Crystal Structures of Fission Yeast Histone Chaperone Asf1 Complexed with the Hip1 B-domain or the Cac2 C Terminus*

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The assembly of core histones onto eukaryotic DNA is modulated by several histone chaperone complexes, including Asf1, CAF-1, and HIRA. Asf1 is a unique histone chaperone that participates in both the replication-dependent and replication-independent pathways. Here we report the crystal structures of the apo-form of fission yeast Asf1/Cia1 (SpAsf1N; residues 1–161) as well as its complexes with the B-domain of the fission yeast HIRA orthologue Hip1 (Hip1B) and the C-terminal region of the Cac2 subunit of CAF-1 (Cac2C). The mode of the fission yeast Asf1N-Hip1B recognition is similar to that of the human Asf1-HIRA recognition, suggesting that Asf1 recognition of Hip1B/HIRA is conserved from yeast to mammals. Interestingly, Hip1B and Cac2C show remarkably similar interaction modes with Asf1. The binding between Asf1N and Hip1B was almost completely abolished by the D37A and L60A/V62A mutations in Asf1N, indicating the critical role of salt bridge and van der Waals contacts in the complex formation. Consistently, both of the aforementioned Asf1 mutations also drastically reduced the binding to Cac2C. These results provide a structural basis for a mutually exclusive Asf1-binding model of CAF-1 and HIRA/Hip1, in which Asf1 and CAF-1 assemble histones H3/H4 (H3.1/H4 in vertebrates) in a replication-dependent pathway, whereas Asf1 and HIRA/Hip1 assemble histones H3/H4 (H3.3/H4 in vertebrates) in a replication-independent pathway.

Eukaryotic genomic DNA forms hierarchical nucleoprotein complex structures in the nucleus. The nucleosome core particle is the basal repeating unit of the complex, which is composed of ~147 bp of DNA wrapped around a core histone particle, comprising a tetramer of H3 and H4 and two dimers of H2A and H2B (1, 2). The precise and regular arrays of nucleosomes is supposed to be a key determinant for the formation of upper hierarchical structures, such as the 30-nm fibers and chromatin fibers. The assembly of the nucleosome is regulated by several groups of chromatin-associated factors involving histone chaperones, chromatin remodeling factors, and histone modification enzymes, which are tightly linked to the regulation of DNA metabolism (2).

Histone chaperones are factors that bind to core histones and facilitate their deposition onto nucleosomes (3). Among the variety of histone chaperones, Asf1 is the most evolutionarily conserved in its primary structure (4–8), and its function as a histone chaperone is conserved throughout the eukaryotes (5, 6, 9–11). Asf1 associates with a variety of chromatin-associated factors, including the histone chaperones CAF-1 (12, 13) and HIR (10, 14, 15), and stimulates both the assembly and disassembly of chromatin (5, 6, 9, 16, 17). Consequently, Asf1 affects most DNA-mediated events, including gene expression (15, 16, 18, 19) and silencing (4, 20–22) as well as DNA repair (5, 13, 23, 24), replication (5) and recombination (25).

Asf1 interacts with a heterodimer of histones H3/H4 through the C-terminal region of H3 (26, 27). Importantly, two human family members of Asf1 (ASF1A and ASF1B) are involved in both the major S-phase histone H3.1- and histone variant H3.3 complexes, whereas the Asf1-interactive histone chaperones CAF-1 and HIRA are detected only in the histone H3.1 and H3.3 complexes, respectively (28). Consistently, Asf1 facilitates both DNA replication-dependent and -independent histone depositions cooperatively with the CAF-1 and HIR complexes, respectively (5, 28, 29), indicating the central role of Asf1 in controlling the state of histone deposition in the nucleus. Recently, the complex structure of human Asf1 with the B-domain of HIRA was reported (30). In addition, biochemical studies suggested that human Cac2, the second largest subunit of CAF-1, interacts with Asf1 at the HIRA-binding region of Asf1 through its B-domain-like motif at the C terminus (30). Hence, human Asf1 is thought to interact mutually exclusively with the histone chaperones HIRA and CAF-1. However, structural evidence for the interaction between Asf1 and CAF-1 has yet to be obtained. In addition, it is not clear how Asf1 recognizes HIRA and CAF-1 for histone assembly in yeast.

