Articles

Effects of Full-Length Borealin on the Composition and Protein−Protein Interaction Activity of a Binary Chromosomal Passenger Complex[†]

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ABSTRACT: The chromosomal passenger complex (CPC) comprises at least four protein components and functions at various cellular localizations during different mitotic stages to ensure correct chromosome segregation and completion of cytokinesis. Borealin, the most recently identified member of the CPC, is an intrinsically unstructured protein of low solubility and stability. Recent reports have demonstrated the formation of binary or ternary CPC subcomplexes incorporating short Borealin fragments *in vitro*. Using isothermal titration calorimetry, we show that full-length Borealin, instead of a Borealin fragment possessing the complete Survivin and INCENP recognition sequence, is required for the composition of a Borealin−Survivin complex competent to interact with INCENP. In addition, we show evidence that full-length Borealin, which forms high-order oligomers in its isolated form, is a monomer in the Borealin−Survivin CPC subcomplex.

The CPC[1] functions in various key mitotic events, including chromatin modification (phosphorylation of histone H3), correction of kinetochore attachment errors, quality control of the spindle assembly checkpoint, the assembly of a stable bipolar spindle, chromosome segregation, and completion of cytokinesis (*1−4*). During the progression of mitosis, the CPC moves to a series of locations in the cell, activating substrates in a coordinated fashion. Specifically, during prometaphase when chromosomes begin to bind to microtubules, the CPC localizes at the centromeres, which are the regions of the chromosomes to which the mitotic spindle fibers attach. At the onset of anaphase when chromosomes start to migrate to opposite poles of the cell, the CPC relocates to the central spindle. A fraction of the CPC then moves to the equatorial cortex just before cleavage furrow assembly and the ensuing cytokinesis (*1−4*). Despite the intriguing localization patterns of the CPC at mitosis, what the localization-specific CPC targets are at various mitotic stages is currently largely a mystery, and the precise mechanistic basis of CPC function remains unclear.

There are four reported chromosomal passenger proteins within the CPC: the serine-threonine kinase Aurora B, the catalytic core of the CPC whose function is regulated by phosphorylation as well as by association with other CPC components (*5−9*); the inner centromere protein INCENP, an activating and targeting subunit of the CPC (*10−13*); Survivin, a protein that plays a clear role in cell mitosis (*14, 15*) and a plausible role in apoptosis which is based principally on the sequence composition of Survivin that includes a baculovirus inhibitor of apoptosis protein (IAP) repeat (BIR), and the observed overexpression of Survivin in a variety of cancer cells (*16−18*); and Borealin/Dasra B, the most recently reported CPC component that is important in kinetochore−microtubule attachment error correction (*19−21*). The Aurora B kinase appears to be at least partially activated at the onset of mitosis following association with INCENP and Survivin, after which further post-translational modification events (including phosphorylation of two serines in the C-terminus of INCENP) proceed and lead to full Aurora B activation (*5−9*). The three remaining CPC components (INCENP, Survivin, and Borealin) are thought to act as
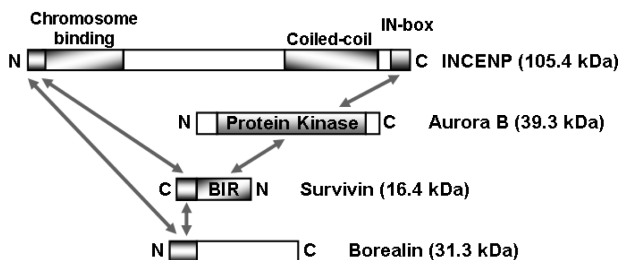
FIGURE 1: Summary of observed protein−protein interactions (illustrated by two-way arrows) within the CPC (*1−4*). Protein sequences of unknown structure and function are shown as blank boxes. The molecular weights of the four CPC components are those from *Homo sapiens*.

adaptor proteins and direct the Aurora B kinase to specific substrates at various localizations during different stages of cell mitosis. For example, Borealin has been shown to be the only CPC component that demonstrates DNA binding and thus has been suggested to be responsible for recruiting the CPC to the inner centromere during prometaphase through direct interaction with centromeric DNA (*22*). Furthermore, a ternary CPC subcomplex comprising Survivin and a short N-terminal fragment each from Borealin and INCENP presents a composite molecular surface of conserved residues essential for central spindle and midbody localization (*23*).

