Siah1 is the central component of a multiprotein E3 ubiquitin ligase complex that targets β-catenin for destruction in response to p53 activation. The E3 complex comprises, in addition to Siah1, Siah-interacting protein (SIP), the adaptor protein Skp1, and the F-box protein Ebi. Here we show that SIP engages Siah1 by means of two elements, both of which are required for mediating β-catenin destruction in cells. An N-terminal dimerization domain of SIP sits across the saddle-shaped upper surface of Siah1, with two extended legs packing against the sides of Siah1 by means of a consensus PXAXVP motif that is common to a family of Siah-binding proteins. The C-terminal domain of SIP, which binds to Skp1, protrudes from the lower surface of Siah1, and we propose that this surface provides the scaffold for bringing substrate and the E2 enzyme into apposition in the functional complex.

Polyubiquitination of specific proteins in cells involves the concerted action of E1, E2, and E3 enzymes. First, E1 covalently binds and activates ubiquitin for subsequent transfer to one of several E2s. The latter recognizes substrates and directs their interaction with E3s, resulting in highly specific regulation of target protein polyubiquitination (1, 2).

Humans carry two highly related genes, siah1 and siah2 (3), that encode the mammalian homologs of the Drosophila Sina protein, which is required for R7 photoreceptor cell differentiation within the sevenless pathway (4, 5). Sina/Siah proteins are E3 ligases, acting either as single proteins or as part of a multiprotein complex that is analogous to the Skp1-cullin-1-F-box (SCF) complex. Among the targets of Sina/Siah are NcoR (6), DCC (7), c-Myb (8), BOB-1/OBF-1 (9, 10), Peg3/Pw1 (11), Kid (12), Numb (13), synaptoophysin (14), group 1 metabotropic glutamate receptors (15), promyelocytic leukemia protein (16), CtIP (17, 34), α-synuclein (18), synphilin-1 (18, 19), PEG10 (20), T-STAR (21), AF4 (22, 23), prolyl-hydroxylase domain proteins (24), and α-ketoglutarate dehydrogenase (25). In addition, Siah interacts with adenomatous polyposis coli, a tumor suppressor involved in colon cancers (26); VAV, a nucleotide exchange factor involved in control of Rho/Rac proteins (27); BAG-1, a Hsp70/Hsc70-binding protein that modulates pathways involved in the control of cell proliferation, death, and migration (28, 29); and Dab-1, an inhibitor of Siah1 (30). However, Sina/Siah does not appear to target phyllopod, adenomatous polyposis coli, VAV, BAG-1, or Dab-1 for polyubiquitination and degradation. Thus, not all Siah-binding proteins are targets of Siah-mediated degradation.

Recently, we discovered a novel pathway for β-catenin degradation involving a complex formed by Siah1, SIP, the adaptor protein Skp1 that is common to the SCF complex, and the F-box protein Ebi that binds β-catenin independent of phosphorylation (31). Siah1 expression is up-regulated by p53, revealing a link between genotoxic injury and destruction of β-catenin, reduced Tcf/LEF activity, and cell cycle arrest (31).

Siah1 is a dimeric protein that contains an N-terminal RING domain (an E2 binding domain) followed by two zinc finger motifs and a C-terminal dimerization domain. The crystal structure of a major fragment of Siah1a lacking the RING domain (Siah1ΔR) has been determined (32); in this structure, the first zinc finger is highly mobile, whereas the second packs tightly against the C-terminal domain, forming a dimeric substrate-binding domain. In the multiprotein E3 complex, Siah1 plays an analogous role to the cullin-1 and Rbx1 domains in the classic SCF complex, and Siah-interacting protein (SIP) provides a novel link between Siah1 and Skp1 (31). We previously showed that SIP binds to Siah1 via determinants in its N-terminal domains (residues 1–72), whereas its C terminus (residues 73–228) binds to Skp1 (31). The murine ortholog of SIP (which is 93% identical) was initially characterized as a calcyclin-binding protein (33, 34). Calcyclin binding does not appear to compete with Skp1 binding, and its functional role has not been determined.

A consensus motif for Siah-binding proteins, comprising the sequence PXAXVP, has been identified (35). This motif is present in 15 of 27 known, functionally diverse, Siah-binding proteins, some of which are targets of degradation. The motif is also present in SIP. Here we present a structural and functional characterization of the interaction between human Siah1 and SIP. Our work demonstrates how SIP interacts with Siah1 in the context of the multiprotein complex. It also provides the first structure of Siah1 bound to the consensus PXAXVP motif, providing a basis for probing a broad range of Siah functions.

