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Interaction Mapping between *Saccharomyces cerevisiae* Smc5 and SUMO E3 Ligase Mms21

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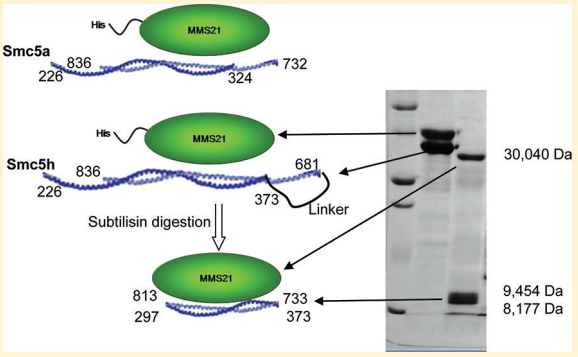
ABSTRACT: The multisubunit Smc5–Smc6 holocomplex (Smc5/6) plays a critical role in chromosome stability maintenance, DNA replication, homologous recombination, and double-stranded DNA damage repair. Smc5 and Smc6 form the core of the holocomplex, along with six non-SMC elements, for which most functions are not yet understood. Mms21 (Nse2), the relatively well-studied subunit in Smc5/6, contains a SP-like-RING finger motif on the C-terminus and was identified as a SUMO E3 ligase. Deletion of Mms21 is lethal; however, while deficient in DNA damage repair, SUMO ligase mutants remain viable. These functions of Mms21 in Smc5/6 are hard to address without understanding the interaction between Smc5 and Mms21. Previously, we systematically examined the architecture of *Saccharomyces cerevisiae* Smc5/6 and, using yeast two-hybrid methods, found that Mms21 interacts with the coiled-coil of Smc5. Later, crystallographic studies revealed the molecular arrangement of Mms21 with Smc5/6. For this study, we use a combination of limited proteolysis, mass spectrometry, and N-terminal sequencing to precisely define the interaction region of Smc5 with Mms21. In addition, using isothermal titration calorimetry, we find that Mms21 interacts with Smc5 in a 1:1 ratio with a K_d of 0.68 μM. This combination of methods would be useful in examining the structure of any large multiprotein complex.



The structural maintenance of chromosome (SMC) protein complexes, including cohesin, condensin, and the Smc5–Smc6 complex (Smc5/6), is crucial for chromosome metabolism.[1−4] Compared to cohesin and condensin, Smc5/6 is the most complicated[2] and contains the most subunits: Smc5, Smc6, and six non-SMC elements (NSEs), including Nse1, Mms21 (Nse2), and Nse3–Nse6.[5−12] Smc5/6 is critical in multiple-chromosome processes, such as efficient homologous recombination, collapsed replication fork restart, DNA double-strand break repair, and telomere and rDNA maintenance.[9,13−20] Defects in Smc5/6 result in increased DNA sensitivity to damage, while in ALT cancer cells, its activity is required for the maintenance of telomere length.[18]

Smc5 and Smc6 form the heterodimeric core of Smc5/6. Similar to all members of the SMC family, Smc5 and Smc6 are large proteins organized into five domains: an N-terminal head domain, a long helical region, a hinge domain, another long helical region, and a C-terminal head domain (Figure 1A, left). The N- and C-terminal head domains associate to form a globular head. The two long helices associate to form the coiled-coil arm. The hinge domain from Smc5 associates with that from Smc6 to form the Smc5–Smc6 heterodimer[3,21] (Figure 1A, right). Studies of cohesin and condensin complexes show that all the non-SMC molecules are located close to the head region of the SMC heterodimer.[3] Smc5/6 *Saccharomyces cerevisiae*, however, has the Nse5–Nse6 subcomplex that localizes to the hinge region of the holocomplex.[22] A similarly named subcomplex exists in *Schizosaccharomyces pombe*; however, those proteins localize to the head region and are not similar at the primary sequence level.[8] The most interesting and relatively well-studied subunit, Mms21, was found to associate with the coiled-coil arm of Smc5.[10,22]

