Structural Analysis of Siah1 and Its Interactions with Siah-interacting Protein (SIP)*

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Seven in absentia homologue (Siah) family proteins bind ubiquitin-conjugating enzymes and target proteins for proteasome-mediated degradation. Recently we identified a novel Siah-interacting protein (SIP) that is a Sg1-related molecule that provides a physical link between Siah family proteins and the Skp1-Cullin-F-box ubiquitin ligase component Skp1. In the present study, a structure-based approach was used to identify interacting residues in Siah that are required for association with SIP. In Siah1 a large concave surface is formed across the dimer interface. Analysis of the electrostatic surface potential of the Siah1 dimer reveals that the β-sheet concavity is predominately electronegative, suggesting that the protein-protein interactions between Siah1 and SIP are mediated by ionic contacts. The structural prediction was confirmed by site-directed mutagenesis of these electronegative residues, resulting in loss of binding of Siah1 to SIP in vitro and in cells. The results provide a structural basis for understanding the mechanism by which Siah family proteins interact with partner proteins such as SIP.

Siah1/Sina family proteins represent mammalian homologs of the Drosophila Sina (seven in absentia) protein. Sina is required for R7 photoreceptor cell differentiation within the sevenless pathway (1). The members of the family are E3 ubiquitin-protein isopeptide ligases that regulate ubiquitina-
tion and protein degradation. For example, Sina binds a ubiquitin-conjugating enzyme (E2). Heterocomplexes of Sina and another protein called Phyllodome form an E3 complex that interacts with a transcriptional repressor called Tramtrack, targeting it for polyubiquitination and proteasome-mediated degradation (2, 3). The destruction of Tramtrack is necessary for differentiation of R7 cells (2, 3).

In humans two genes exist that encode Sina-like proteins, SIAH1 and SIAH2 (4). Like their Drosophila counterpart, the Siah1 and Siah2 proteins contain a N-terminal RING domain that binds E2s followed by a cysteine-rich domain and then a novel domain implicated in binding various substrate proteins and targeting them for degradation. The reported targets of Siah-mediated degradation include DCC (5), Nco-R (6), c-Myb (7), BOB1/OBF1 (8, 9), Peg3/Pw1 (10), APC (11), Kid (12), Numb (13), synaptophysin (14), and group 1 metabotropic glutamate receptor (15). In addition, Siah reportedly interacts with Vav, a GDP-exchange factor for Rac/Rho (16), and BAG1, a Hsp70/Hsc70-binding protein that modulates cellular pathways involved in control of cell proliferation, cell death, and cell migration (17). Interestingly, however, Siah does not appear to target Vav or BAG1 for polyubiquitination and degradation despite its ability to bind these proteins. Thus, not all Siah-binding proteins are targets of Siah-mediated degradation. The physiological basis for the broad range of protein-protein interactions with Siah/Sina family members is as yet not clear.

Recently, we identified a novel Siah-interacting protein (SIP) by using yeast two-hybrid interaction cloning methods (18). SIP is a Sg1-related protein that provides a physical link between Sina/Siah family proteins and the SCF complex component Skp1. Similar to Siah/Sina family proteins, SCF complexes play a critical role in the ubiquitination and degradation of a variety of target proteins including cyclins, p27kip1, p21waf1, E2F, IκB, and β-catenin (19–21). We elucidated a network of protein interactions involving Siah and SIP that regulate levels of β-catenin and thus the activity of β-catenin-dependent Tcf/LEF transcription factors. In this network an evolutionarily conserved pattern exists where Siah binds to SIP, which interacts with Skp1, which in turn binds to the F-box protein Ebi (18). Here, to begin to dissect this network we have used site-directed mutagenesis to identify the contact surfaces on Siah for SIP.

The crystal structure of a fragment encompassing the Cys-rich domain and the substrate-binding domain (SBD) of murine Siah1a has been determined (22). The structure reveals that the SBD of Siah1a bears striking structural similarity to the tumor necrosis factor receptor-associated factor (TRAF) domains of TRAFs, a family of adapter proteins that bridges the cytosolic domains of multiple tumor necrosis factor family receptors to intracellular protein kinases (23). However, the oligomeric state of Siah1a SBD differs from TRAFs. Siah1a exists as a dimer, whereas TRAFs associate as trimers. In Siah1a a large concave surface is formed across the dimer interface. This region as well as two other clefts have been suggested as sites for protein-protein interaction (22, 24). The results of the present study identify SIP contact residues in the large concave surface of the Siah1 dimer, which represent critical sites for the first docking event in the Siah/SIP/Skp1/Ebi network.