**EXPERIMENTAL PROCEDURES**

Protein Expression and Purification—Human SIP-S-(1–80), SIP-(1–47), and Siah1ΔR-(81–282) were cloned into the NdeI/BamHI site of...
pET15b (Novagen) and expressed in Escherichia coli BL21(DE3) as N-terminal His tag fusion proteins. Cell cultures were grown at 37 °C and induced with isopropyl θ-thio-β-D-galactopyranoside. After recovery from the soluble fraction of the cell extract, SIP-S was purified by Zn²⁺ or Ni²⁺ chelating affinity chromatography (Amersham Biosciences), followed by removal of the histidine tag with thrombin (Sigma) and molecular sizing (Superdex 200 10/30; Amersham Biosciences). Typical yields were 25–30 mg/liter. SIP-(1–47) was purified using a similar protocol with yields of 40–50 mg/liter. For NMR studies, isotopically enriched SIP-S and SIP-(1–47) were expressed in the appropriate minimal media and purified according to the same protocol. Siah1ΔR was purified similarly, except that trypsin was used to remove the histidine tag. This digestion also removed nine native residues at the N terminus of Siah1, resulting in a shorter fragment comprising residues 90–282. The final yield was 2–3 mg/liter. All proteins were dialyzed against 150 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol (10 mM dithiothreitol for Siah1), concentrated to 100–200 mg/ml (SIP) or 15–25 mg/ml (Siah1), and incubated with 0.02% dithiothreitol (10 mM dithiothreitol for Siah1), concentrated to 100–200 mg/ml in 1 mM Tris-HCl, pH 8.0, 10 mM NaCl, 1 mM EDTA was equilibrated at room temperature with a reservoir consisting of 2 M urea and 15–25 mg/ml (Siah1). 

**Crystallization and Structure Solution of SIP-(1–47)** — SIP-(1–47) was crystallized by sitting drop vapor diffusion. The protein at 25–35 mg/ml in 1 mM Tris-HCl, pH 8.0, 10 mM NaCl, 1 mM EDTA was equilibrated at room temperature with a reservoir consisting of 2 mM (NH₄)₂SO₄, 0.1 M CAPS, pH 10.5, 0.2 M Li₂SO₄. Crystals grew to a typical size of 100 × 100 × 300 μm³ in 4–8 months in space group C222₁, with unit cell dimensions a = 58.8, b = 152.5, c = 37.0 Å and soaked in a cryoprotectant buffer (2 M (NH₄)₂SO₄, 50 mM CAPS, pH 10.5, 100 mM Li₂SO₄, 20% glycerol) prior to flash cooling in liquid nitrogen. A data set to 1.2 Å resolution (TABLE I) was collected at 100 K at the SSRF facility beamline 9.1 and processed with the HKL package (36). The structure was solved by multiwavelength anomalous dispersion using a crystal soaked for 30 s in cryoprotectant solution saturated with NaBr. A four-wavelength multiwavelength anomalous dispersion data set (TABLE I) was collected at beamline 9.2 to a resolution of 1.5 Å. Initial phases were obtained with SOLVE (37) and the density was improved with ACORN (Accelrys Inc.). Sequential resonances for residues 4–47 were unambiguously assigned and have been deposited in the BioMagnRes data bank. All other spectra were collected at 298 K on a Bruker Avance 600-MHz spectrometer equipped with a 5-mm triple resonance probe and z axis pulsed field gradients. ¹H,¹⁵N HSQC and transverse relaxation optimized spectroscopy (TROSY) spectra were recorded with 2048 (H²⁰)×128 or *256 (¹⁵N) data points and a total of 4 or 16 transients per t₁ increment. Proton and nitrogen spectral widths were set to 12 and 26 ppm, respectively. Spectra were recorded on a sample of 500 μM uniformly ¹⁵N/²H-labeled SIP-S in 100 mM NaCl, 20 mM phosphate buffer H₂O/D₂O (90:10) pH 6.7, 10 μM Zn(OAc)₂. ¹³C HMRC experiments were obtained in the same buffer on a sample of ¹³C/¹²C,³H-Thr, ¹³C/¹²C,¹H-Ile, ³H-labeled SIP-S (200 μM) in the presence of increasing amounts of unlabeled Siah1ΔR protein. ¹²C/¹³C HMRC spectra were recorded as 8192*150 data points with 8 transients per t₁ increment; proton and carbon spectral widths were set to 12 and 20 ppm, respectively. Two-dimensional NOESY spectra were acquired with mixing times of 150 and 200 ms (spectral width 12 ppm along both f₁ and f₂, 4096*320 data points in t₁ and t₂, respectively, and 80 scans per t₁ increment). Water suppression was achieved by the WATERGATE PFG technique. All spectra were processed with Bruker software (Xwinnmr 3.5) and analyzed with XEASY version 1.3.13 (45) and Mestrec (available on the World Wide Web at qobrue.usc.es/). For Kₐ evaluation, normalized intensities from ¹²C/¹³C HMRC spectra were plotted as a function of Siah1 concentration (10, 50, 100, 150, 200, 300, and 400 μM). Normalized intensities were evaluated according to the relationship \( I_{\text{free}} - I_{\text{obs}}/I_{\text{sat}} = I_{\text{free}} \), where \( I_{\text{free}} \) is the measured value of the intensity, \( I_{\text{obs}} \) is the value in the absence of Siah1, and \( I_{\text{sat}} \) is the value at saturation (400 μM Siah1). Intensities were evaluated as average values of the observed peaks with the errors set to the S.D., using XEASY (45). Data were fitted to the equation, \( Y = (X + B + A) - \gamma (X + A + B)^{-2} - 4XB)/(2B) \), where B represents the concentration of SIP-S (200 μM), and A is the Kₐ obtained from the fit.

