The MukF subunit of *Escherichia coli* condensin: architecture and functional relationship to kleisins

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The *Escherichia coli* MukB, MukE, and MukF proteins form a bacterial condensin (MukBEF) that contributes to chromosome management by compacting DNA. MukB is an ATPase and DNA-binding protein of the SMC superfamily; however, the structure and function of non-SMC components, such as MukF, have been less forthcoming. Here, we report the crystal structure of the N-terminal 287 amino acids of MukF at 2.9 Å resolution. This region folds into a winged-helix domain and an extended coiled-coil domain that self-associate to form a stable, doubly domain-swapped dimer. Protein dissection and affinity purification data demonstrate that the region of MukF C-terminal to this fragment binds to MukE and MukB. Our findings, together with sequence analyses, indicate that MukF is a kleisin subunit for *E. coli* condensin and suggest a means by which it may organize the MukBEF assembly.

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Introduction

The appropriate organization and condensation of chromosomes is crucial to all cells. The systematic folding of extended DNA molecules into compact structures protects chromosomes from entanglements that can lead to DNA breakage during segregation and helps cells partition their genomes efficiently and evenly into two daughter cells (Koshland and Strunnikov, 1996; Hirano, 1999, 2000, 2002). Throughout the cell cycle, chromosomes are maintained in a highly organized state, although the degree of compaction varies from organism to organism. For instance, bacteria compress their DNA to approximately 1/1000th of its theoretical free, random-coil size, while eukaryotes compact their chromosomes nearly 100 000-fold (Trun and Marko, 1998; Holmes and Cozzarelli, 2000).

Although chromosome complexity and organization vary enormously among different domains of life, virtually all cells

rely in part on a multiprotein complex known as condensin to achieve chromosome compaction (Koshland and Strunnikov, 1996; Hirano, 2002). The ability to modulate DNA supercoiling appears to be one facet of condensin function. Direct evidence for supercoiling by condensin was first noted in *Xenopus* extracts (Kimura and Hirano, 1997; Kimura *et al.*, 1999). Researchers have since observed that the net superhelical densities of plasmid and chromosomal DNAs also change in condensin-deficient strains of *Bacillus subtilis* and *Escherichia coli* (Weitao *et al.*, 2000; Lindow *et al.*, 2002). In *E. coli*, genetic studies have demonstrated that certain condensin mutants can suppress the phenotypes of other mutations that increase DNA supercoiling levels inside cells (Weitao *et al.*, 1999; Sawitzke and Austin, 2000).

In eukaryotes, the condensin complex consists of an SMC (structural maintenance of chromosomes) heterodimer (XCAP-C/Smc2 and XCAP-E/Smc4) and three non-SMC subunits (XCAP-D2, XCAP-G, and XCAP-H/barren) (Hirano *et al.*, 1997; Sutani *et al.*, 1999; Lavoie *et al.*, 2000; Hirano, 2002). A striking structural characteristic of SMC monomers is that they possess two globular domains connected by an extended coiled-coil segment (Niki *et al.*, 1991; Hirano and Mitchison, 1994; Melby *et al.*, 1998; Anderson *et al.*, 2002; Yoshimura *et al.*, 2002). A globular 'hinge' disrupts the central helical region and reverses the orientation of the polypeptide chain (Haering *et al.*, 2002), creating an antiparallel coiled coil and juxtaposing the N- and C-terminal regions to form a bipartite domain of the ABC superfamily of ATPases at one end of the protein. Although the ATPase domains can dimerize in the presence of nucleotide, SMC proteins principally form dimers through the association of hinge regions (Niki *et al.*, 1992; Melby *et al.*, 1998; Hopfner *et al.*, 2000; Hirano *et al.*, 2001; Haering *et al.*, 2002; Hirano, 2002; Hopfner and Tainer, 2003).

In contrast to eukaryotes, bacteria and archaea rely on a three-subunit condensin complex (Yamanaka *et al.*, 1996; Mascarenhas *et al.*, 2002; Soppa *et al.*, 2002). Most prokaryotes employ an SMC homodimer along with two non-SMC proteins, ScpA and ScpB, although clear ScpB homologs do not appear to be present in all species (Mascarenhas *et al.*, 2002). By contrast, *E. coli* and other members of the γ -proteobacter family use the MukB, MukE, and MukF and proteins for chromosome condensation (Niki *et al.*, 1991, 1992; Yamanaka *et al.*, 1996). Although MukB is a distant relative of the SMC protein family, the architecture and evolutionary lineage of the accessory MukE and MukF subunits have not been determined (Niki *et al.*, 1992; Melby *et al.*, 1998; van den Ent *et al.*, 1999; Cobbe and Heck, 2004).