Within the CPC, the four proteins interact extensively. Figure 1 summarizes the domain structures of the four CPC components and the mapping of their interaction regions. A general feature of most chromosomal passenger proteins is low solubility and stability. Borealin, an intrinsically unstructured protein, appears to be the least soluble CPC component since full-length and N- and C-terminal fragments of Borealin have all exhibited high-order oligomerization characteristics (*19, 22*). Furthermore, our work has shown rapid degradation of recombinant full-length Borealin expressed in *Escherichia coli*. So far, structural and biophysical studies have been reported for only binary or ternary CPC subcomplexes involving short Borealin fragments (*23, 24*), but it is not known whether these complexes functionally behave in the same way as their corresponding native CPC subcomplexes incorporating full-length Borealin.

In this study, we have developed an approach to circumvent the unfavorable solubility and stability characteristics of full-length (fl) Borealin, and we produced sufficient amounts of a binary CPC subcomplex comprising fl Borealin and Survivin (fl Survivin has been used throughout this study) for protein−protein interaction studies using isothermal titration calorimetry. In parallel, we performed identical ITC characterization of a binary CPC subcomplex comprising an N-terminal fragment of Borealin (containing the entire Survivin recognition sequence, a region that Borealin also uses to interact with INCENP) and Survivin. We observed striking differences in the protein−protein interaction behavior between the Survivin−Borealin fragment complex and the corresponding complex that consisted of Survivin and fl Borealin. In addition, our study shows that the two Survivin−Borealin complexes adopt different stoichiometries, providing insight into their respective three-dimensional (3D) structural assembly.

MATERIALS AND METHODS

DNA Cloning and Protein Sample Preparation. The Borealin constructs (the full-length protein and the fragment comprising residues 13−92) were each cloned into a pET21a vector derivative (Gev1, Addgene), which has a C-terminal His$_6$ tag and an N-terminal GB1 (immunoglobulin G binding domain B1 of streptococcal protein G; 56 amino acid residues) tag. Survivin and INCENP$^{1−69}$ were cloned into a pET21a vector (MerckBiosciences) as a nontagged and a C-terminally His$_6$-tagged protein. Proteins were expressed in *E. coli* strain DE3 T1^R (Sigma-Aldrich) overnight at 18 °C for ^{GB1}Borealin^{fl} and ^{GB1}Borealin$^{13−92}$ or at 25 °C for Survivin and INCENP$^{1−69}$. The Survivin−^{GB1}Borealin^{fl} and Survivin−^{GB1}Borealin$^{13−92}$ complexes were produced by copurification of the overnight induction cell cultures containing separately expressed Borealin and nontagged Survivin. The cell lysis buffer contained 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 0.5 mM TCEP, and Roche protease inhibitors (Roche Applied Sciences). The cleared cell lysate was purified using Ni-NTA affinity columns (MerckBiosciences), and the eluents were subjected to extensive dialysis in buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1 mM DTT. A final purification step was then carried out using size-exclusion chromatography (SEC) on a Superdex 200 prep grade column (GE Healthcare). The protein samples of His$_6$-tagged INCENP$^{1−69}$, Survivin, and GB1 were purified using similar procedures.