**Docking** — AutoDock version 3.0.5 (46) was used for the docking simulation. We used the Lamarckian genetic algorithm for SIP-(1–47) positional searching. The protonation states of Siah1 and SIP were set to pH 7.0.
7.4. SIP-(1–47) domain was kept as a rigid body. The docking parameters were as follows: trials of 100 dockings, population size of 150, random starting position and conformation, translation step ranges of 1.5 Å, rotation step ranges of 35°, elitism of 1, mutation rate of 0.02, cross-over rate of 0.8, local search rate of 0.06, and 10 million energy evaluations. Final docked conformations were clustered using a tolerance of 1.5 Å root mean square deviation.

Plasmids, Transfections, and Cell Culture—Mutations in SIP were generated by two-step PCR-based mutagenesis using a full-length human SIP cDNA (31) as a template. Products were purified by QiaQuick gel extraction kit (Qiagen), digested with EcoRI and XhoI, and then directly subcloned into the EcoRI and XhoI sites of pcDNA3 plasmid (Invitrogen) with an N-terminal Myc epitope tag (MEQKLISEEDL), thus creating pcDNA3-Myc. Alternatively, the cDNAs were subcloned into yeast two-hybrid plasmids pGilda and pG4–5, which produce fusion proteins with a LexA DNA-binding domain or a B42 transcription activation domain, respectively, at the N terminus, under the control of a GAL1 promoter. HEK293T cells were maintained in high glucose Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 1 mM L-glutamine, and antibiotics. For transient transfections, cells (~5 × 10^6) in 6-well plates were transfected with plasmid DNAs using Lipofectamine Plus (Invitrogen).

Yeast Two-hybrid Assay—The yeast EGY191 strain was co-transformed with pGilda plasmids encoding Lex A DNA-binding domain fusion proteins, pG4–5 plasmids encoding B42 transactivation domain fusion protein, and β-galactosidase reporter plasmids (pRB1840) as previously described (28).

Isothermal Titration Calorimetry—SIP-S and Siah1ΔΔR were extensively dialyzed against a buffer containing 100 mM NaCl, 20 mM Tris-HCl, pH 7.5, 10 μM Zn(OAc)_2 with or without 10 mM MgCl_2 and concentrated to 1.20–2.90 mM and 100–115 μM, respectively. 7–15-μl aliquots of SIP-S were added to the initial 2 ml of Siah1 in the cell of a Microcal (Northampton, MA) VP-ITC. Values for K_d, ΔH, and ΔS were calculated using the Microcal Origin software provided by the manufacturer. For each titration point, the heat of reaction was plotted against the molar ratio, and the data were fitted as described (47). The SIP-S concentration and SIP-S/Siah1 molar ratio at the end point of the titration were at least 210 μM and 4.2, respectively. Each measurement was performed independently in duplicate and was reproducible within a 4% error margin (K_d).