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The atomic coordinates and structure factors (code 2CU9, 2Z34, and 2Z3F) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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4 The abbreviations used are: Asf1, anti-silencing function-1; CAF-1, chromatin assembly factor-1; HIRA, histone regulatory homolog A; Hip1, HIRA-like protein-1; r.m.s.d., root mean square deviation; GST, glutathione S-transferase; WT, wild type.
In this study, we report the crystal structures of the apo-form of fission yeast Asf1/Cia1 (SpAsf1N; residues 1–161) and its complexes with the B-domain of the fission yeast HIRA orthologue Hip1 (Hip1B) and the C-terminal region of fission yeast Cac2 (Cac2C) (Fig. 1A). Cac2C shows remarkably similar modes of interaction toward Asf1 as compared with those of Hip1B and HIRA. Based on structural and biochemical analyses of the three histone chaperones, we discuss a mutually exclusive binding model of Asf1 to CAF-1 and Hip1/HIRA.

EXPERIMENTAL PROCEDURES

Peptides—All peptides used in co-crystallization and for in vitro binding experiments were purchased from Invitrogen.

Plasmids and Site-directed Mutagenesis—The structural domain of Asf1 from Schizosaccharomyces pombe (SpAsf1N; residues 1–161) was subcloned into the pET15b vector (Novagen) such that the construct consisted of an N-terminal hexahistidine tag followed by a thrombin cleavage site upstream of the expressed protein. For the GST fusion proteins used in the in vitro binding experiments, the SpAsf1N structural domain sequence was subcloned into the pGEX-2T vector (GE Healthcare). Point mutants of Asf1 were generated by subjecting the pGEX-2T-SpAsf1N constructs to mutagenesis using the QuikChange II site-directed mutagenesis system (Stratagene). The following mutagenic oligomers were used (forward primers are shown with mutation codons in boldface, 5′ to 3′): D37A-forward, GTCTGGAGCCGTTAAAAGC; E39A-forward, GGTTTTGGGTGTCACAGTTTTT; V95A-forward, GATGTTTTGGGTGTCACAGTTTTT; and 170 mM NH4F at a concentration of 4 mg/ml protein at 20 °C. The crystals were cryoprotected by briefly soaking them in the mother liquor solution containing 10% glycerol prior to flash-freeze cooling in a cold stream of nitrogen gas.

Preparation of the SpAsf1N-Hip1B Peptide Complex and Its Crystallization—The expression, purification, and crystallization of the functional domain of SpAsf1-N (residues 1–161) were performed. The SpAsf1-N hexahistidine tag was cleaved by overnight digestion with thrombin (Sigma), and the uncleaved proteins were removed by a second passage through the HisTrap column. Finally, another round of purification on the Superdex 75 16/60 column was performed.

For GST-SpAsf1N expression, the constructs were transformed into BL21(DE3), grown to late log phase at 37 °C and then induced overnight with 0.4 mM isopropyl 1-thio-β-D-galactopyranoside at 25 °C. The fusion proteins were each bound to glutathione-Sepharose 4B columns (GE Healthcare), which were washed with 1× phosphate-buffered saline, pH 7.4, and 1 mM dithiothreitol and eluted with 10 mM reduced glutathione in 50 mM Tris–HCl, pH 8.0. Further purification was performed by gel filtration on a Superdex 75 10/30 column (GE Healthcare).

Preparation of the SpAsf1N-Cac2C Peptide Complex and Its Crystallization—The Asf1-Cac2C complex was formed by mixing purified SpAsf1N, at a 4 mg/ml final concentration, with a 5-fold molar excess of the S. pombe Hip1 B-domain peptide (residues 469–497; IPTKFVKVTITKEGKKRVAPQLLTLTSLA) and incubating the mixture for 1 h at room temperature. The complex was then purified as a single peak by size exclusion chromatography on Superdex 75 resin. The purified complex was successfully crystallized in a 96-well plate by the sitting drop vapor diffusion method under 25% polyethylene glycol 3350 and 170 mM NH4F at a concentration of 4 mg/ml protein at 20 °C. The crystals were cryoprotected by briefly soaking them in the mother liquor solution containing 10% glycerol prior to flash-freeze cooling in a cold stream of nitrogen gas.

Data Collection and Structure Determination—Diffraction data were collected at the beamline BL26B1 (SPring-8, Hyogo, Japan) for the native SpAsf1N and on the RAXIS IV++ in-house facilities for the two complexes (Hip1B and Cac2C). All of the data were collected under cryogenic conditions. The data were processed and scaled using the HKL2000 package (32). The structure of the native SpAsf1N structure was determined by the molecular replacement method employing the ScAsf1 structure as a search model, using the program Molrep (33) from the CCP4 suite (34). The structures of the complexes were solved by molecular replacement using the native SpAsf1N structure as a model. Iterative manual model building and initial refinement were performed using the programs O (35) and CNS (36), respectively. Final refinement was carried out using Refmac5, which is incorporated in CCP4. Statistics for data processing and refinement for all three structures are listed in Table 1. The stereochemistry of the structures, as assessed with

Crystal Structures of Asf1 with Hip1 or Cac2
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PROCHECK (37), were excellent. All of the molecular graphics figures were generated using PyMOL.