MATERIALS AND METHODS

Plasmids—Mutations in Siah1 were generated by two-step PCR-based mutagenesis using a full-length human Siah1 cDNA (17) as a template. Products were purified by the Quick gel extraction kit (Qiagen), digested with EcoRI and XhoI, and then directly subcloned into the EcoRI and XhoI sites of pcDNA3 plasmid (Invitrogen) with a N-terminal Myc epitope-tag (MEQKLISEEDL), thus creating pcDNA3-myc. Alternatively, the cDNAs were subcloned into yeast two-
hybrid plasmids pGilda and pJG4–5, which produce fusion proteins with a LexA DNA-binding domain or a B42 transcriptional activation domain, respectively, at the N terminus under the control of a GAL1 promoter.

Transfections and Cell Culture—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 (5×10⁵) in 6-well plates were transfected with plasmid DNAs using LipofectAMINE Plus (Invitrogen).

Immunoprecipitations—HEK293T cells (2×10⁶) in 100-mm plates were used directly or transiently transfected with 6 μg (total) of plasmid DNA. After 24 h, cells were treated with 10 μM MG132 for 8 h and lysed in 1 ml of HKMEN solution containing 10 mM HEPES (pH 7.2), 142 mM KCl, 5 mM MgCl₂, 2 mM EGTA, 0.2% Nonidet P-40, 0.1 μg/ml aprotinin, 1 μg/ml pepstatin and 20 μM MG132. Immunoprecipitations were performed using agarose-conjugated anti-Myc antibody (9E10, Santa Cruz Biotechnology) followed by horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit immunoglobulin (Amersham Biosciences) and detection using enhanced chemiluminescence (Amersham Biosciences).

Transfection reporter gene assays—Tcf/LEF transcriptions were detected exogenous cytosolic Tcf/LEF transcriptional activity was measured at the dimer interface by an antiparallel arrangement of β-sheets from each monomer (Fig. 1). The other two are symmetrically equivalent clefts in each monomer that are located between the Cys-rich domain that contains two zinc fingers and SBD. The electrostatic surface potential of these regions is different (Fig. 1); that is the central large β-sheet concavity is predominately electronegative, whereas the two smaller clefts at the ends of the oligomer are electropositive in nature. Thus, the potential exists for binding at distant sites that differ significantly in overall charge.

Interaction of Siah1 with SIP—Because SIP is a basic protein, we tested the role of the negatively charged residues in the concave surface of the Siah1 dimer for binding of SIP, representing a cluster of glutamic acids and aspartic acids in the concave β-sheet structure. In contrast to wild-type Siah1 protein, mutant Siah1 molecules (mutant A) with alanine substituted for Glu-161, Asp-162, Glu-226, and Glu-237 in the concavity failed to bind SIP in co-immunoprecipitation experiments (Fig. 2A). Because Siah1 exists as a dimer, the large electronegative surface concavity is formed by an antiparallel orientation of identical β-sheets in each monomer. As shown in Fig. 1, this arrangement produces a symmetrical relationship between corresponding structural features of the monomeric subunits. Consequently, eight acidic residues reside in the large concavity; that is four from each monomer. Thus, substitution of alanine for four acidic residues resulted in a loss of eight carboxyl groups at the surface and a dramatic change in the electrostatic character of the concavity. Failure of the mutant with these substitutions to bind SIP identifies this concave region as the SIP-binding interface. Substitution of alanine for Asp-253 and Glu-265 on the surface of the opposite face of the dimer or Asp-142 and Gln-151 did not affect SIP binding.

Two other regions were proposed as sites for protein-protein interactions in Siah1a (22). These are electropositive clefts between the SBD and two zinc fingers on each monomer (see Fig. 1). These distinct sites are located at each end of the elongated dimer, separated by 46 Å. The mutation of Arg-214,
Arg-215, Arg-231, Arg-124, and Arg-232 in these clefts to alanine. The resulting mutant Siah1 molecule retained the capability to recognize, these two arginines were mutated to alanine. The mutations were as follows: A, E161A, D162A, E226A, E237A; B, N253A, Q265A; C, R224A, R233A; D, R214A, R215A, R231A, R232A; E, D142A, Q151A. Lysates were normalized for total protein content and subjected to immunoprecipitation using agarose-conjugated anti-Myc epitope monoclonal antibody (9E10). After recovering immune complexes and washing, the immunoprecipitates were analyzed by SDS-PAGE/immunoblotting using an anti-hemagglutinin monoclonal antibody with ECL-based detection. As a control, 0.05 volume of input cell lysate was loaded directly in the context, we took advantage of our previous findings showing that overexpression of Siah1 induces degradation of β-catenin (18). We performed transient transfection assays in HEK293T cells (Fig. 3A), monitoring β-catenin protein levels by immunoblotting. As shown in Fig. 3A, overexpression of wild-type Siah1 markedly reduced levels of β-catenin protein. In contrast, the Siah1 mutant with alanine substitutions for the carboxylate cluster (mutant A) did not induce degradation of β-catenin; rather, it enhanced levels of β-catenin. Consistent with the binding results, the other mutant molecules retained the ability to reduce levels of β-catenin.