RESULTS

SIP-S Comprises an N-terminal Dimer with a Pair of Widely Separated Flexible Tails—The gene for human SIP encodes two alternatively spliced proteins 228 and 80 amino acids long, called SIP and SIP-S (for short) (31). SIP-S is identical to SIP in its first 72 amino acids (Fig. 1A). Using NMR techniques, we probed the structure of SIP-S and obtained sequential assignments for residues 4–47. The chemical shifts and the NOE patterns are consistent with a folded domain with one helical hairpin for one subunit and the helical hairpin for one subunit of the SIP dimer with selected side chains indicated. The two subunits are in blue and red, with Ni and C termini and the helical hairpin for one subunit labeled. D, close-up view of the hydrophobic core of the SIP dimer with selected side chains indicated E, superposition of two-dimensional 13N,1H HSQC spectra of 15N-labeled SIP (500 μM in 20 mM phosphate buffer H2O/D2O (90:10), pH 6.7, and 0.05% NaN_3) in the absence (black) and presence (red) of 750 μM unlabeled Siah1ΔΔR. Peaks that move and/or disappear after the addition of Siah1 are indicated by sequence numbers or by arrows for residues in the SIP tail. The asterisks indicate nonnative residues at the N terminus from the protein construct. F, one-dimensional 1H traces from two-dimensional 13C-1H HMQC spectra recorded with 13C-1H-red, 13C-1H-AQ-ilie-13C-1H-labeled SIP-S in the absence (blue) or presence (red) of Siah1ΔΔR. Residues in the dimerization domain are labeled D, and those in the tail are labeled T. Resonance broadening is most evident for threonine residues (68, 69, and 72) in the tail region.

FIGURE 1. Structure of SIP-S. A, domain organization of human SIP and SIP-S. B, SIP-S sequence with secondary structure assignment. Residues included in the crystallographic study are in boldface. The PXAAQPXP Siah-binding motif is in blue. C, ribbon representation of the SIP-(1–47) dimer. The two subunits are in blue and red, with Ni and C termini and the helical hairpin for one subunit labeled. D, close-up view of the hydrophobic core of the SIP dimer with selected side chains indicated E, superposition of two-dimensional 13N,1H HSQC spectra of 15N-labeled SIP (500 μM in 20 mM phosphate buffer H2O/D2O (90:10), pH 6.7, and 0.05% NaN_3) in the absence (black) and presence (red) of 750 μM unlabeled Siah1ΔΔR. Peaks that move and/or disappear after the addition of Siah1 are indicated by sequence numbers or by arrows for residues in the SIP tail. The asterisks indicate nonnative residues at the N terminus from the protein construct. F, one-dimensional 1H traces from two-dimensional 13C-1H HMQC spectra recorded with 13C-1H-red, 13C-1H-AQ-ilie-13C-1H-labeled SIP-S in the absence (blue) or presence (red) of Siah1ΔΔR. Residues in the dimerization domain are labeled D, and those in the tail are labeled T. Resonance broadening is most evident for threonine residues (68, 69, and 72) in the tail region.
lapping resonances at typical random coil values and the lack of sequential NOEs, suggesting that the tail is disordered.

Attempts to crystallize full-length SIP or SIP-S were unsuccessful. Limited proteolysis identified a protease-resistant core (residues 4–43), consistent with the NMR data, and we made a new construct encoding SIP residues 1–47 that crystallized and diffracted X-rays to high resolution. We solved the crystal structure of SIP-(1–47) at 1.2 Å resolution (Fig. 1, TABLE I). The protein is dimeric, as expected based on our previous biophysical and biochemical studies (31). There are two independent copies of the dimer within the crystal lattice with essentially identical structure. The dimer forms a curved cylinder, ~50 Å long with a maximum diameter of ~20 Å. Each monomeric subunit forms a helical hairpin in which the two helices, α1 (residues 2–20) and α2 (residues 24–47), are connected by a tight 3-residue turn. Hairpins from two monomers associate as a four-helix bundle (Fig. 1, C and D), with the two C termini at opposite ends of the cylinder, 50 Å apart. The bundle is stabilized by a hydrophobic core across the 2-fold axis that includes hydrophobic residues (Val13, Leu16, Val25, and Leu29) from each chain; further stability arises from a number of surface-exposed salt bridges, burying a total of 1700 Å2. The DCOMPLEX server (available on the World Wide Web at phyyz4.med.buffalo.edu/czhang/complex.html) (48) attempts to discriminate between biological dimers and crystal packing artifacts. It predicts that SIP-S forms a true biological dimer and estimates a $K_d$ of 15.2 kcal/mol (compared, for example, with 18.6 kcal/mol for the Siah1 dimer). The dimerization motif has a novel topology according to SCOP (49) and most closely resembles the dimerization of Siah-SIP Complex Structure.

