | 
|-----------------------------------|----------------|
| Distance violations (d)           | 0.1 Å < d < 0.2 Å | 0.3 ± 0.4 |
| Average maximum d (Å)             | 0.09 ± 0.02      |
| Torsion violations (θ)            | θ < 5.0°          | 3 ± 2     |
| Average maximum θ (°)             | 3.8 ± 1.1        |
| AMBER energies, mean ± S.D. (kcal mol$^{-1}$) | 3.4 ± 0.6 |
| Total energy                      | -1051 ± 24       |
| Precision, root mean square deviations (Å) | All residues | 1.17 ± 0.10 |
|                                    | All heavy atoms  | 0.63 ± 0.10 |
|                                    | Backbone atoms   | 0.89 ± 0.10 |
|                                    | All-heavy-strands| 0.38 ± 0.08 |

Because CS domains have been identified in a variety of other proteins (9), it is logical to ask whether these proteins might also bind HSP90. One such example is the CS domain of SIP, another protein that is part of an E3 protein ubiquitination complex (6, 30). Sgt1 and SIP are homologous not only in their CS domains but also in their C-terminal domains, which bind S100 proteins (8, 29). Consequently, a homology model was constructed of the CS domain from SIP based on the structure of Sgt-CS domain. The electrostatic surface of the SIP-CS domain is shown in the Fig. 4D, lower panel. It is clear from the comparison to the corresponding surfaces of Sgt1-CS and p23 that the SIP CS domain has a similar charge distribution on surface I, which implies that SIP may also be an HSP90-interacting protein. In fact, preliminary NMR experiments suggest that SIP may indeed bind HSP90.

HSQC spectra of Sgt1-CS acquired at substoichiometric ratios of HSP90 reveal that the binding of the 180-kDa HSP90 dimer causes peaks in the HSQC spectrum to diminish in intensity and ultimately disappear. Remarkably, most of the first 20 peaks to disappear correspond to residues on surface I. This observation is very intriguing because this is precisely the region that has very similar electrostatic character in Sgt1-CS, SIP-CS, and p23. The large and intense basic patches on surface I (Fig. 4C) suggest that the interaction between the CS

$^a$ Y.-T. Lee and W. J. Chazin, unpublished observations.
domain and HSP90 may involve a significant electrostatic component. The importance of surface I for HSP90 binding is further supported by recent genetic mapping experiments, which showed that p23 mutations lowering estrogen receptor signaling were localized to the same surface, whereas mutations showing no effect map to surface II (31).

**DISCUSSION**

Although TPR domains are known as heat shock protein-interacting modules (32), the NMR assay showed that Sgt1-TPR does not bind HSP90. Because some TPR domains bind HSP70 (33, 34), we also tested for interaction of Sgt1-TPR with an HSP70. The C-terminal HSC70-(540–650) was used for these experiments because this domain is already known to interact with the TPR domain of CHIP. However, no interaction between HSC70 and Sgt1-TPR was observed. In summary, despite very high sequence homology with TPR domains known to interact with HSP90 or HSP70, the Sgt1-TPR appears not to be involved in interactions with either HSP90 or HSP70.

**TPR domains form a series of antiparallel \( /H_9251\)-helices whose arrangement creates a groove for substrate binding (35). The conserved C-terminal EEVD motif in HSP90 and HSP70 is found to be essential for the interaction with this groove in TPR domains. Scheufler et al. (35) reported that this interaction is mediated by an electrostatically driven dicarboxylate clamp that anchors the EEVD motif to TPR domains. Conserved residues in TPR domains are involved in interactions with the EEVD motif (Fig. 5). However, the Sgt1-TPR is distinguished in this alignment by low homology for these residues. A lack of conservation of these critical residues necessary for forming the dicarboxylate clamp suggests why there is no interaction be-
between Sgt1-TPR and heat shock proteins. In fact, TPR domains are found in a functionally diverse set of proteins including many whose function does not appear to involve heat shock proteins (33).

The β-sandwich fold common to p23 and Sgt1-CS is also found in the small HSPs MjHSP16.5 from *Methanococcus jannaschii* and HSP16.9B from wheat (38). However, in these proteins, the framework is extended by additional structural features including an N-terminal helical motif, an extra loop in the center, and additional residues at the C terminus (37, 38). Despite the conservation of fold among these proteins, the differences in the elaboration of the structural framework result in different functional modes of action and biological activities. For example, although monomeric p23 and CS domain-containing proteins bind HSP90, there are no reports of binding to the multimeric small HSPs. Sgt1-CS and p23 are themselves distinguished structurally by the absence of the C-terminal β-strand in Sgt1-CS (Fig. 3B) and biochemically by Sgt1 binding to HSP90 in the absence of ATP (Fig. 1), whereas ATP binding to HSP90 is strictly required for interaction with p23.

Given the structural and biochemical differences between Sgt1-CS and p23 and the fact that CS domains are seen only in multidomain proteins, we postulate that CS domains function as a general binding module for recruiting heat shock protein activities to multiprotein assemblies, as opposed to providing specific co-chaperone activity as does p23 (13, 39). Although ATP binding to HSP90 is not required for interaction with the Sgt1-CS domain, ATP binding and turnover is required for proper HSP90 function when it is associated with Sgt1, both for disease resistance activity in plants (5) and proper assembly of the core kinetochore (10). Thus, the binding of Sgt1 and the ATP-dependent activities of HSP90 in these processes are functionally coordinated but physically decoupled.

Sgt1 associates with HSP90 (3–5), and because it is necessary for kinetochore function and disease resistance in plants, it is probable that HSP90 also plays a role in these processes. Indeed, HSP90 has been shown to be required for active assembly of the CBF3 kinetochore complex (10) and proper plant disease resistance (3–5, 40). However, the exact molecular mechanism of how HSP90 and Sgt1 work together has yet to be determined.

Sgt1 also appears to be involved in regulation of SCF ubiquitin ligase activity through the recruitment of HSP90. The function of HSP90 in this context would be to stabilize the assembly of proteins associated with the SCF complex. An alternative mode of action for Sgt1 is suggested by recent studies of CHIP (33), a modular protein that also has a role in ubiquitin ligase activity. CHIP contains TPR and U-box domains and is known to bind heat shock proteins. The CHIP TPR domain associates with HSP90 and HSC70, and the CHIP U-box domain mediates interactions with other proteins in ubiquitin ligase assemblies. Thus, in analogy to CHIP, Sgt1 might also function as a molecular bridge using the CS domain to recruit substrates carried by HSP90 and the N-terminal TPR domain to mediate interactions with the other members of the ubiquitin ligase complex. Clearly, additional investigation is urgently needed to distinguish among the various modes of action of HSP90 and Sgt1 and fully elucidate their correlated functions.

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