Isothermal Titration Calorimetry (ITC). ITC experiments were performed using a VP-ITC instrument (MicroCal Inc.). Samples of the Survivin−^{GB1}Borealin^{fl} and Survivin−^{GB1}Borealin$^{13−92}$ complexes and INCENP$^{1−69}$ were dialyzed into buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1 mM DTT for 1 h. Titrations were performed by injecting consecutive aliquots of INCENP$^{1−69}$ (63 μM) into the ITC cell (volume of 1.5 mL) containing the Survivin−^{GB1}Borealin^{fl} complex or the Survivin−^{GB1}Borealin$^{13−92}$ complex (both at 6.3 μM) at 10 °C. A repeat titration of INCENP$^{1−69}$ (63 μM) into the Survivin−^{GB1}Borealin$^{13−92}$ complex (6.3 μM) was carried out at a different temperature of 20 °C to validate the near-zero enthalpy change observed at 10 °C. ITC data were corrected for heats of dilution of the protein solution. Binding stoichiometry, enthalpy, entropy, and binding constants were determined by fitting the corrected data to a one-site binding model. The ITC data were fitted using Origin 7.0 (MicroCal Inc.). ITC experiments with the Survivin−^{GB1}Borealin^{fl} complex were carried out immediately after the 1 h dialysis following the final step of purification using SEC. Each ITC experiment was completed within 1 h.

Similar ITC procedures were carried out for the titrations of GB1 (100 μM) into Survivin (10 μM) at two temperatures (20 and 30 °C) to confirm that the GB1 tag used in the Borealin constructs does not interact with Survivin.

Analytical Ultracentrifugation (AUC). Sedimentation velocity experiments were performed using a Beckman Optima XL-I analytical ultracentrifuge equipped with an interference optical system. The sample of the Survivin−^{GB1}Borealin$^{13−92}$ complex was prepared with dilution buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5 mM TCEP, and 1 mM DTT. The aluminum double-sector centerpieces were filled with 400 μL of the protein complex and 420 μL of the

dilution buffer. Samples were centrifuged at a speed of 40000 rpm at 10 °C using an An50-Ti rotor. Scans were acquired at a wavelength of 280 nm at time intervals of 600 s. The partial specific volume of 0.72 mL/g for a 2:2 stoichiometry of the Survivin$-^{GB1}$Borealin$^{13-92}$ complex was calculated on the basis of the amino acid composition (*25*). Sedimentation velocity data were analyzed using SEDFIT (*26*).

Size-Exclusion Chromatography and Multiangle Laser Light Scattering (SEC−MALLS). The molecular weight (MW) and MW distributions of the Survivin$-^{GB1}$ Borealin$^{13-92}$ complex were determined by SEC conducted using a JASCO chromatography system composed of dual PU1580 pumps, a UV1575 detector, a Dawn HELOS Laser photometer, and an Optilab rEX interferometric refractometer (Wyatt Technology, Santa Barbara, CA), with a Superdex 200 10/300 GL analytical column (Pharmacia) at a flow rate of 0.5 mL/min in buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.5 mM TCEP. Molecular weight was analyzed using Astra (version 5.1.9.1) at a dn/dc value of 0.186 mL/g.

RESULTS

A Specific Protocol for the Preparation of a CPC Subcomplex Comprising Full-Length Borealin and Survivin. The challenges in producing good-quality protein samples of CPC subcomplexes incorporating fl Borealin include severe aggregation problems (most likely due to Borealin) and Borealin degradation. To alleviate these problems with fl Borealin, we introduced an N-terminal GB1 tag based on the highly soluble small immunoglobulin binding domain B1 (56 residues) of streptococcal protein G, a tag that has the potential to enhance the expression and solubility of target proteins (*27*). The addition of the GB1 tag to Borealin significantly improved the protein yield in the soluble fractions compared to Borealin constructs with no GB1 tag. To accurately assess whether GB1 interacts with Survivin, we carried out ITC experiments in which aliquots of purified protein samples of GB1 (100 μM) were titrated into 1.5 mL of a purified Survivin sample (10 μM) at 20 and 30 °C. No heat (enthalpy) change was detected under both experimental conditions (Figure S1 of the Supporting Information). These results are consistent with our initial observations that GB1 does not interact with Survivin using protein pull-down assays and analytical SEC analysis of a mixture of purified GB1 and Survivin in which the two proteins elute separately (data not shown). We then devised a specific protocol for producing tractable protein samples of a binary CPC subcomplex comprising Survivin and fl Borealin (Survivin$-^{GB1}$Borealin^{fl}) for protein−protein interaction studies using ITC. The protocol which includes protein expression and purification using the nickel affinity column is described in Materials and Methods. The eluents from the nickel column require extensive dialysis (at least 48 h) in buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1 mM DTT at 4 °C prior to the final purification of the Survivin$-^{GB1}$Borealin^{fl} complex using SEC. In addition, the Survivin$-^{GB1}$Borealin^{fl} complex is sensitive to high temperatures (>10 °C) and the concentration process using benchtop centrifuges where there are at least two adverse factors: the complex sticking to the concentrator membrane and the appreciable dissociation of the complex at a relative cen-

