Structure of Salmonella Effector Protein SopB N-terminal Domain in Complex with Host Rho GTPase Cdc42*

Received for publication, December 6, 2011, and in revised form, February 17, 2012. Published, JBC Papers in Press, February 23, 2012, DOI 10.1074/jbc.M111.331330

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Background: Salmonella effector protein SopB binds host Rho GTPase Cdc42.

Results: Structural characterization of SopB with Cdc42 reveals that SopB contains a CRIB-like motif and contacts Cdc42 switch regions.

Conclusion: SopB structurally mimics contacts between Cdc42 switch regions and human guanine dissociation inhibitor (GDI) and slows nucleotide exchange.

Significance: This is the first structure of SopB and provides further insight into Salmonella regulation of Cdc42.

SopB is a type III secreted Salmonella effector protein with phosphoinositide phosphatase activity and a distinct GTPase binding domain. The latter interacts with host Cdc42, an essential Rho GTPase that regulates critical events in eukaryotic cytoskeleton organization and membrane trafficking. Structural and biochemical analysis of the SopB GTPase binding domain in complex with Cdc42 shows for the first time that SopB structurally and functionally mimics a host guanine nucleotide dissociation inhibitor (GDI) by contacting key residues in the regulatory switch regions of Cdc42 and slowing Cdc42 nucleotide exchange.

Salmonella enterica is a Gram-negative bacterium that causes typhoid fever and gastroenteritis. Typhoid fever resulted in ~22 million illnesses and 200,000 deaths during 2000 (1), whereas Salmonella-induced gastroenteritis results in an estimated 93.8 million illnesses and 155,000 deaths each year (2). Such numbers emphasize the importance of understanding the molecular mechanisms of Salmonella pathogenesis. Salmonella gains entry into human host phagocytic and intestinal epithelial cells by secreting virulence effector proteins into the host cytoplasm via a type III secretion system (T3SS)3 (3). Inside the host cell, Salmonella delivers additional effector proteins via a second distinct T3SS and creates a replication niche termed the Salmonella-containing vacuole (SCV).

SopB (also known as SigD) is a T3SS secreted Salmonella effector protein that contains a GTPase binding domain (residues 117–168) and a phosphoinositide phosphatase domain (residues 357–561). Upon T3SS-mediated delivery of SopB into host cells, the phosphoinositide phosphatase domain promotes host membrane fission by hydrolysis of phosphatidylinositol 4,5-bisphosphate, a process that facilitates bacterial entry (4). Subsequently, SopB is multi-monoubiquitinated and translocated to the SCV (5, 6), where SopB phosphatase activity is proposed to help prevent lysosomal degradation of the bacteria by reducing the negative surface charge of the SCV (7).

The potential function of the SopB GTPase binding domain was suggested when phosphatase-deficient SopB R468A expressed in yeast interfered with actin dynamics (8), cell cycle progression, and MAP kinase signaling through direct interaction with yeast Cdc42 (9). Cdc42 is an essential Rho GTPase that regulates cytoskeleton organization and membrane trafficking during cell motility, proliferation, and cytokinesis in eukaryotes (10, 11). Active Cdc42 (GTP-bound) and inactive Cdc42 (GDP-bound) differ in conformation in the flexible regulatory regions, switch I and II (Fig. 1A) (12). Downstream host effector proteins such as p21-activated kinase (PAK) serine/threonine kinases (13) and the Wiscott-Aldrich syndrome protein (WASP) (14) recognize and bind the switch regions of active Cdc42, and this interaction subsequently alters the signaling activity of the host effector (Fig. 1B) (12). The interaction of the Salmonella SopB virulence effector with Cdc42 was delineated by stable isotope labeling of amino acids in cell culture (SILAC) mass spectrometric analysis in mammalian cells with the resulting Cdc42 binding region (residues 117–168) shown to down-regulate Cdc42-dependent signaling (9, 15). SopB binds specifically to Cdc42 and no other related GTPases (15). SopB binds both active and inactive Cdc42, and this interaction is important for translocation of SopB to the SCV (16). Furthermore, disrupting interaction between SopB and Cdc42

Salmonella-containing vacuole; WASP, Wiscott-Aldrich syndrome protein; SILAC, stable isotope labeling of amino acids in cell culture.
during a Salmonella invasion assay reduces the efficiency of Salmonella replication within the SCV (16).