### TABLE ONE

Crystallographic data collection and refinement statistics

|                  | SIP-(1–47) | Br $\lambda_1$ | Br $\lambda_2$ | Br $\lambda_3$ | Br $\lambda_4$ | Siah1/SIP-(58–70) (native) |
|------------------|------------|----------------|----------------|----------------|----------------|----------------------------|
| Space group      | C222       |                |                |                |                | C222                       |
| Unit cell (Å)    | $a = 58.77$ | $b = 152.52$  | $c = 36.97$    |                |                | $a = 47.7$                 |
| Resolution (Å)   | 50–1.2     | 50–1.5         | 50–1.5         | 50–1.5         | 50–1.5         | 30–2.2                     |
| Wavelength (Å)   | 0.82700    | 0.91833        | 0.83208        | 0.92014        | 0.95369        | Cu $K_{\text{nl}}$         |
| $R_{\text{merge}}$ (%) | 4.6        | 4.4            | 4.5            | 3.1            | 2.8            | 4.3                        |
| Overall          |            |                |                |                |                |                            |
| Last shell (limits) | 38.1 (1.22–1.20) | 35.8 (1.52–1.50) | 39.1 (1.52–1.50) | 21.8 (1.52–1.50) | 19.0 (1.52–1.50) | 36.5 (2.28–2.20) |
| $I/\sigma$       |            |                |                |                |                |                            |
| Overall          | 65.0       | 39.5           | 37.3           | 43.7           | 44.9           | 27.0                       |
| Last shell (limits) | 2.0 (1.22–1.20) | 5.8 (1.52–1.50) | 5.0 (1.52–1.50) | 9.6 (1.52–1.50) | 10.9 (1.52–1.50) | 4.0 (2.28–2.20) |
| $\sigma$ cut-off | None       | None           | None           | None           | None           | None                       |
| Unique reflections | 50,565   | 27,011         | 27,059         | 26,997         | 26,975         | 10,567                     |
| Redundancy       | 8.3        | 7.2            | 7.2            | 7.2            | 7.2            | 3.9                        |
| Completeness (%) | 96.7       | 99.2           | 99.3           | 99.4           | 99.4           | 99.4                       |
| Mosaicity        | 0.6        | 0.5            | 0.5            | 0.5            | 0.5            | 0.6                        |
| $R_{\text{work}}$ |            |                |                |                |                |                            |
| Overall          | 17.4       |                |                |                |                | 21.3                       |
| Last shell (limits) | 19.2 (1.20–1.23) |            |                |                |                | 29.6 (2.32–2.20) |
| $R_{\text{free}}$ |            |                |                |                |                |                            |
| Overall          | 19.8       |                |                |                |                | 25.2                       |
| Last shell (limits) | 24.5 (1.23–1.20) |            |                |                |                | 34.4 (2.32–2.20) |
| $\sigma$ cut-off | None       |                |                |                |                | None                       |
| Nonhydrogen atoms |            |                |                |                |                |                            |
| Protein          | 1244       |                |                |                |                | 1220                       |
| Solvent          | 230        |                |                |                |                | 100                        |
| Ion              | 5          |                |                |                |                | 1                          |
| Average B factors (Å²) |            |                |                |                |                |                            |
| Main chain       | 14.4       |                |                |                |                | 24.9                       |
| Side chains      | 16.9       |                |                |                |                | 27.4                       |
| Solvent          | 31.4       |                |                |                |                | 34.9                       |
| Wilson           | 16.8       |                |                |                |                | 39.7                       |
| rmsd bond lengths (Å) | 0.17      |                |                |                |                | 0.15                       |
| rmsd bond angles (degrees) | 1.94    |                |                |                |                | 1.55                       |
| Ramachandran plot (residues) |            |                |                |                |                |                            |
| Most favored     | 130        |                |                |                |                | 120                        |
| Additionally allowed | 4        |                |                |                |                | 12                         |
| Generously allowed | 0         |                |                |                |                | 0                          |
| Disallowed       | 0          |                |                |                |                | 0                          |

*a* Residual isotropic.  
*b* Residual after TLS.  
$c$ rmsd, root mean square deviation.
tion-anchoring domain of cAMP-dependent type II protein kinase regulatory subunit, but with a left-handed twist and a more open configuration. Recently, the NMR structure of SIP-S was reported (50), and the authors proposed that SIP-S is monomeric based on the absence of putative intermolecular NOEs in a sample comprised of labeled and unlabeled SIP-S at 1:1 ratio. However, it is not clear if mixing equal amounts of labeled and unlabeled samples is sufficient to obtain a differentially labeled dimer, especially if the dissociation constant is in the nanomolar range. Also, in comparing the NMR and X-ray structures, there are significant differences in the relative disposition of the two helices that might be explained if some of the intermolecular NOEs have been interpreted as intramolecular. Clearly, further biophysical data are required to settle this issue.