Mms21, with an SP-like-RING finger motif on its C-terminus, is a SUMO E3 ligase. Mutational studies suggest that the sumoylation via Mms21 mediates the participation of Smc5/6 in DNA damage repair[5,10,12,23] and telomeric recombination.[18] Sumoylation is a three-step enzymatic process requiring three sets of enzymes: E1, a SUMO activation enzyme; E2, a conjugation enzyme; and E3, a SUMO ligase.[24] In *S. cerevisiae*, the E1 function is performed by the Uba2–Aos1 complex and the E2 function by Ubc9.[25,26] The function of SUMO E3 ligase is to recognize activated E2 conjugase as well as the targeting substrates.[27] In Smc5/6, Smc5 has been identified as a substrate of Mms21; in addition, Mms21 has other substrates such as YKu70, Smc6, and Shelterin.[5,12,23,28] Interestingly, deletion of full-length Mms21 leads to lethality for both budding yeast and fission yeast, while mutations to inactivate the signature motif affect the DNA damage response[5,12,23] and reduce the growth rate in unstressed cells.[29] This suggests that in addition to the SUMO E3 ligase function, Mms21 has another unknown role that is important for cell viability.

Because of its central role in Smc5/6 function, Mms21 has been intensively studied. Studies in *S. pombe* and in *S. cerevisiae* localize Mms21 to the coiled-coil arm of Smc5.[10,22] In our previous study of the architecture of *S. cerevisiae* Smc5/6 that

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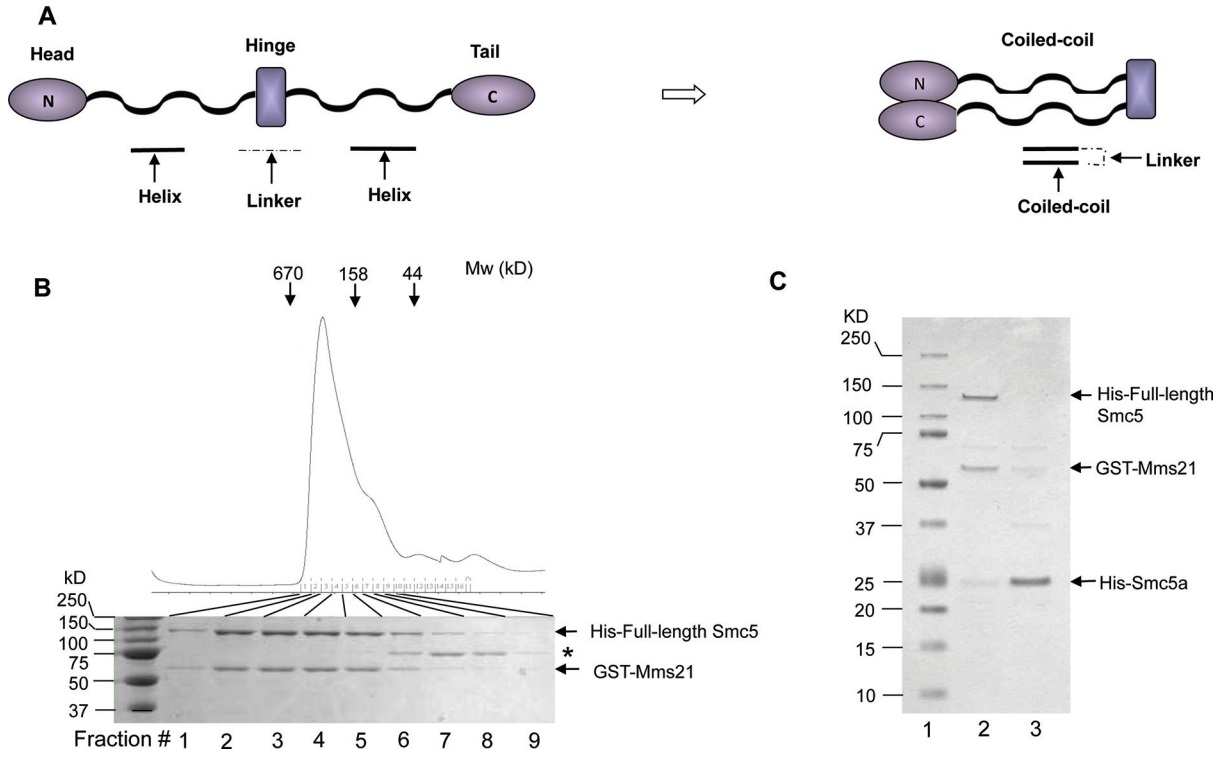

Figure 1. Full-length Smc5 and Mms21 form a tight complex. (A) Schematic representation of Smc5 containing five domains (left). The N- and C-terminal domains associate together (right). The chimeric protein constructs used in these studies were built up by connecting the two segments together as shown. (B) Gel filtration profile showing that the coexpressed and purified full-length Smc5–Mms21 complexes comigrate together (top). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis shows the fractions from gel filtration. The bottom band with a molecular mass of approximately 60 kDa is GST-Mms21, and the top band is full-length Smc5. * denotes a non-specific binding protein. (C) The affinity of the construct Smc5a (residues 226–323 linked with residues 731–836, lane 3) for Mms21 is much weaker than that of full-length Smc5 (lane 2).