Deletion of accessory condensin subunits causes defects similar to those of SMC knockouts in all systems studied to date (Bhat *et al.*, 1996; Yamanaka *et al.*, 1996; Sutani *et al.*, 1999; Lavoie *et al.*, 2000; Mascarenhas *et al.*, 2002). In bacteria, the phenotype of null mutations includes diffuse nucleoids, a high incidence of anucleate cells, elongated or filamentous cells, and an inability to form colonies at temperatures above 30°C (Niki *et al.*, 1991; Yamanaka *et al.*, 1996;

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Britton *et al*, 1998; Mascarenhas *et al*, 2002; Jensen and Shapiro, 2003; Volkov *et al*, 2003). Nonetheless, the precise biochemical functions of the non-SMC components of condensins are still poorly understood. In general, these subunits bear little or no homology to other proteins, although HEAT repeats have been identified in eukaryotic XCAP-D2 and XCAP-G (Neuwald and Hirano, 2000). More recently, phylogenetic analyses have indicated that at least one non-SMC subunit is conserved across many diverse complexes that employ SMC proteins. These factors, known as 'kleisins', include bacterial ScpA, eukaryotic condensin XCAP-H (barren), Rec8, and Scc1/Mcd1/Rad21 (Schleiffer *et al*, 2003). Of these, the functional role of Scc1 has been best delineated; in the cohesin complex, it anchors together the ATPase domains of the Smc1/Smc3 heterodimer and is selectively cleaved by the separin protease to initiate anaphase (Uhlmann *et al*, 1999, 2000). Although no Muk subunits have been categorized as kleisin members, Schleiffer *et al* have suggested that *E. coli* MukF might function as a kleisin, since it associates with MukB (Yamanaka *et al*, 1996; Schleiffer *et al*, 2003).

To better understand the structure and function of the MukBEF condensin, as well as the relationship of its non-SMC subunits to those of other condensins, we have carried out a series of structural, informatic, and biochemical studies on the Muk system. We report here the crystal structure of a 33 kDa N-terminal fragment of MukF(1–287), and show that this region forms a doubly domain-swapped dimer of a novel overall architecture. Protein interaction assays reveal that a domain of MukF C-terminal to this dimerization module binds specifically to MukB and MukE, while sequence analyses show that two domains at the extreme N- and C-termini

of MukF are helix–turn–helix (HTH) folds homologous to the extreme termini of kleisin proteins. Together, these data indicate that MukF serves as a kleisin, and highlight the organizational basis by which it links together MukB subunits.

Results

MukF(1–287) monomer structure

Using limited proteolysis and deletion mapping, we identified a stable 33 kDa N-terminal fragment of MukF comprising residues 1–287 of the 440-amino-acid protein. When purified, this fragment crystallized in the space group $P6_1$, with unit cell dimensions $a = b = 58.7 \text{ \AA}$ and $c = 307.5 \text{ \AA}$. Preliminary crystals suffered from perfect merohedral twinning (Yeates and Fam, 1999); however, the addition of divalent metals to crystallization drops, notably Ca^{2+} ions, reduced the fraction of twinned crystals. We solved the structure of MukF(1–287) by multiwavelength anomalous dispersion (MAD) methods and refined it to a resolution of $2.9 \text{ \AA}$ with a free R -value of 27.3% and a working R -factor of 23.3% (Figure 1 and Table I). Despite the presence of Ca^{2+} in the crystallization conditions, and reports that MukF may be a Ca^{2+} -binding protein (Yamazoe *et al*, 1999), we observed no electron density for divalent metals.