Cdc42 is also targeted by Salmonella effector proteins SopE (and its parologue SopE2) and SptP. SopE/E2 mimic eukaryotic guanine exchange factors (GEFs) to catalyze the exchange of GDP for GTP and activate Cdc42 and Rac1, a closely related Rho GTPase, to drive actin cytoskeleton assembly (Fig. 1B) (17, 18). SptP inactivates Cdc42 and Rac1 by mimicking a GTPase-activating protein (GAP) and catalyzing hydrolysis of GTP to GDP by Cdc42 and Rac1 (Fig. 1B) (19). The structures of SopE in complex with Cdc42 (20) and SptP in complex with Rac1 (21) have been solved, but a lack of structural information for SopB has impeded analysis and understanding of its interaction with and modulation of Cdc42.

Here, we present the crystal structure of the N-terminal GTPase binding domain of SopB in complex with its host tar-
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get, Cdc42. This structure shows that SopB contacts Cdc42 by mimicking a eukaryotic CRIB (for Cdc42 and Rac-interactive binding)-like motif, which was previously unidentified due to a lack of sequence conservation. This is the first time a bacterial effector protein has been shown to contact a Rho GTPase through a CRIB-like motif. Furthermore, our structure shows that SopB mimics key interactions between Cdc42 and host guanine dissociation inhibitor (GDI), a regulatory protein that sequesters Cdc42 in the inactive state by preventing exchange of GDP for GTP. In *in vitro* nucleotide exchange assays confirm that the N-terminal domain of SopB slows intrinsic Cdc42 nucleotide exchange as well as Cdc42 nucleotide exchange catalyzed by the *Salmonella* GEF, SopE.

**EXPERIMENTAL PROCEDURES**

- **Isothermal Titration Calorimetry (ITC)**—ITC was done using an *ITC200* (MicroCal GE Healthcare). Cdc42 and SopB(29–181) were each expressed in *Escherichia coli* BL21 (DE3) cells and purified from cell lysate by injection over a 1-ml His-trap column (GE Healthcare) and a Superdex 75 26/60 column (GE Healthcare). Protein samples were dialyzed into 20 mM Hepes, pH 7.5, with 50 mM NaCl. The concentrations of SopB(29–181) and Cdc42 were determined by Bradford assay. SopB(29–181) and Cdc42 were determined by Bradford assay. SopB(29–181) was concentrated to 774 mg/ml and 100 μM Cdc42. Titration experiments were repeated in triplicate. The dissociation constant was determined by fitting the raw data to a bimolecular interaction model.

- **Static Light Scattering**—SopB(29–181)-Cdc42 complex was expressed and purified (described below) and concentrated to 2 mg/ml. The protein complex (100-μl volume) was injected over a Superdex 75 10/300 GL column (GE Healthcare) and analyzed by miniDAWN multangle static light scattering device (Wyatt Technologies) and ASTRA program.

- **Analysis of SopB and Cdc42 Interaction by Gel Filtration**—SopB(29–181), SopB I49A(29–181), and Cdc42 were expressed and purified (as described below). Protein was concentrated to 2 mg/ml and injected over a Superdex 75 10/30 column (GE Healthcare) for gel filtration analysis. To determine the effect of the SopB I49A point mutation on interaction with Cdc42, WT SopB(29–181) and SopB I49A(29–181) (2 mg/ml) were incubated with Cdc42 (2 mg/ml) on ice for 1 h and then injected over a Superdex 75 10/30 column for gel filtration analysis.