Surface Plasmon Resonance Binding Assay—Steady-state binding analyses between GST-SpAsf1N and the Hip1B or Cac2C peptides were carried out using a Biacore-3000 apparatus. Experiments were performed at 25°C, using a CM5 sensor chip and HBS-EP buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20). The GST-Asf1N proteins were captured at final densities of 14,000 to 16,000 resonance units. The peptides in the HBS-EP buffer were passed over the GST-SpAsf1N surfaces at a rate of 20 μl min⁻¹. The concentrations of the wild-type peptides were 62.5, 125, 250, 500, 1000, 2000, 4000, and 8000 nM, and those of the mutant peptides were 250, 500, 1000, 2000, 4000, and 8000 nM. A saturated response value at each concentration of the peptide was used as the equilibrium response value, and the steady-state data were fit to a simple 1:1 interaction model to obtain the dissociation constants.

RESULTS

Sequence Comparison of Hip1 and Cac2—The Asf1 protein associates with HIRA through the Asf1 binding domain of HIRA (namely, the B-domain) (38, 39). The HIRA homolog from S. pombe, Hip1/HIRL, is a 932-residue protein containing eight WD40 repeat domains. The putative Hip1 B-domain lies in the region between WD40 repeats 6 and 7, within residues 450–500. A sequence alignment of the B-domains from a diverse array of organisms shows a high degree of conservation (Fig. 1B). The “consensus sequence” consists of a conserved glycine (Gly-483) at position −3 followed immediately by three positively charged residues, with a conserved arginine (Arg-486) at position 0, and a proline residue (Pro-489) occupying position +3.

The Cac2/p60 subunit of the CAF-1 complex similarly binds to Asf1 (12, 13, 15). A sequence alignment of the Cac2/p60 C-terminal residues from various organisms is shown in Fig. 1C. The alignment reveals that the C-terminal region of p60 exhibits significant similarity to the B-domain of HIRA (Fig. 1D). An arginine (Arg-504) at position 0 is the only strictly conserved residue in the aligned sequences; this is preceded by another basic residue at position −1 and is followed by hydrophobic residues at positions +1, +3, and +6. In vertebrate sequences, a pair of overlapping Asf1-binding sites is predicted to lie at the Cac2/p60 C termini (30). In S. pombe, the putative Cac2/p60 orthologue corresponds to SPAC26H5.03, a WD40 repeat protein that shares 34% sequence identity with the Cac2/p60 orthologue corresponds to SPAC26H5.03, a WD40 repeat protein that shares 34% sequence identity with Cac2/p60 along its entire length. Interestingly, in S. pombe there is greater similarity between the Hip1 B-domain and the C-terminal region of the putative Cac2 protein than in most of the other organisms examined, with the Lys-Lys-Arg (484–486) residues at positions −2 to 0 and the Ala-Pro (488–489) residues at positions +2 to +3 being conserved (Fig. 1D).

The Native SpAsf1N Structure—For the structure determination, we expressed and purified the highly conserved, N-terminal structural domain of S. pombe Asf1/Cia1 (SpAsf1N; residues 1–161), which corresponds to the functionally active globular domain of S. cerevisiae Asf1. The native spAsf1 protein was crystallized in the C2 space group, with one molecule in the asymmetric unit. The structure was solved by molecular replacement, using the ScAsf1/Cia1 structure (40) as a model (Table 1). The native structure of SpAsf1N is similar to those of ScAsf1 (10, 40) and human Asf1 (41). Briefly, the core of the spAsf1 structure consists of 10 β-strands forming an elongated β-sandwich of two anti-parallel sheets packed against each other (Fig. 2). The topology falls into the “switched” immunoglobulin class of proteins. The “front” face is composed of the strands βv, β3, β5, βp, and β1p, and the “back” face is formed by the strands β1, βv, and β6. The front face forms a highly hydrophobic concave surface. In contrast to the well conserved β-strands, the three loop/helical regions connecting the β-strands (loops β3−βv, β5−β5, and β5−β6) are less conserved. These loops are positioned above the hydrophobic surface and exhibit a greater degree of flexibility (see below).