Table 1. Diagram of the Different Regions of Smc5 Constructs Used To Identify the Mms21–Smc5 Complex-Binding Site

Constructs	Amino acid	Interaction	Constructs position on Smc5
Smc5	M1-D1093	Yes	
Smc5a	Q226-K323 & Q731-E836	No	
Smc5h	Q226-I373 & S681-E836	Yes	
Smc5j	L180-K323 & Q731-A886	No	
Smc5m	K303-I373 & S681-I751	No	
Smc5x	D302-L369+K733-Q813	Yes	

aimed to determine the location of Mms21, we found Mms21 localized on the coiled-coil arm of Smc5 using the yeast two-hybrid system.[22] In that study, we used a full-length Mms21 as bait, and neither the head nor the hinge of Smc5 was able to bind to Mms21; only the piece containing the coiled-coil arm was able to interact. In the studies we present here, we have used a combination of methods, including various reconstituted chimeric constructs of Smc5, subtilisin digestion, N-terminal sequencing, and mass spectrometry, to precisely identify the Mms21 interaction region on Smc5. The binding ability of this identified Smc5 region is similar to that of full-length Smc5. The strategy we used here not only lays the foundation for further studies of the Mms21–Smc5 subcomplex but also provides an approach for studying other protein complex interactions.

MATERIALS AND METHODS

Plasmid Constructs. Full-length Smc5 and Mms21 DNA sequences were amplified by polymerase chain reaction (PCR) from *S. cerevisiae* chromosomal DNA and verified by DNA sequencing. Full-length Smc5 and segments of Smc5 were cloned into the pET24D vector (Novagen) (Table 1). A short flexible linker with repetitive amino acids of Gly-Ser at various lengths was inserted in the middle of two segments of Smc5, which linked them together to form a chimeric protein. The schematic representation of how to construct the chimeric proteins is shown in Figure 1A. Full-length Mms21 was cloned into the pGEX4T3 vector (GE Healthcare).

Protein Expression and Purification. All Smc5 and Mms21 proteins were expressed in Rosetta BL21(DE3) cells

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(Novagen). The cells for six-His-tagged proteins were grown at 37 °C to an OD_{600} of 0.5 and induced with 0.4 mM isopropyl β-thiogalactopyranoside (IPTG) for 16 h at 20 °C. The cells for the GST-tagged protein were grown at 37 °C to an OD_{600} of 0.5 and induced with 0.4 mM IPTG for 4 h at 37 °C. The cells were harvested and lysed in buffer containing 100 mM Tris-HCl (pH 8.0) and 200 mM NaCl. The proteins containing the six-His tag were purified by Ni affinity chromatography (Qiagen), and the protein containing the GST tag was purified by glutathione affinity chromatography (GE Healthcare) according to a standard protocol. Superdex 200 gel filtration chromatography (GE Healthcare) was used for further purification with buffer containing 50 mM Tris-HCl (pH 7.5) and 100 mM NaCl.

Mass Spectrometry. Mass spectrometry was conducted at the University of Louisville Mass Spectrometry core facility. The protein sample was digested by subtilisin for 2 h, and the digestion was stopped by addition of phenylmethanesulfonyl fluoride (PMSF) to a final concentration of 1 mM. The digested product was further purified by Superdex 200 gel filtration. The purified product was desalted against H_2O. A 20 μL sample collected from the desalting column was loaded into the mass spectrometry instrument for analysis. The precise molecular mass was measured by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry using a TofSpec 2E MALDI-TOF mass spectrometer (Micromass).

N-Terminal Sequencing. N-Terminal sequencing was performed at the M. D. Anderson Cancer Center core facility (Houston, TX). The protein samples were digested by subtilisin, the reactions stopped by PMSF as described above, and the samples purified by Superdex 200 gel filtration. The fractions collected from gel filtration were separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). Proteins in the SDS−PAGE gel were then electroblotted to a polyvinylidene fluoride (PVDF) membrane and stained with Coomassie blue. After the blotted PVDF membrane had been destained, a large amount of deionized water was used to rinse the membrane thoroughly. The target bands were excised and sequenced with an Applied Biosystems Precise Protein Sequencer using a chemical process based on the technique developed by Edman.[30] Five amino acid residues were identified on each band, and the sites were mapped on full-length Mms21 and Smc5.