- **Protein Cloning, Purification, and Crystallization**—Cdc42 with a deletion of seven C-terminal amino acids (Cdc42(1–181)) was amplified from *Homo sapiens* DNA and cloned into pET28 vector (Novagen) with restriction sites NdeI and SacI. Residues 29–181 of SopB were amplified from *Salmonella typhimurium* DNA and cloned into pET21 vector (Novagen) with restriction sites NdeI and SacI. Expression constructs were transformed into *E. coli* BL21 (DE3) cells. Cells were grown in LB medium with 50 μg/ml ampicillin and 100 μg/ml kanamycin and 100 μg/ml ampicillin to an *A*<sub>x</sub> of 0.6, and expression of Cdc42 and SopB(29–181) was induced with 1 mM IPTG for 20 h at 30 °C. Cells were harvested by centrifugation, resuspended in buffer with 20 mM Hepes, pH 7.5, 300 mM NaCl, and 50 mM imidazole and lysed by French press. The cell suspension was centrifuged at 45,000 rpm for 1 h, and supernatant was filtered and injected onto a 1-ml His-trap column. The column was washed with buffer (20 mM Hepes, pH 7.5, 300 mM NaCl, 50 mM imidazole), and His-tagged Cdc42 and bound SopB(29–181) were eluted with elution buffer (20 mM Hepes, pH 7.5, 300 mM NaCl, 500 mM imidazole). Fractions containing the protein complex were concentrated and further purified by gel filtration with a Superdex 75 26/60 column in 20 mM Hepes, pH 7.5, 300 mM NaCl. The final yield of protein was 100 mg of purified SopB(29–181) and Cdc42 complex for 2 liters of bacterial culture. Protein was concentrated to 12 mg/ml for crystallization trials. The SopB(29–181) and Cdc42 complex was crystallized using the sitting drop vapor diffusion method by mixing 0.5 μl of 12 mg/ml protein solution with 0.5 μl of well solution, 0.2 mM sodium chloride, 0.1 mM phosphate-citrate, pH 4.2, 20% (w/v) PEG 8000 (JCSG suite, Qiagen). The crystals were diamond-shaped and appeared after 14 days at 4 °C.

- **Data Collection, Structure Determination, and Refinement**—Crystals were cryoprotected in a solution of mother liquor with 20% glycerol and flash frozen in liquid nitrogen. Crystals were screened at the CMCF-2 of the Canadian Light Source and diffracted to 2.35 Å resolution. The crystals were space group P4<sub>2</sub>2<sub>1</sub>2 and had cell dimensions of 106.7 × 106.7 × 87.5 Å. Data were reduced and scaled using MOSFLM (22) and SCALA (23). The phases of the SopB(29–181)-Cdc42 complex were solved by molecular replacement using Phaser_MR (24), with the search model Cdc42-GDP (PDB code 1AN0), COOT (25) was used to perform model building and the model was refined with REFMAC5 (26) from the CCP4 suite and Phenix (27). Ligand and ion atoms were refined at an occupancy of one. The structure was analyzed using Molprobity (28) with 96.7, 3.3, and 0% of residues in the favorable, allowed, and disallowed regions of the Ramachandran plot. The SopB-Cdc42 structure was deposited in the Protein Data Bank (4DID). Figures were generated by PyMOL (29).

- **Cdc42 Nucleotide Exchange Assay**—Cdc42 nucleotide exchange assay was adapted from Ref 30. Cdc42 was preloaded with a fluorescent GDP analog, N-methylnaphthalenoyl (Man)-GDP, by incubating 100 μM Cdc42 with 300 μM Man-GDP in buffer (20 mM Tris pH 8.0, 150 mM NaCl, 5 mM EDTA, 1 mM DTG) at 37 °C for 30 min. The reaction was transferred to ice and stopped by addition of 10 mM MgCl<sub>2</sub>. A desalting column (GE Healthcare) was used to remove free Man-GDP and exchange the Man-GDP-loaded Cdc42 into reaction buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 5 mM MgCl<sub>2</sub>). Man-GDP-loaded Cdc42 was diluted with reaction buffer at a final concentration of 2.5 μM. To initiate nucleotide exchange, unlabeled GTP was added to Man-GDP-loaded Cdc42 at a final concentration of 500 μM. The intrinsic exchange rate was measured by exciting Man-GDP at 355 nm and monitoring emission at 448 nm. The fluorescence intensity at 448 nm decreases as Man-GDP is released from Cdc42 into solution. To determine the effect of SopB on nucleotide exchange rate, 2.5 μM Man-GDP-loaded Cdc42 was incubated with a 1.5 μM or 3 μM excess of SopB(29–181) in buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 5 mM MgCl<sub>2</sub>) for 10 min on ice. The exchange reaction was initiated by addition of 500 μM GTP and measured as described.
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![Graph](image)