Structure of the SpAsf1N-Hip1B Complex—As described under “Experimental Procedures,” the SpAsf1N protein was co-purified with the Hip1 B-domain peptide (Hip1B; residues 469–497), yielding a crystal in the P21 space group containing two complexes per asymmetric unit (Table 1). The electron density for the peptide was clearly visible in the regions corresponding to residues 474–494 of one molecule (chain E in the Protein Data Bank code 2234) and residues 475–496 of the other molecule (chain F). The peptide adopts a β-hairpin conformation that binds more or less perpendicularly to the cleft formed between the two faces of the Asf1N structure, between strands β5 and β6 (Fig. 3A).

The turn in the β-hairpin is located between the Glu-482 residue and the strictly conserved Gly-483 of the Hip1 B-domain. Antiparallel β-sheet interactions, which stabilize the structure between residues 476–480 and 484–488, are characterized by five intramolecular main-chain hydrogen bonds and additional hydrogen bonds between the side chain of Thr-480, the main-chain nitrogens, and the Glu-482 side chain.

The characteristics of the SpAsf1N-Hip1B interactions are as follows. The Hip1 B-domain peptide binds to SpAsf1N via a combination of van der Waals contacts, main-chain hydrogen bonds, and polar interactions. The conserved residue Hip1B Pro-489 is nestled in the hydrophobic cleft formed at the interface of strands β5 and β6 of Asf1N, bounded by the conserved residues Val-62, Pro-64, Pro-66, and Phe-72. The Hip1B residues Val-487 and Leu-491 further stabilize this hydrophobic interface. Meanwhile, a network of main-chain hydrogen bonds facilitates the β-sheet interactions between the Hip1B residues 485–487 and the Asf1N residues 61–63 and between the Hip1B residues 490–493 and the Asf1N residues 69–71. Polar interactions between side chains are also seen, with the most prominent being a salt bridge between the conserved Hip1B Arg-486 and Asf1N Asp-37. Additional electrostatic interactions are possible between Hip1B Lys-485 and an acidic patch on Asf1N, consisting of Asp-58, Glu-75, and Asp-77, and between Lys-484 of Hip1B and Glu-39 of Asf1.

A comparison of the recently reported structure of the human Asf1–HIRA complex (30) with our present SpAsf1N-Hip1B complex yielded an r.m.s.d. value of 0.91 Å for the Cα atoms. The mode of SpAsf1N-Hip1B domain recognition is
similar to the human Asf1-HIRA binding scheme, which is unsurprising, given the high degree of sequence conservation (Figs. 1B and 3B). Using the numbering scheme from Fig. 1B, the conserved Arg at position 0 and Pro at position +3 of Hip1/HIRA occupy almost identical positions within Asf1. Likewise, the basic side groups at positions −2 to −1 (Lys-Lys and Arg for Hip1 and HIRA, respectively) superimpose very closely. In addition, the orientations of the Asf1 side chains involved in Hip1/HIRA contacts, including those of residues 37, 39, 58, 75, and 77, are similar between the S. pombe and human structures.

**FIGURE 1.** Sequence conservation of Hip1 and Cac2. The red and yellow boxes denote strictly and highly conserved residues, respectively. Numbers below the alignments pertain to the numbering scheme used in the text to denote conserved residue positions. A, sequence alignment of five Asf1N structural domains from S. pombe (Sp_Asfl), S. cerevisiae (Sc_Asfl), Drosophila melanogaster Asf1 (Dm_Asfl), human Asf1a (h_Asfla), and human Asf1b (h_Asflb). The secondary structural features from the budding yeast structure (10) and the S. pombe structure (this study) are indicated above the alignments. **Blue dashed lines** below the alignments indicate the flexible L1, L2, and L3 helical/loop regions on SpAsf1N. B, sequence alignment of the B-domain regions of HIRA orthologues: Hip1/HIRL from S. pombe (Hip1 Sp); Hir1 from S. cerevisiae (Hir1 Sc); HIRA from D. melanogaster (HIRA Dm), Xenopus laevis (HIRA XI), mouse (HIRA Mm), and human (HIRA Hs); and nucleotide-binding protein from Arabidopsis thaliana (Nucb At). C, sequence alignment of B-domain-like regions from the C termini of CAF-1 Cac2/p60 orthologues: hypothetical protein SPAC26H5.03 from S. pombe (Cac2 Sp); Cac2 from S. cerevisiae (Cac2 Sc), and Candida albicans (Cac2 Ca); Fasciata2 from A. thaliana (Fas2 At); p105 from D. melanogaster (p105 Dm); and p60 from mouse (p60 Mm) and human (p60 Hs). The vertebrate p60 proteins have two overlapping B-domain-like sequences, denoted as 1 and 2. D, sequence comparison of the S. pombe Hip1 B-domain and the Cac2 orthologue C-terminal region.
Hence, we speculate from these structural observations that Asf1, from yeast to human, associates with Hip1/HIRA in a similar fashion.

