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DNA-binding properties of Smc6, a core component of the Smc5–6 DNA repair complex

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ABSTRACT

The Smc5–6 complex is an essential regulator of chromosome integrity and a key component of the DNA damage response. As an essential DNA repair factor, the Smc5–6 complex is expected to interact with DNA and/or chromatin during the execution of its functions. How the Smc6 protein promotes the binding of the Smc5–6 complex to DNA lesions is currently unknown. We show here that Smc6 is a strong DNA-binding protein with a clear preference for single-stranded DNA substrates. Importantly, Smc6 associates with DNA in the absence of other Smc5–6 complex components and its activity is modulated by nucleotides. Our results also show that the minimal size of single-stranded DNA required for tight association with Smc6 is ~60 nucleotides in length. Taken together, our results suggest that Smc6 contributes to DNA repair *in vivo* by targeting the Smc5–6 complex to single-stranded DNA substrates created during the processes of homologous recombination and/or DNA replication.

1. Introduction

The ability to maintain genome integrity is fundamental to all living organisms. To achieve this, several families of proteins have evolved in eukaryotes to promote effective detection and repair of DNA damage. Among these, structural maintenance of chromosomes (SMC) family of proteins forms a unique group that acts at diverse levels to ensure chromosome homeostasis (reviewed in [1,2]). Inactivation of any SMC family member leads to genome instability and lethality, a phenotype that illustrates the critical importance of SMC function for cellular vitality.

There are six conserved SMC family members in eukaryotes, Smc1–6. These are involved in diverse processes including chromosome condensation, sister-chromatid cohesion, and repair of DNA lesions [1]. How these proteins promote such distinct functions is unclear at present. Insights into the mode-of-action of SMC family proteins are suggested by their unique organization as well as their pattern of association with other proteins. Indeed, as they acquire their mature configuration, SMC proteins are known to fold into highly elongated structures containing an asymmetrically-localized ATPase activity [3,4]. Electron microscopy experiments revealed that the resulting SMC "rod" can spend as much as 60 nm in length and shows limited flexibility. In addition to these physical properties, SMC proteins act as dimers with distinct and predetermined SMC family members [1]. Specifically, Smc1 and Smc3 associate to form a complex known as cohesin, whereas binding of Smc2 to Smc4 results in the formation of the condensin complex [1]. Two other SMC proteins initially discovered in fission yeast [5,6] – Smc5 and Smc6 – are now known to form a novel and evolutionarily-conserved DNA repair complex in eukaryotes (reviewed in [2,7,8]). Ultimately, the three conserved pairs of Smc dimers associate with several additional proteins to form large complexes with multiple biochemical activities.

It has been proposed that the combination of unique structural configuration together with the presence of an asymmetric ATPase domain results in mechano-chemical activities in SMC proteins. Moreover, the ability of cohesin and condensin to create topological links in chromosomal DNA [9,10] suggests that the molecular function of SMC complexes is to hold two distinct DNA molecules (or distant regions of a specific chromosome) in close proximity. How SMC complexes first identify and then bind to various chromosomal sites *in vivo* is less clear. In particular, little is known about the nature of the mechanism used by the Smc5–6 complex to interact with DNA substrates in live cells [11–14]. Furthermore, the fact that the Smc5–6 complex is involved in DNA double-strand break (DSB) repair may impose special requirements on its interaction with DNA substrates. Indeed, it has been demonstrated that association of SMC complexes to DNA molecules through topological linkages cannot be maintained in the presence of DSBs [9]. The fact that the Smc5–6 complex must act in proximity to DNA lesions, including

Abbreviations: CV, column volume; DSB, DNA double-strand break; EMSA, electrophoretic mobility shift assay; HST, 9xHIS-3xStreptagII tag; SMC, Structural maintenance of chromosomes; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA.

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DSBs, suggests that the association of this complex with chromosomal DNA may depend on alternative DNA-binding mechanisms relative to condensin and cohesin. In this regard, it is interesting to note that, unlike other SMC proteins, Smc5 and Smc6 appear to work independently of each other under certain circumstances *in vivo* [15].

To better understand how the Smc5–6 complex might regulate the repair of chromosomal DNA, we previously undertook an extensive biochemical analysis of purified Smc5 [16]. We now report the first biochemical characterization of the DNA-binding activity of Smc6, the core binding partner of Smc5.

2. Materials and methods

2.1. Yeast strains and molecular biology

All yeast strains used in this study are derivatives of W303. Standard procedures were used for yeast culture, genetic manipulations and molecular biology [17]. For protein overexpression, *SMC6* coding sequence was fused at its N-terminus to a tandem affinity purification tag (9x His-3x Streptag II; hereafter referred to as the HST tag) and subcloned downstream of the *GAL1* promoter in a 2μ-derived plasmid (p718).