**FIGURE 2. Analysis of SopB(29–181) binding to Cdc42 by ITC and light scattering.** A, SopB(29–181) was concentrated to 774 μM, and 1-μl aliquots were injected into 94.7 μM Cdc42. The dissociation constant (K_d) of 6 μM was calculated as the mean value of the K_d determined. Experiments were repeated a minimum of three times and error is represented as S.D. B, purified SopB(29–181)-Cdc42 complex was analyzed by injection over a Superdex 75 10/300 GL column followed by static light scattering (Wyatt Technologies). The complex eluted from the column in a monodisperse peak and a molecular mass of ~42 kDa, corresponding to a 1:1 binding stoichiometry.

above. Each reaction was repeated in three independent experiments, and the mean value of relative fluorescence was plotted versus time. S.D. was calculated in Microsoft Excel and is represented by error bars. The SopE-catalyzed nucleotide exchange reactions were prepared and measured as described above. GFP was added to reaction mixture, and measurements were recorded for 30 s. The spectrophotometer was paused as SopE was added to the reaction at a final concentration of 25 nM, and measurements resumed immediately after.

**RESULTS**

**Biochemical Analysis and Crystallization of Bacterial SopB and Host Cdc42 Complex**—To determine how the SopB N-terminal domain binds Cdc42 to down-regulate activity, SopB(29–181) in complex with Cdc42 bound to GDP (K_d of 6 μM) was determined by ITC analysis; Fig. 2A) was co-purified for crystallization. SopB(29–181) and Cdc42 formed a 1:1 complex (as determined by static light scattering; Fig. 2B) and yielded crystals that diffracted to 2.35-Å resolution (one complex in the asymmetric unit; Table 1). Density could be observed for residues 45–171 of SopB and residues 1–179 of Cdc42 as well as its GDP ligand. SopB consists of a β-strand (β1) that forms an intermolecular β-sheet with Cdc42 and five α-helices (α1–5), three of which (α2, α3, and α4) contact Cdc42 switch I and II regions (Fig. 1, C and D). The interface of SopB and Cdc42 forms an area of ~1770 Å² as calculated by PISA (31).

**SopB Contacts Host Cdc42 by Mimicking Eukaryotic CRIB-like Intermolecular β-Sheet**—SopB residues 48–52 and Cdc42 β2 form an intermolecular β-sheet that extends the three-stranded β-sheet of Cdc42 by an additional strand (Fig. 1C). SopB residues flanking the intermolecular β-strand interact with Cdc42 α5 and α1 (Fig. 1C). Specifically, SopB residue Arg-46 side chain forms hydrogen bonds with side chains of Cdc42 α5 residues Asn-167 and Glu-171. Additionally, hydrophobic interactions are formed between SopB residues Pro-47 and Ile-49 and Cdc42 residue Leu-174 in α5. SopB residue Arg-53 forms a hydrogen bond with Cdc42 residue Tyr-23 in α1. Furthermore, SopB residues 58–62 form a short α-helix (α1) that packs against the intermolecular β-sheet such that SopB residues Thr-58 and Tyr-62 hydrogen bond with Cdc42 residues Ala-41 and Thr-43, respectively.

Eukaryotic effector proteins such as PAK, which couples activation of Cdc42 to cytoskeletal function, and cell migration (32) form an intermolecular β-sheet with Cdc42 β2 and contact Cdc42 α1 and α5 through a CRIB motif with the conserved sequence ISXp(X)_2–4FXHXXHV (Fig. 3A) (13). The SopB region (resides 46–53) that forms the intermolecular β-sheet contacts the same regions of Cdc42 (β2, α1, and α5) as the PAK CRIB motif. The canonical CRIB motif is not conserved in SopB except for residue Ile-49, which remarkably superimposes closely with the PAK residue Ile-12 (Fig. 3B). In both the eukaryotic PAK and Salmonella SopB, this Ile residue contacts Cdc42 Ile-46 and additional hydrophobic residues contained within Cdc42 α5. As such, SopB is the first example of a bacterial effector protein that mimics a host CRIB-like interaction to contact and mediate the action of a mammalian Rho GTPase, despite lacking the identifying CRIB sequence motif. These data indicate a key structural role of the Ile residue in the context of Cdc42 binding. This observation is supported by earlier site-specific mutagenesis studies (33) of an I75N mutant within the eukaryotic αPAK CRIB that binds Cdc42 with ~3-fold less affinity as well as our gel filtration analysis of an I49A point mutant of SopB that disrupts interaction with Cdc42 (Fig. 3C).