Isothermal Titration Calorimetry Experiments. The Microcal VP-ITC instrument (Microcal, Northampton, MA) was used to perform the measurement. All proteins were prepared in 50 mM sodium phosphate buffer (pH 7.5) and 150 mM NaCl. Smc5x was loaded in a syringe as the ligand by its higher solubility and titrated into a sample cell filled with Mms21. A total of 250 μL of Smc5x was injected with 10 μL each into a 1.4 mL sample cell. Twenty-five injections were performed at 4 °C for determining the binding affinity. The heat change resulting from dilution was measured by injecting an Smc5 sample into the buffer that was used to dialyze both Smc5 and Mms21 samples.

RESULTS AND DISCUSSION

Identification of the Mms21-Binding Region on Smc5 with a Similar Binding Affinity for Full-Length Smc5. To find the Mms21-binding site on Smc5, we first examined the binding affinity between full-length Smc5 and Mms21. The coding region of Mms21 was cloned into a pGEX4T3 vector containing a GST tag with ampicillin resistance, and the full-length coding region of Smc5 was cloned into the pET24D vector with kanamycin resistance. The two proteins were coexpressed in BL21(DE3) cells and purified by Ni-NTA affinity chromatography and gel filtration. The coexpressed full-length Smc5 and GST-Mms21 proteins form a tight complex, shown by the comigrated peak from the gel filtration profile (Figure 1B).

The studies of Smc5/6 in *S. pombe*[10] provided evidence that the binding site for Mms21 is formed by a head-to-tail coiled coil of Smc5 at amino acids 226−324 with amino acids 731−836. In searching for the Mms21-binding site on *S. cerevisiae*, we first tested whether Mms21 shares similar binding sites with Smc5 in *S. cerevisiae* and *S. pombe*. To create and test such a construct, we used a flexible 10-amino acid spacer, Gly-Ser-Gly-Ser-Gly-Ser-Gly-Ser-Gly-Ser, to covalently link the two helical segments containing residues 226−324 and 731−836 of Smc5 and named it construct "Smc5a". To compare the binding affinity of Mms21 for Smc5a and full-length Smc5, both Smc5a and full-length Smc5 were cloned as His-tagged fusion proteins while Mms21 was cloned as a GST-tagged protein. The proteins were expressed separately. Ni-NTA affinity resin was used for the pull-down assay (Figure 1C). Lane 2 shows that Mms21 binds strongly to full-length Smc5. However, the same amount of Smc5a only pulls down a faint band of Mms21 (lane 3). This indicates that some affinity might exist between Smc5a and Mms21 but that it is dramatically weaker than that of full-length Smc5 with Mms21.

In an effort to identify the Mms21-binding region of Smc5 that interacts with Mms21 with a higher affinity, or with an affinity similar to that of full-length Smc5, we created a series of Smc5 constructs containing various domains of Smc5. Table 1 shows a diagram of these different constructs. Weak affinity similar to that of Smc5a is considered "no binding" in Table 1.

GST-Mms21 and various Smc5 constructs were expressed separately and copurified. Ni-NTA affinity resin was used to pull down Smc5 in a manner similar to that used for Smc5 and Smc5a. The association of Smc5 with Mms21 was evaluated by SDS−PAGE. Figure 2A shows the selected constructs used to test the binding ability with Mms21 via the Ni-NTA pull-down assay. Our results indicate that construct Smc5h, which contains amino acid residues 226−373 and 681−836, has the highest affinity for Mms21 among all the constructs. This construct shows binding affinity for Mms21 similar to that of full-length Smc5. Gel filtration of these two proteins from copurification showed a comigrated peak (Figure 2B). Deletions of 50 residues from the ends of each fragment on this coiled-coil construct (Smc5a and Smc5j) severely disrupted the interaction with Mms21, implying that the end residues of the construct are important for binding.