trifugation force (RCF) of 3112g (4000 rpm on a Heraeus Labofuge 400R benchtop centrifuge with a swinging bucket rotor) as assessed using SEC (data not shown). Therefore, the concentration process needs to be avoided or carried out at a lower RCF of 778g.

ITC Reveals an Active Survivin$-^{GB1}$Borealin^{fl} Complex and an Inactive Survivin$-^{GB1}$Borealin$^{13-92}$ Complex. Previous studies have shown clear biochemical evidence for *in vitro* formation of a complex between Borealin and INCENP, and also between Survivin and INCENP, an interaction that is enhanced by the presence of Borealin (*19, 22*). Since the Survivin$-^{GB1}$Borealin^{fl} complex aggregates severely at a concentration above approximately 7 μM and shows signs of degradation <2 h after the final SEC purification step, we chose to use ITC to characterize the protein−protein interaction activity based on its speed and sensitivity. The following ITC experiments were carried out within 2 h of passing the Survivin$-^{GB1}$Borealin^{fl} complex sample through the final SEC column. In the ITC experiments, 1.5 mL of the Survivin$-^{GB1}$Borealin^{fl} or Survivin$-^{GB1}$Borealin$^{13-92}$ complex sample (both at 6.3 μM) was titrated with aliquots of 63 μM purified protein samples of an N-terminal fragment of INCENP (INCENP$^{1-69}$, containing the entire Borealin−Survivin recognition sequence). Strikingly, the ITC experiments suggest no detectable interaction between the Survivin$-^{GB1}$ Borealin$^{13-92}$ complex and INCENP$^{1-69}$, based on the observation of no observed heat (enthalpy) change at two different temperatures [10 and 20 °C (Figure 2)]. This observation is consistent with our protein pull-down assay results where nontagged INCENP$^{1-69}$ failed to bind to the His$_6$-tagged Survivin$-^{GB1}$Borealin$^{13-92}$ complex that was prebound to the nickel column (data not shown). On the other hand, the Survivin$-^{GB1}$Borealin^{fl} complex demonstrated a strong interaction with INCENP$^{1-69}$ [27 nM affinity (Figure 2)]. These data suggest that the Survivin$-^{GB1}$Borealin^{fl} complex, rather than the Survivin$-^{GB1}$Borealin$^{13-92}$ complex, is an active CPC subcomplex that is competent to interact strongly with INCENP. The stoichiometry of the interaction between INCENP$^{1-69}$ and the Survivin$-^{GB1}$Borealin^{fl} complex is presumed to be 1:1 despite the fitted ITC data giving an N value of ~0.75. The lower value of N is likely due to a finite level of degradation of Borealin over the duration of the ITC experiment since our independent observations of freshly purified protein samples of the Survivin$-^{GB1}$Borealin^{fl} complex show a small percentage of degradation of Borealin within a 2 h time scale (Figure S2 of the Supporting Information).