**SopB Contacts Key Cdc42 Regulatory Switch Residues to Mimic a Rho GDI**—SopB helices (α2, α3, and α4) insert between Cdc42 regulatory loop regions, switch I and II (residues 26–45 and 59–74, respectively) (Fig. 1D). When our structure of Cdc42 in complex with GDP and SopB is superim-

### TABLE 1

| Data collection and refinement statistics |
|-----------------------------------------|
| Crystal parameters                      |
| Space group                             | P4_2_2 |
| a, b, c (Å)                             | 107.00, 107.00, 87.75 |
| α, β, γ (%)                             | 90.0, 90.0, 90.0 |
|                                      |
| Data collection                         |
| Resolution (Å)                          | 45.7-2.35 |
| Completeness (%)                        | 99.9 (100)* |
| Redundancy                              | 14.5 (14.7) |
| Rmerge                                  | 0.081 (0.578) |
| I/σ                                     | 18.8 (4.7) |

*Values in parentheses are for highest resolution shell.
*Calculated by MolProbity (33).
posed with that of Cdc42 in complex with GDP (PDB 1AN0), the overall structure of Cdc42 is similar (average r.m.s.d. of 1.5 Å on 179 Cα atoms). However, our comparison shows that there is a localized change in conformation of the Cdc42 switch I (r.m.s.d. of 2.5 Å Cα atoms of residues 26–45) and that it is the SopB interaction with Cdc42 Val-36 that is predominantly responsible (Fig. 1D; Fig. 4A). Significantly, our observed conformation of Cdc42 switch I in complex with GDP and Salmonella SopB is similar to that of Cdc42 switch I in complex with GDP and human Rho GDI, an inactivator of Cdc42 activity (Fig. 1B). At switch I, the main contact between Cdc42 and SopB is a hydrogen bond between the hydroxyl group of SopB residue Ser-100 and the main chain carbonyl of Cdc42 residue Val-36 and van der Waals interactions between SopB residue Val-97 and the side chain atoms of Cdc42 Thr-35. In addition, the main chain carbonyl of Cdc42 switch I residue Thr-35 and the terminal phosphate group of GDP are coordinated by magnesium. Similarly, in the human Rho GDI, a serine residue contacts Cdc42 Val-36, and the main chain carbonyl of Cdc42 Thr-35 and the terminal phosphate group of GDP are coordinated by magnesium (34). Additional observations from our data show that in the presence of SopB, Cdc42 switch I residue Phe-37, which is solvent-exposed in Cdc42-GDP, is reoriented to a hydrophobic pocket that Tyr-40 occupies in the Cdc42-GDP structure (Fig. 4A). This flip of Phe-37 also occurs when Rho GDI is bound to Cdc42 (Fig. 4B) (34). Collectively, our structure shows that Salmonella SopB mimics several contacts between host Rho GDI and Cdc42 to reposition Cdc42 switch I residues such that Thr-35 forms a coordination network with magnesium and GDP, an interaction that prevents dissociation of bound nucleotide from Cdc42 (34, 35).

Our structural data also show that SopB makes extensive contacts with Cdc42 switch II; however, the conformation of switch II in our structure is similar to that of Cdc42 in complex with GDP (r.m.s.d. 0.6 Å on Cα atoms of residues 59–74), suggesting that SopB uses switch II as an anchoring point to further facilitate binding. SopB α2, α3, and α4 form a helical bundle with a hydrophobic groove composed of several leucine residues (Leu-84, Leu-98, Leu-127, Leu-131, Leu-134) that contact Cdc42 switch II residues Leu-67 and Leu-70 (Fig. 5). SopB mutant L84P has previously been shown to disrupt SopB binding to Cdc42 (16). These interactions are complemented by additional polar interactions (Fig. 1D). Like host Rho GDI (25) and the GDI domain of the Yersinia effector YpkA (35), two SopB residues, Arg-138 and Asp-95, hydrogen bond to the hydroxyl group of switch II residue Tyr-64. Two salt bridges are formed between SopB residues Lys-111 and Arg-138 and...
When Cdc42 is bound to SopB the conformation of Cdc42 switch I residues aligns with the conformation of Cdc42 switch I residues when bound to human Rho GDI, and the GTP exchange activity of Cdc42 is down-regulated. 