2.2. Protein overexpression and purification

Smc6 was overexpressed in yeast strain D2131 (*Mat**a** lys2::Pgal1-GAL4 pep4::HIS3 bar1::hisG* p718 [2μ *URA3 leu2-d* P_{GAL1}*-HST-SMC6*]). Purification of Smc6 by Ni–NTA and Streptactin chromatography was performed according to previously-described procedures [16]. Following Streptactin chromatography, the eluate was diluted 15-fold with buffer A (50 mM KH_2PO_4/K_2HPO_4 pH 6.4, 5% glycerol, 0.2% Tween 20, 1 mM EDTA, 2 mM 2-mercaptoethanol) and loaded on a HiTrap SP FF column (GE Healthcare). The column was washed with 10 CV of buffer B (50 mM KH_2PO_4/K_2HPO_4 pH 6.4, 50 mM NaCl, 15% glycerol, 0.2% Tween 20, 1 mM EDTA, 2 mM 2-mercaptoethanol) and eluted with 5 CV of buffer C (50 mM KH_2PO_4/K_2HPO_4 pH 8.0, 1 M NaCl, 15% glycerol, 0.2% Tween 20, 1 mM EDTA, 2 mM 2-mercaptoethanol). Final fractions containing Smc6 were concentrated to ~0.5 mg/mL and stored at −80 °C.

2.3. DNA binding and pelleting assays

All DNA binding assays were performed according to Roy et al. [16] with minor modifications. Specifically, DNA binding experiments using oligonucleotide templates were performed in reaction buffer containing 10 mM Hepes pH 7.5, 75 mM NaCl, 7 mM $MgCl_2$, 2 mM 2-mercaptoethanol, and 7.5 pmol DNA oligonucleotides. The sequences of the 75-mer oligonucleotides used in EMSA experiments are: oligo1635: 5′-6-carboxyfluorescein (FAM)-CCAGTGAATTGTAATACGACTCACTATAGGGCGAATTGGAGCTCCACCGCGGTGGCGGCCGCCGGCGCACCTGTC-3′; oligo1636: 5′-GACAGGTGCGCCGGCGGCCGCCACCGCGGTGGAGCTCCAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGG-3′. All other substrates and conditions are as described in [16]. Pelleting assays were performed as before [16] except that DNA was omitted from the reaction mixture and Smc6 was visualized by Western-blotting using an anti-StreptagII antibody (Qiagen).

3. Results

3.1. Purification of Smc6

To characterize the biochemical properties of Smc6, we first needed to purify large amounts of the protein. Although small fragments of eukaryotic Smc6 have been successfully purified from bacteria in the past [18,19], large amounts of the full-length protein have never been purified from any organism so far. Protein overexpression is likely necessary for this because Smc6 is normally expressed at low levels – 339 copies per cell – in yeast [20]. Initial tests demonstrated that addition of an affinity tag at the carboxy terminus of Smc6 was ineffective for purification because it reduced the solubility of the protein (data not shown). We therefore fused the amino-terminus of Smc6 to the HST tag sequence, a short tandem affinity tag for purification. This tag allows for rapid and effective two-step purification of overexpressed proteins in yeast [16,17]. Strains carrying the HST-tagged version of *SMC6* at its endogenous locus were viable and grew well (Fig. 1A; compare with *smc6–56* mutant), which indicates that the tagged protein is functional. Importantly, expression of *HST-SMC6* from a multicopy plasmid resulted in high levels of soluble Smc6 in yeast lysates. The amino-terminal tag on Smc6 was both accessible and effective for purification of the soluble fraction of Smc6. Indeed, we could purify Smc6 to more than 95% homogeneity when using consecutive steps of metal-chelate and streptactin chromatography, followed by an additional step of ion exchange chromatography (Fig. 1B). Using this procedure, we obtained a typical yield of ~0.32 nmol of pure Smc6 per 6 L of culture. This purification yield is modest compared to the yield obtained previously with Smc5 [16], but is nevertheless sufficient to conduct key biochemical assays. Interestingly, although the calculated molecular weight of Smc6 is very similar to that of Smc5 (128 vs 126 kDa, respectively), both proteins migrated at distinct positions during SDS–PAGE (Fig. 1C). This allowed us to confirm that the purified Smc6 fraction was largely devoid of contamination from Smc5, which is consistent with the fact that the purification was performed under ionic conditions that are unlikely to be compatible with complex formation.

3.2. DNA-binding activity of Smc6

We next determined the specific contribution of Smc6 to the overall DNA-binding properties of the Smc5–6 complex. As an essential DNA repair factor, Smc6 is expected to interact with DNA and/or chromatin during the execution of its functions. However, we previously observed that Smc6-binding partner, Smc5, has strong DNA-binding activity in the absence of all other components of the complex [16]. This raises the possibility that Smc5 might be sufficient on its own to mediate all DNA-binding activity within the Smc5–6 complex. Alternatively, Smc6 could contribute to the overall DNA-binding activity of the complex by providing a separate high-affinity interaction site for DNA.

To discriminate between these two possibilities, we investigated the putative DNA-binding activity of purified Smc6 using electrophoretic mobility shift assays (EMSAs). Specifically, various concentrations of Smc6 were allowed to interact with a set amount of DNA molecules and the resulting complexes were separated by agarose gel electrophoresis [16]. Interactions between proteins and DNA in this assay typically cause a retardation in the migration of DNA molecules in the gel. We tested the interaction between Smc6 and DNA substrates of various configurations, including circular dsDNA plasmids, linear dsDNA fragments, and circular ssDNA plasmids. Remarkably, these EMSA experiments revealed that Smc6 interacts quantitatively with all types of DNA molecules tested (Fig. 2A). Specifically, increasing the amount of Smc6 in DNA-binding reactions resulted in a concentration-dependent formation of protein–DNA complexes that had reduced mobility during electrophoresis (i.e., see DNA signal next to asterisk in Fig. 2A). The low mobility of Smc6–DNA complexes in gel is a general characteristic observed with a large number of SMC proteins, as previously noted (see [21] and references cited therein). This behavior could be ex-