Different Stoichiometries Are Adopted by the Survivin$-^{GB1}$Borealin$^{13-92}$ and Survivin$-^{GB1}$Borealin^{fl} Complexes. Why do the two Survivin−Borealin complexes behave differently with respect to INCENP binding? We characterized their respective solubility, stability, and stoichiometry. The Survivin$-^{GB1}$Borealin$^{13-92}$ complex is highly soluble [up to 9 mg/mL without detectable aggregation (assessed using SEC, data not shown)] and stable (>2 months at −80 °C). Previous 3D structural studies show that Survivin is a homodimer in its isolated form and adopts an elongated shape due to the presence of a protruding C-terminal ~65 Å long α-helix in each protomer (*28−31*). In our SEC analysis, purified protein samples of Survivin (predicted MW of 35.6 kDa as a homodimer including a 14-residue sequence derived from the pET21a vector) elute at a volume corresponding to a
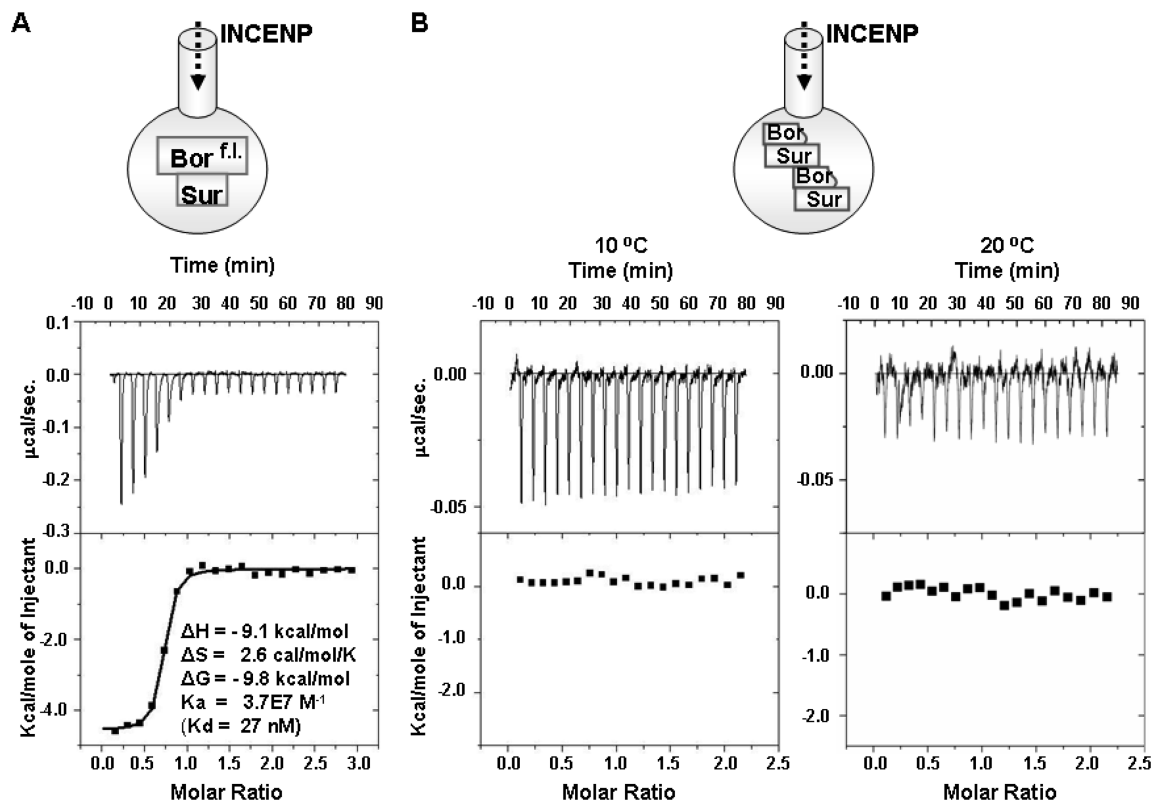
FIGURE 2: Isothermal titration calorimetry. (A) Titration of the Survivin−GB1Borealin[fl] complex with INCENP[1−69] in buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1 mM DTT at 10 °C suggests a strong binding interaction. (B) Titrations of the Survivin−GB1Borealin[13−92] complex with INCENP[1−69] at 10 (left) and 20 °C (right) in buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1 mM DTT suggest near zero enthalpy change implying no detectable interaction.