**SIP-S Engages Siah1 via the PXAXVP Consensus Motif within Its Flexible Tails**—We previously showed that SIP binds to Siah1 via its N-terminal 72 residues (31). We carried out a series of binding studies of SIP-S to Siah1ΔR. Siah1ΔR binds SIP-S with a K_d of 10 ± 5 µM as determined by isothermal titration calorimetry and NMR. Using NMR, we mapped the interactions between 15N-SIP-S and Siah1ΔR (15 µM), showing the α-H correlations. Intraconnectivities and sequential connectivities are indicated by the arrows. D, the Siah1ΔR dimer (blue and gray) bound to the SIP 59PAAVVAPI65 motif (red and green) in two orthogonal views. Dotted lines represent loops assumed to be disordered in the crystal structure. Zn²⁺ ions are shown as cyan spheres. β-Strands interacting with the peptide are marked. E, close-up of the interactions between SIP (yellow carbon atoms) and Siah1 (white carbon atoms) showing main-chain intermolecular hydrogen bonds with selected residues labeled.
Although we were unable to co-crystallize Siah1ΔR with either SIP or SIP-S, we did succeed in crystallizing Siah1ΔR in complex with a synthetic peptide comprising SIP residues 58EKPAAVVAPITTG70. The synthetic peptide comprising SIP residues 58EKPAAVVAPITTG70. The peptide binds to Siah1ΔR with a Kd of 24 ± 4 μM as determined by isothermal titration calorimetry. We solved the structure at 2.2 Å resolution by molecular replacement (TABLE I, Fig. 2). The crystal symmetry and packing are different for the peptide-bound versus unliganded Siah1ΔR; nevertheless, the overall structure and organization are very similar, and as observed in the unliganded structure, the first zinc finger is poorly ordered. Good electron density is observed for the 60PAAVVAP66 motif, and some density is observed for one flanking residue at each end of the motif (Fig. 2B). NMR-transferred NOE measurements using a similar peptide confirmed that the peptide binds to Siah1ΔR in solution and adopts an extended conformation as indicated by strong sequential HN-Hα NOEs that span from residue Glu56 to Ala66 (Fig. 2C). One SIP peptide binds at each edge of the saddle-shaped upper surface of the Siah1 dimer, which is formed by an eight-stranded antiparallel β-sheet, with the N termini of the two peptides separated by 35 Å (Fig. 2D). Each 60PAAVVAP66 motif forms a β-strand that augments the β-sheet, making parallel β-strand main-chain interactions from Pro50 to Val64 with strand β1 of Siah1; in addition, Ile67, outside the central motif, makes a main-chain hydrogen bond to Asp177 in strand β3, part of the lower sheet of the Siah β-sandwich (Fig. 2E). The side chains of the consensus residues Ala66 and Val64 pack against the hydrophobic core exposed at the edge of the β-sandwich. The main chain of the tripeptide 60PAA62 packs around an exposed hydrophilic group, Leu158, from strand β3 of Siah; this strand is the connector between the β-sandwich and the second zinc finger motif. When compared with the unliganded structure, peptide binding induces small (1–2 Å) changes in the local structure of Siah1ΔR in this region. The conserved Pro66 stacks against the side chain of Trp178 (strand β3) in Siah1. Residues Ala61 and Val63 point out into solution, consistent with their lack of conservation among Siah-binding proteins.

High affinity peptides derived from phyllopod and plectin have been shown to bind very strongly to Siah1 (Kd ~ 100 nM) and to compete effectively with a range of Siah-binding proteins, including SIP (35). The phyllopod and plectin sequences contain arginines flanking the central motif that have been shown to be important for binding. We note that two acidic patches on Siah1 (Glu161/Asp162 and Asp177/Glu194) are appropriately placed to make salt bridges with the arginines; these additional interactions may explain their stronger binding.