The Minimal Mms21−Smc5 Complex Identified by Limited Proteolysis. To further narrow the Mms21-binding site on Smc5, we reasoned that the region of Smc5 that forms a complex with Mms21 might afford structural resistance to enzymatic digestion. To test this hypothesis, we digested the complex with subtilisin. The original concentration for subtilisin was 1 mg/mL, and it was diluted into 1:10, 1:100, 1:1000, and 1:10000 solutions. Each 1 μL sample of original and diluted subtilisin was incubated with 100 μL of 1 mg/mL proteins. The subtilisin was incubated with either Smc5 or Mms21 alone or with the complex for 2 h at 4 °C. The enzymatic reaction was stopped by addition of PMSF along with SDS loading buffer. After boiling for 5 min, samples were separated by SDS−PAGE. Mms21 (Figure 3A) or Smc5 alone (not shown) was completely digested under these conditions. However, two resistant bands

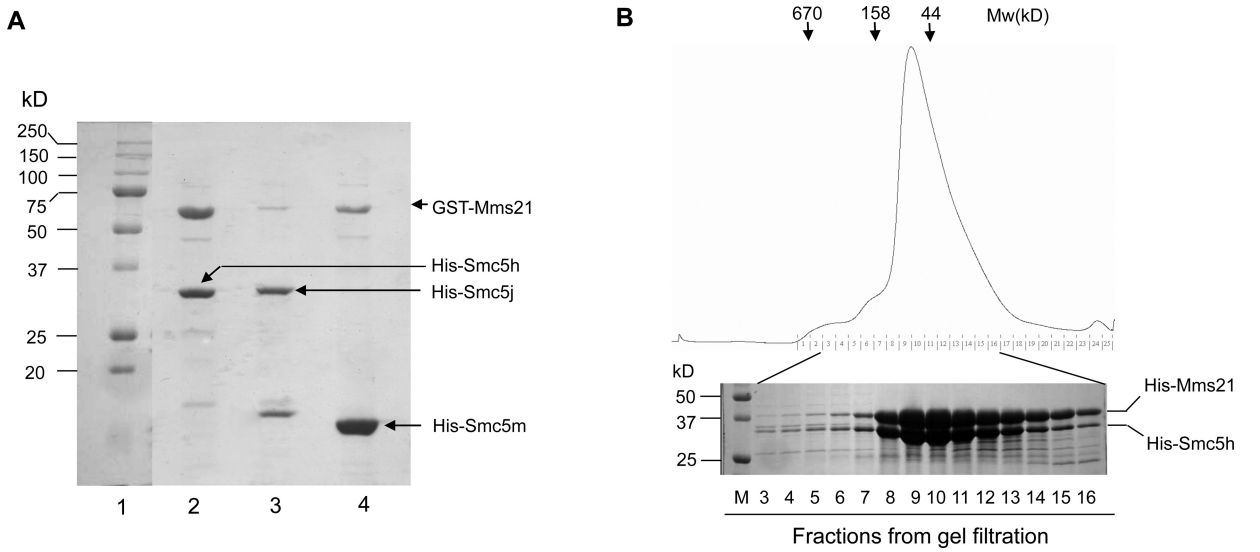

Figure 2. Searching for regions on Smc5 that have high affinity for Mms21. (A) SDS–PAGE gel from the pull-down study shows the interaction between Mms21 and the selective Smc5 constructs. Construct Smc5h has the highest binding affinity for Mms21. (B) Copurification of Smc5h and Mms21. Gel filtration profile (top) and SDS–PAGE gel (bottom) showing that the two proteins comigrate together.