A, alignment of Cdc42 switch I (blue) when bound to SopB and Cdc42 switch I (gray) in the absence of SopB (PDB 1AN0). Both Cdc42 structures contain GDP (yellow).

B, alignment of Cdc42 switch I (blue) when bound to SopB with Cdc42 switch I (gray) when bound to Rho GDI (PDB 1DOA).

C, intrinsic Cdc42 nucleotide exchange monitored by measuring change in emitted fluorescence when Mant-GTP is exchanged for unlabeled GTP. SopB was added in excess to the nucleotide exchange assay, and the change in fluorescence was measured. SopE-catalyzed Cdc42 nucleotide exchange was measured as for intrinsic Cdc42 nucleotide exchange. The experiment was repeated with SopB I49A. The arrow indicates the addition of SopE to the reaction mix. Experiments were repeated in triplicate, and error bars represent S.E.

SopB leucine residues (orange) form van der Waals contacts with Cdc42 switch II residues Leu-67 and Leu-70 (blue). A similar set of interactions is also observed when eukaryotic Rho GDI (PDB 1DOA) binds Cdc42.
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switch II residues Asp-76 and Arg-138, respectively. SopB residue Gln-104 contacts switch II residue Asp-57, which is also coordinated in Rac1 by YpkA.

SopB Prevents Cdc42 Nucleotide Exchange in Vitro—To support our observation that SopB structurally mimics Rho GDI, we used an in vitro nucleotide exchange assay to test whether SopB can slow dissociation of bound GDP from Cdc42 and subsequent exchange for GTP (Fig. 4C). Our data show that SopB significantly decreases the rate of in vitro intrinsic Cdc42 nucleotide exchange in a dose-dependent manner. In addition, we tested whether SopB slows nucleotide exchange in the presence of the potent Salmonella GEF, SopE. As seen with the intrinsic nucleotide exchange reaction, SopB slows the rate of Cdc42 nucleotide exchange catalyzed by SopE (Fig. 4C). To test whether the SopB mutant I49A can still inhibit Cdc42 nucleotide exchange, we repeated the intrinsic and catalyzed Cdc42 exchange assay with SopB I49A. SopB I49A could inhibit nucleotide exchange at levels similar to wild-type SopB for the intrinsic exchange assay. However, in the presence of the potent GEF SopE, the SopB I49A mutation significantly decreased the ability of SopB to inhibit nucleotide exchange.

DISCUSSION

Rho GTPases regulate a multitude of cellular processes such as cytoskeleton organization, cell division, and cell motility (36). Members of the Rho GTPase family (for example Cdc42, Rac1, and RhoA) are low molecular weight proteins containing two flexible regulatory switch regions, termed switch I and II, that bind and hydrolyze GTP. As the name implies, they function as molecular switches, which are active in the GTP-bound state and inactive in the GDP-bound state (36). Cycling between the active and inactive state causes a change in conformation at the switch regions critical to function. In the active state, Rho GTPases interact with effector proteins, such as kinases, lipases, and oxidases to alter their activity (12). The activity of Rho GTPases is regulated by GEFs, which catalyze exchange of GDP for GTP to activate the GTPase; GAPs, which catalyze hydrolysis of GTP to inactivate the GTPase; and GDI, which prevent dissociation of bound nucleotide to prevent reactivation of the GTPase (36).

Because Rho GTPases play a fundamental role in many cellular processes, they are common targets of bacterial effector proteins and toxins. Whereas bacterial toxins often inhibit or activate Rho GTPases by irreversible covalent modification (37), Salmonella uses a T3SS to deliver multiple effector proteins and toxins. This is the first time that a pathogenic bacterium has been shown to mimic all three eukaryotic regulators of small GTPases.

Acknowledgments—We thank M. Vuckovic for cloning of protein constructs; T. Spreter for help with multiangle light scattering; and R. Gruninger for helpful discussion. We thank the Canadian Light Source staff at the CMCF-2 beamline for synchrotron data collection.
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