Because β-catenin is required as a cofactor for activation of the transcription factor Tcf/LEF (26), we explored the effects of wild-type and mutant Siah1 on Tcf/LEF reporter activity. A, HEK293T cells were transiently transfected with 0.2 µg of plasmids encoding Myc-β-catenin with full-length wild-type Siah1 (0.5 µg)/H9262 (wt), various mutant Siah1 (0.5 µg), or fragment Siah1-191–282(ΔR) as indicated (total DNA amount normalized). After 24 h, cell lysates were prepared from duplicated dishes of transfectants, normalized for total protein content (20 µg per lane), and analyzed by SDS-PAGE/immunoblotting using antibodies specific for Myc-β-catenin (top) or Myc-Siah (bottom) with ECL-based detection. B, HEK293T cells were transiently transfected with a reporter gene plasmid (0.1 µg), which contains a Tcf/LEF responsive element cloned upstream of a luciferase reporter gene together with 0.01 µg of pCMV-β-gal as a transfection efficiency control, and 0.1 µg of the indicated plasmids encoding β-catenin, wild-type (WT), Siah1, or various mutants as indicated, normalizing the total amount by the addition of empty pCDNA3 as necessary. Luciferase activity was measured in cell lysates 24 h later and normalized relative to β-galactosidase (mean ± S.D., n = 3).
Separate and Distinct Protein-Protein Interaction Sites on Siah1—Siah1 also binds to the molecular co-chaperone BAG1, which has been reported to abrogate growth arrest by Siah1 (17). Therefore, we tested the effects of mutations in the acidic clusters on Siah1 interaction with BAG1.

In contrast to SIP, BAG1 interacted with all of the mutant Siah1 proteins in the tested samples (Fig. 2), suggesting that SIP and BAG1 bind to different surfaces of the Siah dimer. The Siah1-binding domain of BAG1 is an α-helical bundle with an overall electronegative charge (28). Interestingly, when basic residues at the two positively charged crevices on Siah1 (Fig. 1) were mutuated to alanine, binding to BAG1 was retained. Thus the BAG1 binding site that is distinct from the SIP interaction region has yet to be elucidated.

Recently, it was reported that the Drosophila protein Phyllopod interacts with the SBD region of SINA, a close homologue of Siah1 (29). Deletion of five residues from the C terminus of the SBD region of SINA completely abolished Phyllopod association, suggesting that Phyllopod might interact with the concave cleft in the SINA dimer because these residues are located in this large concavity (22). Moreover, the authors showed by deletion analysis that a 19-amino acid sequence in Phyllopod is necessary for binding to SINA (29). If this segment in Phyllopod assumes ordered secondary structure, it could easily be accommodated in a concave surface-like region in the Siah1 dimer that we have defined for SIP binding. Interestingly, there is no apparent sequence similarity between SIP and the SINA-binding domain of Phyllopod. The molecular basis for recognition of SIP and Phyllopod by the Siah1/SINA homologues awaits future structural analyses of complexes.

The human SIP protein shares 93% identity at the protein level with a mouse protein, previously identified as a calcyclin (S100A6)-binding protein (CacyBP) (30). Interestingly, the CacyBP binds to S100 family proteins through its C-terminal region in a Ca²⁺-dependent fashion (31). Moreover, CacyBP can be phosphorylated by protein kinase C in vitro (32). It is still unclear whether Ca²⁺ or phosphorylation of CacyBP/SIP affects its interactions with Siah1, Skp1, or other proteins. Furthermore, the physiological effects of S100 family proteins on Siah-induced degradation of β-catenin should be addressed.

Because the large SIP-binding crevice identified here is formed by a symmetrical arrangement of identical residues from the two Siah1 subunits, a question arises as to whether protein-protein interactions with SIP involved the entire concave surface or intermolecular contacts with just one monomer, that is, a half-site. We observed a similar antiparallel arrangement of β-sheets in the dimeric MS2 translational repressor (33, 34). In the repressor, residues that are required for binding the RNA operator are located on six adjacent β-strands contributed by residues in three strands from each monomer (35). As in Siah1, a large concave crevice is formed across the dimer interface. In the case of MS2, the dimeric repressor binds one RNA operator. One RNA hairpin binds across the face of the dimer making asymmetric contacts with residues from both monomers (36). For the Siah1 dimer it is possible that the contact surface accommodates a single SIP molecule, or alternatively, those two SIP molecules are bound to each symmetric half-site of the dimer. Further experiments are needed to determine the binding stoichiometries of Siah-SIP interactions.

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