Structure of the SpAsf1N-Cac2C Complex—The SpAsf1N-Cac2C complex was obtained by the co-crystallization of SpAsf1N with a 20-residue peptide, corresponding to the C-terminal sequence of the \textit{S. pombe} Cac2 homolog. The sample crystallized in the \textit{I}4\textit{1} space group, with eight SpAsf1N-Cac2C complexes per asymmetric unit (Table 1). The subunits are arranged as two groups of “tetramers” of SpAsf1N-Cac2C complexes (Fig. 4C). The four SpAsf1N monomers in each tetramer are arranged in roughly square-like arrangements, with two monomers lying along the plane of the square (e.g., chains A and F) and two monomers positioned perpendicular to it (e.g., chains C and H). The middle of each tetramer contains a large central cavity measuring $\sim$20 $\times$ 15 Å. Although the structural refinement could be carried out only at the relatively modest resolution of 2.7 Å, strong electron density was observed for the entire length of all eight monomers. Despite the variations in the crystal

### Table 1
Summary of data collection and refinement statistics

| Data collection parameters | Native | Hip1B complex | Cac2C complex |
|---------------------------|--------|---------------|---------------|
| Diffraction data          |        |               |               |
| Space group               | C2     | \textit{P}2\textit{1} | \textit{I}4\textit{1} |
| a = 78.62, b = 41.30, c = 67.21, $\beta$ = 115.65 | a = 62.01, b = 48.09, c = 63.25, $\beta$ = 99.25 | a = b = 151.51, c = 144.21 |
| Resolution (Å)            | 1.80   | 2.30          | 2.6           |
| Wavelength (Å)            | 1.00   | 1.5418        | 1.5418        |
| Measured reflections      | 62,387 | 51,572        | 30,7285       |
| Unique reflections        | 17,836 | 16,188        | 50,092        |
| $R_{merge}$ (%)\(^a\)    | 7.6 (19.8) | 6.9 (23.8) | 7.2 (48.8) |
| Completeness (%)          | 97.7 (87.4) | 97.6 (98.7) | 99.9 (100) |
| Redundancy                | 3.5 (2.4) | 3.2 (3.2) | 6.1 (6.0) |
| Overall $I/\sigma$        | 28.2 (5.3) | 19.6 (5.0) | 25.2 (3.9) |
| Refinement statistics     |        |               |               |
| Resolution range          | 60–1.80 | 31–2.40      | 32–2.70       |
| Working set               | 16,920 | 13,543       | 42,342        |
| Test set (5.0%)           | 914    | 736          | 2,252         |
| Proteins (SpAsf1 + peptide) | 1,274 (no peptide) | 2,778 | 10,996 |
| Ligands                   | 10 (PEG)\(^c\) |               |               |
| Water molecules           | 248    | 129          | 312           |
| R-factor (%)\(^d\)       | 19.1   | 19.4         | 20.8          |
| $R_{free}$ (%)\(^e\)     | 22.9   | 25.4         | 26.3          |
| Mean B-factor (Å\(^2\))  | 26.9   | 29.6         | 39.0          |
| r.m.s.d. Bond lengths (Å) | 0.025  | 0.021        | 0.022         |
| Bond angles (°)           | 1.966  | 1.739        | 1.749         |
| Ramachandran plot (%)     | 92.7   | 90.7         | 91.1          |
| Most favored regions      | 6.6    | 8.9          | 8.5           |
| Allowed regions           | 0.7    | 0.3          | 0.3           |
| Generously allowed regions|         |               |               |
| Overall $B$-factor (%)    | 0.0    | 0.0          | 0.0           |

\(^a\) Numbers in parentheses are values in the highest resolution shell.  
\(^b\) $R_{merge}$ = $\Sigma|I_{obs} - \langle I \rangle|/\Sigma I_{obs}$ summed over all observations and reflections.  
\(^c\) PEG, polyethylene glycol.  
\(^d\) $R_{cryst}$ = $\Sigma |F_{o} - F_{c}|/\Sigma F_{o}$.  
\(^e\) $R_{free}$ was calculated with 5% of the data omitted from refinement.