Within the crystal, there are two copies of the MukF(1–287) protomer per asymmetric unit. Each adopts a highly helical and extended structure (Figure 1B). The fragment contains nine α -helices and three short β -strands partitioned between two domains. Helix α_1 and strand β_1 form an N-terminal extension that stands apart from the other regions

Figure 1 MukF(1–287) structure. (A) Superposition of MukF(1–287) amino-acid sequence and secondary structure. Helices (cylinders) and strands (arrows) are labeled and colored as follows: N-terminal extension, gold; domain I WHD, red; domain II coiled coil, blue. (B) Ribbon diagram of the MukF(1–287) monomer. Secondary structural labels correspond to those in panel A. (C) Experimentally phased electron density (F_{obs} , $\alpha_{\text{flattened}}$, 1.5σ contour level) for strand β_1 looking down the noncrystallographic two-fold axis and showing the unambiguous separation between protomers. (D) Superposition of WHDs from MukF(1–287) (red) and the C-terminal domain of Scc1 (green) (Haering *et al*, 2004). HTH and wing (W) motifs are labeled. Molecular figures in the paper are rendered with PYMOL (DeLano, 2002).

Table 1 Structure determination statistics

	Remote	Peak
<i>Data collection</i>		
Radiation (Å)	1.1272	0.9797
Resolution (Å) (last shell)	20–2.9 (3.1–2.9)	20–2.9 (3.1–2.9)
Completeness (%) (last shell)	95.5 (93.0)	94.7 (95.5)
R_{sym}^a (%) (last shell)	7.7 (29.2)	8.2 (39.0)
I/σ (last shell)	12.2 (4.2)	11.3 (4.2)
Multiplicity (last shell)	5.3 (4.2)	5.5 (5.4)
<i>Phasing</i>		
No. of sites (identified/total)	8/12	
Mean FOM (SOLVE)	0.47	
<i>Structure refinement^b</i>		
Resolution range (Å)	20–2.9	
No. of protein atoms per dimer	4228	
No. of reflections (overall)	13152	
No. of reflections (test set)	625	
$R_{\text{work}}/R_{\text{free}}^c$ (%)	23.3/27.3	
R.m.s.d.-bonds/angles	0.009 Å/1.14°	
<i>Ramachandran analysis</i>		
Most favored	92.5	
Allowed	6.6	
Generously allowed(%)	0.9	

^a $R_{\text{sym}} = \sum_j \sum_i |I_j - \langle I \rangle| / \sum_j I_j$, where I_j is the intensity measurement for reflection j and $\langle I \rangle$ is the mean intensity for multiply recorded reflections.

^bCoordinates for MukF(1–287) have been deposited in the RCSB database under accession number 1T98.

^c $R_{\text{work, free}} = \sum ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum |F_{\text{obs}}|$, where the working and free R -factors are calculated using the working and free reflection sets, respectively. The free reflections (5.1% of the total) were held aside throughout refinement.

of the protomer and makes no intra-subunit contacts. A six-amino-acid linker region between helices $\alpha 5$ and $\alpha 6$ connects domains I and II.

Domain I spans residues 26–115 and is roughly globular in shape. This region is composed of helices $\alpha 2$ – $\alpha 5$ and strands $\beta 2$ – $\beta 3$. Residues 92–99, which fall between $\beta 2$ and $\beta 3$, were disordered in our electron density maps and are absent from the model. A search of the PDB using DALI and SSM (Holm and Sander, 1996; Krissinel and Henrick, 2004) found an extensive number of proteins similar to domain I. Of these, all top hits corresponded to winged helix domains (WHDs) within nucleic acid-binding proteins or DNA-associated assemblies. Interestingly, the closest spatial match to the MukF N-terminal WHD was the C-terminal domain of Scc1 (top Q-score match from SSM, r.m.s.d. = 1.6 Å over 59 C_α residues; Figure 1D) (Haering *et al*, 2004), a kleisin that binds to the Smc1/Smc3 heterodimer of cohesin.

In WHDs, the second and third helices ($\alpha 3$ and $\alpha 4$ in MukF) form an HTH motif. These elements are immediately followed by two β -strands that comprise the signature wing region ($\beta 2$ – $\beta 3$ in MukF). Typically, the second helix of the HTH motif (often called the recognition helix) and the wing interact with DNA, although other binding modes have also been observed, both to nucleic acids and to other proteins (Gajiwala and Burley, 2000; Mer *et al*, 2000; Haering *et al*, 2004). Despite extensive screens using gel mobility shift, filter-binding, and fluorescence polarization assays, we have detected no association between linear or supercoiled

duplex DNA and either full-length MukF or MukF(1–287) (data not shown), suggesting that the primary purpose of this region may not be to bind nucleic acids.