**The PXAXXP Motif Is Required for β-Catenin Regulation**—Consistent with our structural results, it has previously been shown that mutation of the invariant residues within the 60PXAXXP66 motif, Val64 and Pro66, reduced the binding of SIP to Siah1 in an in vitro pull-down assay (35). We confirmed the importance of these residues by probing the binding of SIP and Siah1 in co-immunoprecipitation assays in HEK293T cells. As shown in Fig. 3A, Siah1 co-immunoprecipitated with wild-type but not with mutant SIP. Similar results were obtained using yeast two-hybrid assays (Fig. 3B). Overexpression of Siah1 induces
ubiquitin-dependent degradation and reduction of β-catenin levels in cells, which can be inhibited by co-expression of loss-of-function mutants of SIP (31). We took advantage of this knowledge by monitoring β-catenin levels in cells transfected with Siah1 and wild-type or mutant SIP. HEK293 cells were transiently transfected with plasmids encoding Siah1, alone or in combination with wild-type or mutant full-length SIP. As shown in Fig. 3C, overexpression of full-length Siah1 caused a marked reduction in cellular levels of β-catenin. Co-transfection of wild-type SIP with Siah1 had no substantial effect on β-catenin levels, since endogenous SIP is abundant in HEK293 cells. In contrast, a mutant SIP with Asn substitutions in the Siah-binding site acted as a dominant negative mutant abrogating the effect of Siah1, perhaps because it retains Skp1-binding activity. Since β-catenin is a co-factor for the transcriptional activator Tcf/LEF (53), the effects of wild-type and mutant SIP on Tcf/LEF activity were probed using transient transfection reporter gene assays (54, 55). Whereas transfection with β-catenin induced a >10-fold increase in Tcf/LEF activity in HEK293T, and co-transfection of an equivalent amount of a Siah1-encoding plasmid reduced such enhancement by about half, co-transfection of wild-type SIP with Siah1 did not lead to a significant change in Tcf/LEF activity (Fig. 3D). In contrast, co-transfection of mutant SIP failed to suppress β-catenin-mediated activation of Tcf/LEF and rather increased transactivation of the Tcf/LEF-responsive reporter gene plasmid. Taken together, these results show that Val64 and Pro66 are required for interaction of SIP with Siah1 in vitro and for the function of the full-length proteins in cells.

The SIP Dimerization Domain Sits across the Siah1 Saddle—The location of the 60PAAVVP66 motifs, with their N termini protruding at the two edges of the central β-sheet of Siah1, places strong constraints on the location of the SIP N-terminal dimerization domain. Consideration of shape complementarity suggested to us that the SIP dimerization domain might use its concave face to sit across the saddle formed by the β-sheet on the upper surface of Siah1. Indeed, our NMR data show that the SIP residues most affected by binding to Siah1 map to its lower concave surface (Fig. 3A), and we previously showed by mutagenesis that acidic residues on the surface of the Siah1 saddle are important for SIP-Siah1 association (56). Computational molecular docking further supports this location: the coordinates of the SIP dimer were subjected to a conformational search over the entire surface of the Siah1 saddle. In the top solution, the concave surface of SIP-(1–47) docked onto the Siah1 saddle with the 2-fold axes of SIP and Siah1 approximately aligned (no symmetry restraints were applied).

To explore this hypothesis, we mutated lysines and arginines protruding from the surface of the concave dimerization domain of full-length SIP. Most single site mutations had a limited effect (not shown), although a K35A mutation reduced Siah1 binding significantly. However, a triple mutant, K23A/R24A/R26A, completely abrogated binding to Siah1ΔR in co-immunoprecipitation experiments (Fig. 4B). Similar results were obtained using yeast two-hybrid assays (Fig. 4C). The triple mutant is folded correctly, as judged by its ability to homodimerize and to heterodimerize with wild-type SIP (Fig. 4C). Therefore, our data support a function for the SIP N-terminal domain in stabilizing the interaction with Siah1 in addition to its role as a dimerization module.

The SIP-Saddle Interaction Is Also Required for Cellular Function—To assess the importance of the SIP-saddle interaction for β-catenin regulation in cells, we examined the effect of the mutant SIP, K23A/R24A/R26A, on β-catenin levels in transient transfection assays in HEK293T cells. Overexpression of Siah1 alone markedly reduced levels

FIGURE 4. The dimerization domain of SIP is directly involved in Siah1 binding and is essential for β-catenin regulation. A, structure of SIP-5 with residues affected by binding to Siah1ΔR shaded in red as determined by NMR (see Fig. 1E). The proposed binding site for the Siah1 saddle is indicated. The C-terminal tails are shown schematically in white (disordered) and blue (ordered in the presence of Siah1ΔR). B, full-length wild-type (wt) SIP or mutant mt1 (K23A/R24A/R26A) or mt2 (K35A) was assayed for binding to Siah1 by co-immunoprecipitation. Details for A–D, respectively. C, yeast two-hybrid analysis. D, β-catenin degradation. E, in vivo Tcf/LEF activity.
of β-catenin (Fig. 4D). Co-transfection of wild-type SIP with Siah1 did not substantially alter Siah1-mediated degradation of β-catenin, since endogenous SIP is abundant in HEK293 cells. In contrast, mutant SIP abrogated the effect of Siah1 overexpression, acting in a dominant negative fashion. Similarly, in a transient transfection reporter gene assay, co-expression of wild-type SIP with Siah1 had no effect on the suppression of β-catenin-induced Tcf/LEF activity. These results thus suggest that a second SIP-Siah1 interface is required for the assembly and function of the Siah1-SIP-Skp1-Ebi complex.