### Figure 2
Overall tertiary structure of SpAsf1N in the apo- and complex forms. A, schematic representation of the apo-form of the SpAsf1N tertiary structure (blue to red). B, the Cα trace of the SpAsf1N structure is colored in gray for the apo-form, blue for the Hip1 peptide complex (chain A), green for the Hip1 peptide complex (chain B), and yellow for the Cac2 peptide complex. The three flexible loop/helix regions are indicated: loop $\beta$4–$\beta$5 (residues 48–53), loop $\beta$6–$\beta$7 (residues 81–92), and loop $\beta$8–$\beta$9 (residues 119–134). The N and C termini and the hydrophobic front face of SpAsf1N are also indicated.
The Cac2C peptide adopts an extended chain conformation and binds to the cleft on Asf1 between strands $\beta 5$ and $\beta 6$ (Fig. 4A). As predicted from the sequence comparison, it shares a common binding site on the Asf1 surface with the Hip1 B-domain peptide. In all chains, only the C-terminal half of the Cac2C peptide was clearly visible in the electron density maps, and thus residues 493–502 were not included in the final model. Main-chain hydrogen bonds were observed between residues 61–63 in Asf1N and residues 503–505 in Cac2C and between Asf1N residues 69–71 and Cac2C residues 508–510. In Cac2C, the Pro-507 residue is lodged into a hydrophobic pocket on the surface of Asf1, which is lined by Val-62, Pro-64, Pro-66, and Phe-72. The conserved Cac2 residue Arg-504 makes a crucial salt bridge with Asf1 Ile-69, and Cac2 Lys-503 makes a salt bridge to Asf1 residue Asp-58, located in an acidic cluster that also includes Glu-75 and Asp-77. The Cac2C peptide actually adopts two distinct conformations at the two final residues (Fig. 4B). In half of the chains, the side chain of the penultimate residue, Tyr-511, points away from the N-terminal part of the peptide, whereas the last residue, Pro-512, protrudes at an angle away from the rest of the linear peptide backbone. This conformation allows extra main-chain hydrogen bonds between Cac2 Tyr-511 and Asf1 Ile-69. In the other half of the chains, the Tyr-511 side chain points back toward Pro-509, and the last residue, Pro-512, is more or less aligned with the rest of the peptide backbone.

Unexpectedly, the maps revealed some extra density residing in the space in the middle of each Asf1N tetramer. These densities were determined to belong to extra Cac2C peptides corresponding to residues 507–511 (not shown). Four such stretches of density were observed (two per Asf1N tetramer), and three short chains could be built confidently. Each extra peptide chain is oriented diagonally within the cavity, making hydrophobic contacts with two molecules of Asf1N. The Pro-507 residue of the extra peptide is oriented within a hydrophobic cleft formed between the N-terminal and C-terminal strands ($\beta 1$ and $\beta 10$) of one Asf1N molecule, whereas the Tyr-511 side chain of the extra peptide is stacked against the side chains of the Asf1N-Cac2C complex on the opposite side of the cavity. There is no evidence for a higher level of association in solution between Asf1N and the Cac2C peptide, and gel filtration experiments clearly indicated a 1:1 stoichiometry (data not shown). Therefore, we conclude that the presence of the extra peptide chains is an artifact from the crystallization process.

Comparison of the Structures of SpAsf1N-Hip1B and SpAsf1N-Cac2C Complexes—The Hip1 B-domain peptide and the Cac2 C-terminal peptide show remarkably similar modes of interaction with Asf1N (Fig. 4D). The backbones of Hip1B residues 485–489 and Cac2C residues 503–508 align very closely, as do the side chains for the conserved residues Hip1-Lys-485/Cac2C-Lys-503 (position −1), Hip1-Pro-489/Cac2C-Arg-504 (position 0), and Hip1-Pro-489/Cac2C-Pro-507 (position +3). The same combination of hydrophobic contacts, main-chain hydrogen bonds, and salt bridge interactions is predicted to play an important role in recognition and binding. One key difference between the two complex structures is that the Cac2 C-terminal sequence does not form the same hairpin structure as the Hip1 B-domain. This is probably because Cac2 lacks the conserved glycine at the −3 position that is necessary to make the $\beta$-turn (Fig. 1). In terms of Asf1N, most of the residues involved in making contacts with the Hip1B/Cac2C peptides overlap very closely. One exception is Asf1 Glu-39, which in the Hip1B complex structure points toward the Hip1B Lys-484 side-chain, whereas in the Cac2C complex structure, it is oriented away from the peptide. This correlates with the lack of electron density at position −2 for Cac2 (Lys-502) and indicates greater disorder in the N-terminal half of the Cac2C peptide.