58 kDa globular protein according to MW calibrations using 29−700 kDa protein MW standards (Sigma-Aldrich; data not shown). To accurately determine the MW and the stoichiometry of the Survivin−GB1Borealin[13−92] complex, we applied AUC and SEC−MALLS, two techniques that allow estimation of the MW in a manner broadly independent of the hydrodynamic anisotropy of the molecules under investigation. The AUC and SEC−MALLS analyses of the Survivin−GB1Borealin[13−92] complex suggested MWs of 73.9 ± 4.3 and 80.9 ± 1.0 kDa, respectively (Figure 3). The data are consistent with a 2:2 stoichiometry between Survivin (predicted MW of 17.8 kDa) and GB1Borealin[13−92] (predicted MW of 19.9 kDa) in the Survivin−GB1Borealin[13−92] complex (predicted MW of 75.4 kDa as a 2:2 complex).

In contrast, the Survivin−GB1Borealin[f.l.] complex has low solubility (severe aggregation above approximately 7 μM) and low stability (degradation starts <2 h after the final SEC purification), therefore precluding MW characterizations using AUC. We have attempted to use SEC−MALLS to determine the MW of the Survivin−GB1Borealin[fl] complex at a concentration of 6.3 μM at 4 °C using freshly made samples. However, no reliable data could be obtained due to the low signal-to-noise level in the elution profile detected by the refractive index detector (whose sensitivity is 10−100 times lower than that of a UV detector).

According to analytical SEC, the Survivin−GB1Borealin[13−92] 2:2 complex elutes at 12.92 mL, while the Survivin−GB1Borealin[fl] complex elutes at a later volume of 13.27 mL (Figure 4). The difference in the SEC retention volumes of the two Survivin−Borealin complexes suggests
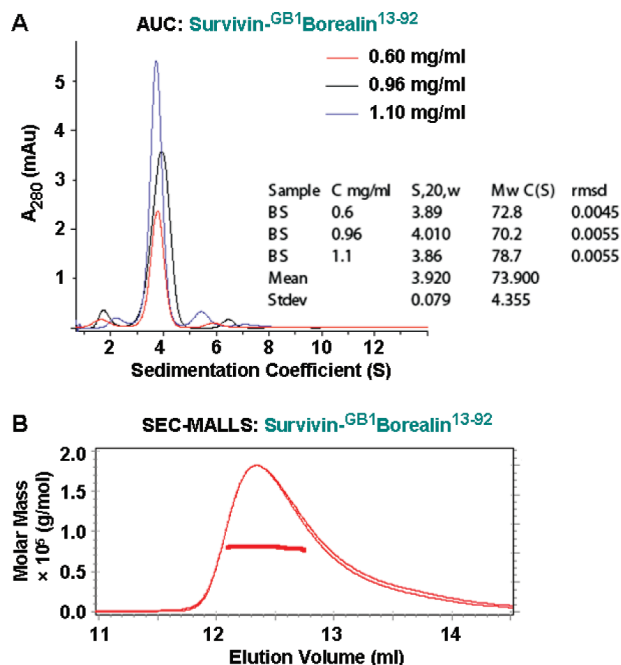

FIGURE 3: (A) Sedimentation velocity AUC analysis of the Survivin−GB1Borealin[13−92] complex at three concentrations (0.6, 0.96, and 1.1 mg/mL) suggests a mean MW of 73.9 ± 4.3 kDa. (B) SEC−MALLS analysis of the Survivin−GB1Borealin[13−92] complex at 0.44 mg/mL suggests a MW of 80.9 ± 1.0 kDa.

that the MW of the Survivin−GB1Borealin[fl] complex is lower than that of the Survivin−GB1Borealin[13−92] complex whose MW has been determined to be ~70−80 kDa using AUC and SEC−MALLS. On the basis of the computed molecular
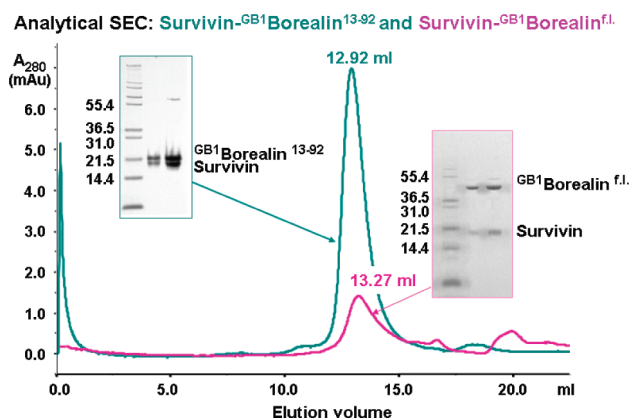
FIGURE 4: Analytical SEC reveals that the Survivin−GB1Borealinfl complex (magenta) elutes at a later volume than the Survivin−GB1Borealin13−92 complex (green). The SDS−PAGE results show that stable complexes are formed (the Survivin−GB1Borealin13−92 complex at 4.5 and 9.0 mg/mL and the Survivin−GB1Borealinfl complex at 0.18 and 0.36 mg/mL or 6.3 μM) and can be eluted by SEC.