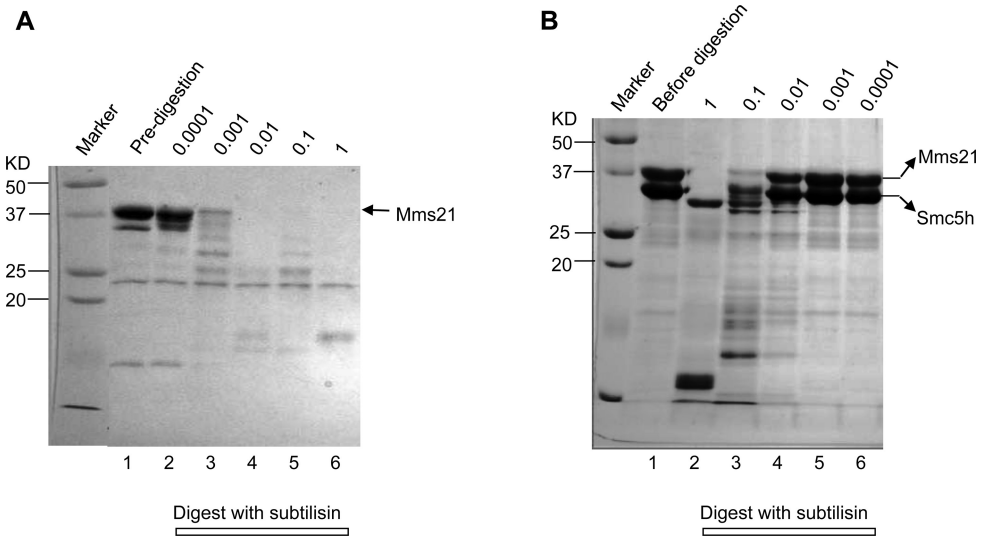

Figure 3. Enzymatic digestion to identify the core interacting complex between Smc5 and Mms21. (A) Mms21 alone is more easily digested by subtilisin. SDS–PAGE shows the digestion of Mms21. Lane 1 shows Mms21 before digestion, while lanes 2–6 show digestion products with progressively increasing concentrations of subtilisin: 1, 0.1, 0.01, 0.001, and 0.0001 mg/mL, respectively. One microliter of subtilisin was mixed individually with 100 μL of 1 mg/mL Mms21 protein. (B) SDS–PAGE shows the digestion of the Smc5–Mms21 complex. Lane 1 shows the Mms21–Smc5 complex before digestion, while lanes 2–6 show digestion products with progressively decreasing concentrations of subtilisin. The concentrations were the same as in panel A.

were evident following digestion of the complex (Figure 3B). Comparison with the molecular mass marker shows the top band is ~30 kDa and that the bottom smeared band is ~9 kDa.

The digested products were subjected to N-terminal sequencing, to identify the beginning residues of each protein, and to mass spectrometry, to determine the precise molecular mass of each digested component. N-Terminal sequencing of the larger digestion product was unambiguous, identifying five residues from the top band that were matched to Mms21 sequence. Mass spectrometry analysis showed that the top band has a molecular mass of 30040 Da (Figure 4A). The results from these two methods led to the conclusion that aside from the N-terminal six-His-tag Mms21 had undergone a deletion of only two residues from its N-terminus during the digestion. However, N-terminal sequencing of the 9 kDa band indicated multiple digestion products. The data gave four top possibilities for each residue (Table 2), indicating a mixture of amino acid sequences. Mass spectrometry showed the two highest peaks for the lower band as 8177 and 9454 Da (Figure 4B). After analysis of the construct sequence of Smc5h in combination with the information obtained from N-terminal sequencing and mass spectrometry, we concluded that the resistant fragments of Smc5 consisted of residues 302–369 and 733–813. The 10-amino acid spacer, designed to connect the two helices of Smc5, was digested, leaving two fragments of ~8 and 9 kDa. After identifying the Mms21-binding site on Smc5, we next created a construct containing Smc5 residues 302–369 and 733–833 and examined the Mms21 binding ability using a Ni-NTA