A Different Surface of Siah1 May Direct the Assembly of the E3 Ligase Complex—The C termini of the SIP-S dimer protrude on the lower surface of Siah1 (i.e. opposite from the saddle that binds the SIP dimerization domain) adjacent to the second zinc finger domain and several conserved loops (Fig. 5A). We previously showed that the C-terminal domain of full-length SIP, residues 73–228, contains the binding determinants for Skp1 (31). To test the hypothesis that this lower surface is involved in assembly of the E3 multiprotein complex, we made mutations in two of the surface loops. The mutants retained the ability to bind SIP, as revealed by yeast two-hybrid assays and co-immunoprecipitation (Fig. 5, B and C), indicating that they do not adversely affect folding. However, the mutants failed to mediate degradation of β-catenin or to suppress Tcf/LEF-dependent transcription (Fig. 5, D and E), suggesting that they play a functional role in directing the ubiquitination reaction.

DISCUSSION

By analogy to the well studied SCF multiprotein E3 ligase complex, the Siah1-SIP-Skp1-Ebi complex is believed to function by assembling a scaffold that orchestrates the ubiquitination reaction by bringing the E2 enzyme and its substrate into apposition with the appropriate geometry. Our results allow us to develop a model of the structure and function of the multiprotein Siah1 complex.

We identified two binding surfaces for SIP on Siah1, both of which are necessary for the Siah1-SIP complex to form, and showed that disruption of either of these two interactions abrogates functional activity in a cellular context, indicating that the full interaction is required for the assembly of the Siah-SIP-Skp1-Ebi E3 ligase complex. The helical N-terminal domain of SIP/SIP-S forms a dimer, and our data are consistent with a model in which the SIP-S dimer sits atop the saddle formed by the large β-sheet on the upper surface of Siah1, making chiefly ionic interactions, while two “legs” containing the 60PAAVVAP66 sequence pack against both sides of the Siah1 dimer, making specific β-sheet interactions (Fig. 6A). The binding mode, in which a β-strand from the ligand augments a β-sheet, is reminiscent of the interactions between PTB and PDZ domains and their ligands, as well as those of the structurally related tumor necrosis factor receptor-associated factor-associated factor with ligands (32, 57). The distance (~18 Å) between the C termini of the SIP dimer (residues 1–47) and the N termini of the SIP peptides (residues 59–67) can be readily bridged by the 11-residue linker (residues 48–58).

Our structural data provide a rationale for the role of the PXAXVXP motif in Siah1-SIP interactions. More than half of SIP-binding proteins identified thus far contain this motif (Fig. 6B). High affinity peptides
from phyllopod and plectin containing the motif have been shown to compete effectively with a range of Siah-binding proteins, including SIP (35). It thus seems very likely that many Siah-binding proteins will bind in the same mode that we have observed for SIP. The importance of this is underscored by studies of the AF4 gene, which is disrupted in childhood leukemia (58) and mutated in the "robotic" mouse (59). The human AF4 gene binds Siah1 (23), and genetic mapping identified a point mutation (V280A) in AF4 within the consensus PXAXVXP motif (22, 23) that correlated with accumulation of the AF4-proteins and increased transcriptional activity in cells, suggesting that the AF4 activity is controlled by Siah1-mediated degradation.

We previously showed that the C-terminal domain of full-length SIP, residues 73–228, contains the binding determinants for Skp1 (31). In our model of SIP-Siah1 binding, the C terminus of SIP-S emerge on the "lower" surface of the Siah1 dimer, on the opposite side from the binding site for the SIP dimerization domain. This suggests that the C-terminal domain of full-length SIP (residues 68–228) and its ligand Skp1 lie adjacent to this lower surface. This surface includes three prominent loops that are highly conserved but mobile in the Siah1 crystal structures (except when stabilized by fortuitous crystal contacts), as well as an $\alpha$-helix ($\alpha2$) that packs against the $\beta$-sheet. We showed that mutation of one of the mobile loops, as well as of a conserved methionine residue exposed on the surface of helix $\alpha2$, abrogated $\beta$-catenin degradation without affecting SIP binding. Thus, our studies support a role for the lower surface of Siah1 in organizing the E3 ligase assembly into a functional complex. Indeed, a conservative point mutation (I208L) in a residue exposed on the surface of helix $\alpha2$ against the $\beta$-sheet (60).

Finally, we note that the inherent binding affinity for SIP to Siah1 (~11 $\mu M$) is relatively low, consistent with the ability of peptides derived from phyllopod to readily disrupt this interaction. Therefore, we propose that the selection between construction of a single protein E3 ligase versus an SCF-like multiprotein complex could be made at the level of competition between SIP and substrate proteins binding at the PXAVXP site on Siah1 and modulated by the availability of other components of the multiprotein complex that would enhance binding.

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