masses of the expressed polypeptides, the predicted MW of a Survivin−GB1Borealinfl heterodimer complex is 57.3 kDa. Therefore, the later retention volume of the Survivin−GB1Borealinfl complex compared to that of the Survivin−GB1Borealin13−92 complex suggests that the former is most likely to adopt a 1:1 stoichiometry in which fl Borealin is a monomer.

DISCUSSION

The observed differences in stability, solubility, stoichiometry, and protein−protein interaction activity between the two Survivin−Borealin complexes imply dramatic structural differences. According to the recently determined crystal structure of a core Survivin−Borealin10−109−INCENP1−58 1:1:1 complex, INCENP residues 1−47 and Borealin residues 15−62 form two long helices that bind to the hydrophobic C-terminal helix of Survivin, thus forming a three-helix bundle adjacent to the globular Survivin BIR domain (23; Figure S3 of the Supporting Information). Our data suggest that the Survivin−GB1Borealin13−92 2:2 complex forms a structure in which the INCENP binding surface is no longer presented, perhaps by forming a heterotetrameric four-helix bundle. Our Survivin−GB1Borealin13−92 2:2 complex, as well as other recently reported Survivin−Borealin N-terminal fragment 2:2 complexes (23, 24), yielded only poorly diffracting crystals, possibly implying conformational heterogeneity. Despite the observations that full-length Borealin as well as N- and C-terminal Borealin fragments form high-order oligomers *in vitro* (19, 22), our analyses suggest that full-length Borealin is likely to be a monomer when complexed with Survivin. Structurally, the Survivin−GB1Borealinfl complex is predicted to adopt an assembly similar to that within the ternary Survivin−Borealin1−58−INCENP1−58 1:1:1 complex (23), in which the INCENP binding surface is clearly exposed. Overall, our study emphasizes the importance of the presence of the C-terminal region within fl Borealin for the composition of a fully active Survivin−Borealin complex, possibly by providing steric hindrance to the formation of an otherwise stable structure in which the INCENP recognition site is buried when a shorter Borealin fragment is used. In addition,

our study provides an example in which a protein complex composed of protein fragments that possess complete protein−protein interaction sequences demonstrates stoichiometry and protein−protein interaction behavior different from that of the corresponding protein complex of the full-length proteins.

So far, 3D structural studies of CPC subcomplexes involving full-length Survivin and fragments of Aurora B, Borealin, and INCENP have all demonstrated monomeric protein−protein interactions (6, 23, 24). Our study provides evidence that full-length Borealin is likely to interact with Survivin as a monomer despite previous observations that Borealin and its N- and C-terminal fragments form high-order oligomers *in vitro* (19, 22). It is plausible that the quaternary CPC adopts a 1:1:1:1 stoichiometry. However, there are still extensive protein sequence regions in Borealin and INCENP (depicted as blank boxes in Figure 1) that await characterization of their 3D structure and analysis of their involvement in protein−protein interactions. In addition to the four reported CPC components, the possibility that new members of the CPC assembly might still come to light cannot be ruled out. Understanding the structural geometry of the CPC represents an important step toward deciphering the functional mechanism of the CPC during cell mitosis, a question that also requires knowledge of the presently unidentified specific targets and/or substrates of the CPC at various localizations of the mitotic cell.

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SUPPORTING INFORMATION AVAILABLE

Additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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