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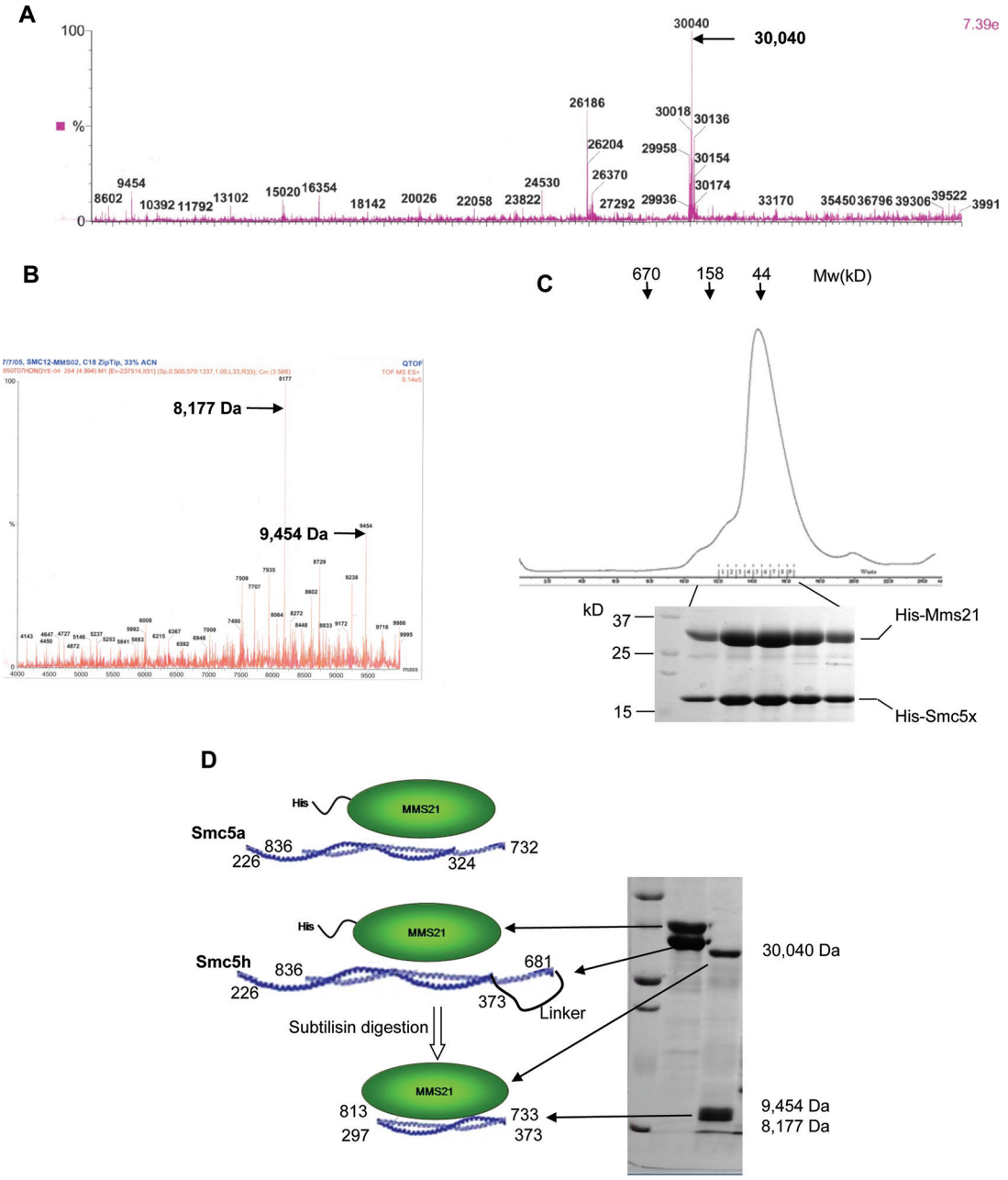

Figure 4. Analysis of the molecular mass of the digested product from the Mms21−Smc5 complex. (A) Mass spectrometry shows a molecular mass of 30040 Da from the top band of the digested product. (B) The two highest peaks indicate molecular masses of 8177 and 9454 Da from the lower smearing band of the digested product. (C) Copurification of Smc5x and Mms21. Gel filtration profile (top) and SDS−PAGE gel (bottom) show the two proteins comigrate together. (D) Model of Mms21 interacting with the coiled-coil region of Smc5: fragment of Smc5a, which has a much weaker affinity than full-length Smc5, fragments of Smc5h identified by our studies, and a tight complex that was the result of subtilisin digestion.

Table 2. N-Terminal Sequencing Identifies the Top Possibilities of the First Five Residues from the Bottom Band That Resulted from the Subtilisin Digestion of the Smc5h−Mms21 Complex

residue number	amino acid	pmol	amino acid	pmol	amino acid	pmol	amino acid	pmol
1	Leu	38.3	Ala	33.6	Asp	22.7	Lys	14.5
2	Arg	62.5	Ile	31.7	Lys	23.9	Glu	7.6
3	Lys	32.3	Leu	31.1	Ala	25.9	Glu	13.2
4	Lys	29.5	Ile	22.2	Pro	16.9	Ala	13.2
5	Asp	28.7	Leu	26.5	Phe	17.1	Arg	15.3

pull-down assay. The result confirmed that binding was similar to that of full-length Smc5. When copurified, the two proteins coeluted via gel filtration (Figure 4C).

Quantitative Measurement of the Affinity between Mms21 and Smc5 Using Isothermal Titration Calorimetry (ITC). To explore the interaction between Mms21 and

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Smc5 after identifying the Mms21-binding site on Smc5, we next determined the affinity between Mms21 and Smc5 using ITC. A recombinant preparation of the Mms21-binding domain on Smc5 containing amino acids 302−369 and 733−813 linked by a relatively shorter linker of four amino residues (Gly-Ser-Gly-Ser, named Smc5x) was expressed and purified to homogeneity. The short linker was designed on the basis of the assumption that the two ends stay close together. Because Smc5x has better solubility, it was chosen to be injected into the cell. Exactly 10 μL of Smc5x in the sample syringe was injected into 1.4 mL of Mms21 in the cell and equilibrated at 300 rpm with 120 s intervals. The heat of Smc5x dilution was measured by titrating Smc5x into buffer. A total of 25 injections were performed, and the heat released during the equilibration of Smc5x into Mms21 agrees well with ideal binding for a heterodimer (Figure 5).