Comparison of the Structures of SpAsf1N Complexes with Its Native Form—Each Asf1N molecule in the Hip1B/Cac2C peptide complex structures adopts the immunoglobulin-like domain fold seen in the apo-form. However, there are several significant differences. In both complex structures, deviations from the wild-type fold can be seen in the three loop/
helical regions $\beta_4-\beta_5$, $\beta_6-\beta_7$, and $\beta_8-\beta_9$, which indicate general flexibility in these regions and correlate with the higher local B-factors (Fig. 2B). In particular, in the Asf1N-Hip1B complex structure, the $\beta_6$-$\beta_7$ loop/helix region dramatically opens up, away from the main body of the protein. This conformational change pivots on "hinges" located at the Gly-120 residue and around Lys-135. It is not clear whether this change is caused by the binding of the Hip1B peptide or if it is merely due to the specific packing interactions in the $P_{2_1}$ crystal form. Perhaps because of this excessive flexibility, the electron density was missing from the regions corresponding to residues 127–130 for Asf1N chain A and 120–131 for Asf1N chain B; thus these regions were omitted from the final Asf1N-Hip1B model.

The two molecules of Asf1N in the Hip1B complex structure have a Cα r.m.s.d. of 0.66 Å (0.35 Å, when excluding the three flexible loop/helix regions). Compared with the native structure, the average r.m.s.d. value of the Cα atoms in the Asf1N-Hip1B complex is 0.61 Å. In contrast, in the Asf1N-Cac2C complex structure there is less variation among the eight Asf1N subunits, with a Cα r.m.s.d. of 0.29 Å, whereas the average r.m.s.d. with respect to the SpAsf1N native form is 0.64 Å along its entire length.

In Vitro Binding Analysis—Surface plasmon resonance (SPR) binding techniques were used to probe the specific molecular interactions between Asf1N and Hip1B/Cac2C. Wild-type and mutant Asf1N proteins were expressed and purified as GST fusion proteins. Following an analysis of the complex structures, the following point mutants of Asf1N were created: D37A, E39A, D58A, L60A/V62A double mutant, E75A, D77A, V95A, and D104A. The V95A mutant serves as a control; this residue was shown to be involved in interactions with the histone H3 C-terminal region. The D104A mutant was also chosen because in the Asf1N-Hip1B complex asymmetric unit, the nonconserved Asp-104 residue from one subunit of Asf1N makes a polar contact with Lys-484 of the Hip1B chain from the opposite complex. The wild-type GST-Asf1N and the eight mutant proteins showed nearly identical levels of expression, solubility, and final purity (data not shown).

The GST-Asf1N wild-type and mutant proteins were bound to CM5 sensor chips. The results of binding assays with the wild-type Hip1 B-domain peptide (residues 469–497) are shown in Fig. 5A.
P489A mutant peptide showed dramatically reduced levels of binding. The Hip1B mutant peptides K485A and K484A also displayed decreased levels of binding to Asf1N, with $K_D$ values of $59 \pm 20$ and $16.9 \pm 1.7 \, \mu M$, respectively, indicating the relative importance of the two lysine residues in Asf1 binding. On the other hand, the Hip1B mutant E482A demonstrated almost wild-type levels of binding to Asf1N.

In the equivalent experiments for Asf1N-Cac2C peptide binding, similar results were observed (Fig. 5, C and D). The wild-type Cac2C peptide interaction with the wild-type Asf1N displayed an almost equivalent level of binding as the wtAsf1N/ wtHip1B interaction, with a $K_D$ value of $1.46 \pm 0.01 \, \mu M$. Consistently, Cac2C peptide binding was abolished for both the Asf1N mutants D37A and L60A/V62A. In addition, reduced levels of binding were observed for the Asf1N mutants D58A and E75A, with $K_D$ values of $5.9 \pm 0.2$ and $3.3 \pm 0.1 \, \mu M$, respectively, whereas the E75A and D77A mutants displayed almost wild-type $K_D$ values. For the converse set of interactions (between wild-type Asf1 and mutant Cac2C peptide), the following Cac2C peptides were tested: K499A, K502A, K503A, R504A, and P507A. As predicted, the R504A mutant showed virtually no binding to wild-type Asf1N. The Cac2C mutant P507A also displayed a greatly reduced level of binding to Asf1N, although the effect was not as drastic as for the equivalent mutant in Hip1B, P489A. The Cac2C mutant K503A also showed reduced binding, with a $K_D$ value of $11.5 \pm 0.3 \, \mu M$. Interestingly, the K502A mutant of Cac2C displayed a smaller decrease in binding ($K_D = 4.5 \pm 0.1 \, \mu M$) as compared with the equivalent Hip1B peptide mutant, K484A. This is consistent with the prediction that the Hip1 Lys-484 residue plays a greater role than Cac2 Lys-502 in the overall binding to Asf1N, as evidenced by the lack of polar contacts between Asf1 Glu-39 and Cac2 Lys-502 in the Asf1N-Cac2 complex structure.