Domain II is formed by amino acids 122–287, which fold into an extended and slightly bent helical bundle that includes helices $\alpha 6$ – $\alpha 9$. Three of these helices ($\alpha 6$, $\alpha 8$, and $\alpha 9$) contain several glycines that allow them to curve around $\alpha 7$, which is essentially straight except for a slight bend near its N-terminus induced by Pro159. The tertiary organization of secondary structural elements within domain II is such that it forms a three-helix coiled coil at one end and transitions into a four-helix coiled coil at the other. Comparisons of MukF(1–287) to the structural database reveal that this domain is globally similar in structure to numerous highly helical proteins of diverse function. None of these relationships appear to bear significantly on function.

The architecture of domain II provides a structural rationale for the null phenotype of the *mukF233* mutation, which changes Leu233 to proline (Yamanaka *et al*, 1996). Leu233 lies on helix $\alpha 8$ and is buried in the core of the four-helix region of the coiled coil. Substitution of this amino acid by proline would most likely distort $\alpha 8$ and compromise the packing and stability of the domain, either impairing the ability of MukF to accommodate a specific aspect of condensin function or promoting protein misfolding and degradation.

MukF(1–287) dimer structure

In the asymmetric unit of the crystal, two protomers of MukF(1–287) associate with each other to form a dimer of dimensions 70 Å × 85 Å × 55 Å (Figure 2A and B). The shape of the dimer is reminiscent of a ‘skull-and-crossbones’ in which the coiled coils of domain II form an ‘X’ that cradles the globular WHDs. Remarkably, inspection of experimental electron density maps unambiguously revealed that two sets of secondary structural elements are exchanged between the two protomers to form a doubly domain-swapped quaternary arrangement (Figure 2). Helix $\alpha 1$ of each protomer nestles against domain I of its partner subunit, burying a portion of $\alpha 2$ on the opposite monomer and sealing off this region’s hydrophobic core. C-terminal to $\alpha 1$, strand $\beta 1$ extends across the molecular two-fold axis and forms a short, antiparallel sheet with $\beta 1$ of the neighboring protomer. Following domain I, a second swapping event occurs as the polypeptide chain between $\alpha 5$ and $\alpha 6$ winds back around the molecular two-fold axis. Conserved residues Leu24 from $\beta 1$ and Tyr113/Tyr114 from $\alpha 5$ form the hydrophobic domain I•domain I interface. As a consequence, domain I of one protomer abuts domains I and II of its partner, but does not contact its own domain II region. In contrast to the domain I•domain I interface, contacts between domains I and II are largely polar and are mediated between $\alpha 7$ of one subunit and the undersides of strands $\beta 2$ and $\beta 3$ of the other.

The domain-swapped structure we observe for the MukF(1–287) dimer is likely to be the physiological state of the molecule. Previous co-immunoprecipitation and sucrose gradient sedimentation experiments with MukF (Yamazoe *et al*, 1999), as well as our own findings from size-exclusion chromatography and light scattering (data not shown), indicate that MukF forms stable dimers in solution. To further validate this finding for MukF(1–287), we performed analytical ultracentrifugation studies of the purified protein used

Figure 2 MukF(1–287) forms a stable, domain-swapped dimer. **(A)** Stereo diagram of the MukF(1–287) dimer. Protomer A is colored as per Figure 1, while the other subunit is colored gray. **(B)** The MukF(1–287) dimer viewed orthogonally to panel A and looking down on the WHDs. The two protomers are colored as in panel A, with one shown as a surface representation and the other shown as a ribbon to highlight the entwined structure of the oligomer. **(C)** Analytical ultracentrifugation data of MukF(1–287). Data (circles) fitting to a single species model calculated a mass of 69.1 kDa, consistent with a dimeric species in solution (see Materials and methods). Theoretical models for the monomeric, dimeric, and trimeric species of the MukF(1–287) protein are shown as lines indicated in the legend. Residuals to the fitted data are shown above.

for crystallization. As can be seen in Figure 2C, data obtained for the particle fit cleanly to a stable dimer model, but not to models predicted for monomeric or trimeric oligomeric states. An inspection of the contacts between protomers reveals that inter-subunit interactions arising from domain swapping account for more than three-quarters of the total surface area buried in the dimerization interface ($\sim 3700 \text{ \AA}^2$ per protomer). Moreover, the linker region between helices $\alpha 5$ and $\alpha 6$ is too short to permit domains I and II within one monomer to associate with each other using the interdomain interactions we observe in the MukF(1–287) dimer. The extensive, intertwined character of its oligomerization interface suggests that this interaction is obligate (Nooren and Thornton, 2003) and probably imparts a high degree of stability to the MukF dimer.