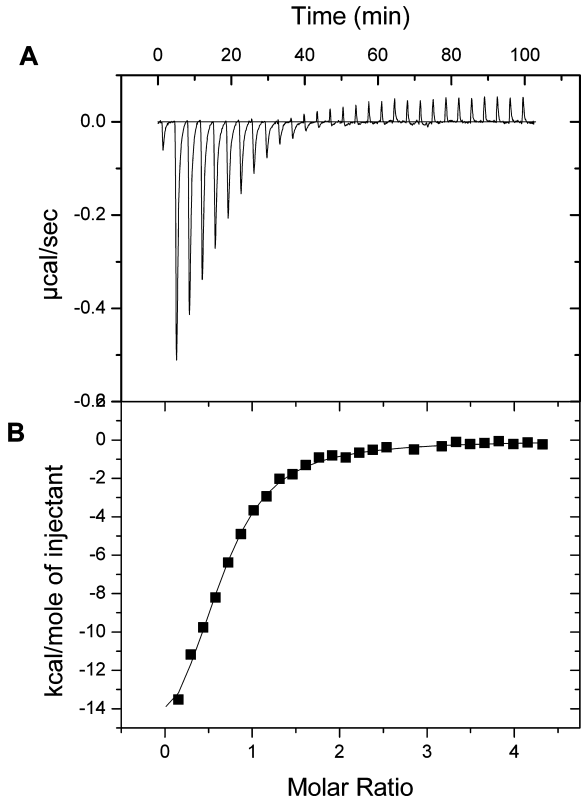

Figure 5. ITC for analyzing the affinity between Mms21 and Smc5. (A) Representative titration data analysis. Smc5x was titrated into Mms21. A total of 25 injections were performed. (B) Data were subtracted with the dilution data and fit into ORIGIN to yield ΔH and K_a.

The data were fitted with ORIGIN provided by the Microcal facility. The experiment directly measured enthalpy (ΔH) and binding affinity (K_a). The remaining thermodynamic parameters, including the binding free energy (ΔG) and the entropy (ΔS), were calculated using the following relationship.

$$\Delta G = -RT \ln K_a = \Delta H - T\Delta S$$

where R is the gas constant and T is the absolute temperature. The dissociation constant (K_d) at 4 °C was 0.68 μM from the average of two measurements.

To improve our understanding of the interaction between Mms21 and Smc5, we analyzed the stoichiometry between the two proteins. First, we examined the gel filtration profile of full-length Smc5 with Mms21. The SDS−PAGE gel showed an approximate 1:1 ratio. We also reran SDS−PAGE with construct Smc5x with which we studied the affinity by ITC, which showed a 1:1 ratio similar to that of full-length Smc5. In addition, gel filtration from Superdex 200 showed the molecular mass of the complex was ~50 kDa, which is consistent for one Mms21 and one Mms21-binding region of Smc5. From characterization of the interaction between Mms21 and Smc5x by ITC, the heat released during the titration of substrate Smc5x into the Mms21 solution exhibited excellent agreement with ideal binding by fitting as one-site binding (Figure 5A), giving additional evidence of the presence of a single type of binding site for the Smc5−Mms21 interaction. Therefore, we concluded that the interaction between Smc5 and Mms21 has a 1:1 stoichiometry.

Mms21 plays a critical role in Smc5/6, not only for responding to DNA damage repair but also for DNA replication and recombination. As such, cellular viability is highly dependent on Mms21. Disruption of the SUMO ligase activity of Mms21 slows growth and increases DNA sensitivity to damage,[5,29] but cells remain viable. Δ*mms21* is lethal in yeast, suggesting another role for the protein, most likely within Smc5/6. In addition to Smc5, Mms21 binds other members of Smc5/6 (Nse3−Nse5)[31,32] as well as Mph1 (unpublished observation). This suggests the essential function of Mms21 within the complex is structural in nature. Here, we have defined the Mms21-binding site on Smc5, determined the affinity, and characterized their thermodynamic interaction, which provide insight into the functional study of Smc5/6. The agreement of these results with the crystallographic data demonstrates the usefulness of the combined approach we used to study this multiprotein complex.

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ABBREVIATIONS

SMC, structural maintenance of chromosome; Ni-NTA, nickel-nitrilotriacetic acid; GST, glutathione *S*-transferase; SUMO, small ubiquitin-like modifier.

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