**DISCUSSION**

In this study, we determined the crystal structures of the apo-form of fission yeast Asf1 and the Asf1-Hip1B and Asf1-Cac2C histone chaperone complexes. In addition, we defined the specific residues responsible for the binding interactions. The modes of recognition between the Asf1N-Hip1B and Asf1N-Cac2C complexes are essentially identical. The same combinations of hydrophobic contacts, main-chain hydrogen bonds, and salt bridge interactions are observed between Asf1N and the Hip1B or Cac2C peptide. On the Asf1N surface, a conserved hydrophobic binding pocket, consisting of residues Phe-28, Val-62, Pro-64, Pro-66, and Phe-72, provides a binding site for hydrophobic residues on Hip1B/Cac2C. Furthermore, the conserved Asf1 residue Asp-37 makes a crucial salt bridge with the conserved arginine at position 0, whereas Asp-58 and Glu-75, and Asp-77 appear to play auxiliary roles in binding. The *in vitro* binding experiments confirmed the observations from the crystal complex structures.

More subtle differences between the Asf1-Hip1B and Asf1-Cac2C interactions were observed, which could play an impor-
Crystal Structures of Asf1 with Hip1 or Cac2

TABLE 2
Calculated $K_D$ values for binding between the WT and mutant forms of SpAsf1N and the Hip1 B-domain peptide or the Cac2 C-terminal peptide.

| SpAsf1 | $K_D$ (μM) | WT Hip1 peptide | WT Cac2 peptide |
|--------|------------|----------------|-----------------|
| Wild type | 0.89 ± 0.01 | 1.46 ± 0.01 |
| D37A | ND* | ND |
| E39A | 2.5 ± 0.1 | 3.3 ± 0.1 |
| D58A | 6.8 ± 0.2 | 5.9 ± 0.2 |
| L60A/V62A | ND | ND |
| E75A | 1.55 ± 0.04 | 2.1 ± 0.06 |
| D77A | 1.36 ± 0.03 | 2.4 ± 0.05 |
| V95A | 0.71 ± 0.02 | 1.2 ± 0.8 |
| D104A | 0.97 ± 0.02 | 1.59 ± 0.04 |

| Hip1 peptide | WT SpAsf1 | Cac2 peptide | WT SpAsf1 |
|-------------|-----------|--------------|-----------|
| Wild type | 1.2 ± 0.06 | 1.5 ± 0.04 | 3.4 ± 0.07 |
| E482A | 2.7 ± 0.07 | 4.5 ± 0.1 | ND |
| K484A | 16.9 ± 1.7 | 11.5 ± 0.3 | 1.7 ± 0.07 |
| K485A | 59 ± 20 | 3.4 ± 0.7 | ND |
| R486A | ND | ND | ND |
| F489A | ND | ND | ND |

* ND, not determined because of weak or absent interaction.

A comparison of the Asf1N structures determined in the present study revealed significant changes in the three flexible loop regions, depending on the bound ligand state. As all three structures were from crystals obtained in different space groups, one cannot rule out the possibility that these conformational changes are due to differences in crystal packing. However, these regions were previously shown to be important for binding to histones H3/H4 (26, 27). Whether the significant structural variations observed in the above-mentioned loops of all known Asf1N structures are functionally important has yet to be clarified. Recently, the structures of Asf1 complexes with H3/H4 heterodimers from yeast and human were reported (26, 27). Superpositioning these structures with the SpAsf1N-Hip1B/Cac2C complex suggests that the H3/H4 heterodimer and the Hip1B/Cac2C peptides bind to Asf1N exclusively, on opposite sides of the sandwich domain of the Asf1N structure.

In summary, we have determined the crystal structures of the N-terminal domain of fission yeast Asf1 complexed with the Hip1B and Cac2C domain peptides. Our results, along with biochemical studies, provided a structural basis for the exclusive interactions of Hip1 and Cac2 with Asf1 in the replication-independent pathway and the replication-dependent pathway, respectively, of nucleosome assembly.
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