The N- and C-termini of MukF are homologous to kleisin domains

Kleisins are a recently identified superfamily of proteins that are distributed throughout many SMC-based complexes

(Schleiffer *et al*, 2003). Kleisins include bacterial ScpA, XCAP-H/Brn1 of eukaryotic condensin, Scc1/Mcd1/Rad21 of cohesin, and Rec8 of the Smc5/Smc6 DNA repair system. These proteins all appear to associate with the ATPase domains of SMC proteins. In the case of bacterial condensin, ScpA specifically associates with Smc subunits (Volkov *et al*, 2003; Dervyn *et al*, 2004; Hirano and Hirano, 2004), while for eukaryotic cohesin, the N- and C-termini of Rad21/Mcd1/Scc1 have been demonstrated to bind directly to Smc3 and Smc1, respectively (Haering *et al*, 2002, 2004).

Given that MukB is part of the SMC superfamily (van den Ent *et al*, 1999; Lowe *et al*, 2001; Cobbe and Heck, 2004), it has been suggested that the functions and perhaps structures of its accessory subunits may overlap with those of the non-SMC components found in other SMC complexes. MukF has been put forth as a potential kleisin in this regard (Schleiffer *et al*, 2003). Given the close structural similarity between the MukF N-terminal WHD and the C-terminal Scc1 WHD, coupled with the fact that MukF forms a stable complex with MukB and MukE, we decided to investigate more closely

Figure 3 Conservation between MukF and kleisins. Sequence alignment of several kleisin N- (upper panel) and C-terminal (lower panel) domains with MukF using MAFFT (Katoh *et al*, 2002) and Jalview (Clamp *et al*, 2004). Secondary structural elements for the N-terminal domain of MukF (red) and the C-terminal domain of Scc1 (green) are highlighted as seen in their respective crystal structures (Figure 1) (Haering *et al*, 2004).

whether MukF might be related to kleisin proteins at the amino-acid sequence level.

To accomplish this, we aligned sequences from multiple members of the MukF and kleisin families (Figure 3). Our analysis shows that the N- and C-termini of MukF align well with the equivalent N- and C-terminal regions of ScpA, as well as with those of eukaryotic kleisins. An examination of our alignments in light of the structural data for the MukF N-terminal region and the Scc1 C-terminus (Haering *et al*, 2004) shows that the two regions of homology correspond to WHDs that form the extreme termini of both proteins. There is particularly good overlap between secondary structural elements predicted to form HTH motifs from primary sequence analysis and the regions now known to contain such architectures from structural studies (Figures 1D and 3) (Haering *et al*, 2004). Significantly, our alignment of MukF with other kleisin proteins independently recapitulated the homology between members of the kleisin family (Schleiffer *et al*, 2003), even though we did not use this information to guide or bias our comparisons. Taken together, this analysis supports the idea that MukF is a member of the kleisin superfamily.

Identification of MukF regions responsible for binding MukE and MukB

To further ascertain the extent to which the behavior of MukF reflects that of kleisins, we set out to define more narrowly the regions of MukF that associate with MukB and MukE. Previous findings from Hiraga and co-workers (Yamazoe

et al, 1999) had already shown that MukF binds both MukB and MukE, and that MukE interacts only weakly, if at all, with MukB in the absence of MukF. Some of these associations were proposed to be mediated by the acidic linker that lies beyond MukF(1–287), as well as by a predicted coiled-coil region in MukF that our structure shows to comprise a portion of domain II (Yamanaka *et al*, 1996; Yamazoe *et al*, 1999). However, the boundaries and relative relationship of MukB- and MukE-binding sites on MukF have remained unclear.

To determine whether MukF(1–287) could bind either MukB or MukE, we first performed pull-down assays with lysates containing N-terminally hexahistidine-tagged MukF(1–287) (bait) and either untagged MukB or MukE(1–209) (prey). MukE(1–209) is a construct of the 243-amino-acid MukE protein that recapitulates the functions of the full-length protein *in vitro* but that lacks several poorly conserved and hydrophilic residues from its C-terminus to improve solubility (Materials and methods). Bait and prey proteins were expressed independently of one another in different cell cultures and, after mixing the lysates together for a short incubation time, binding was assayed batch-wise using Ni-NTA resin (Materials and methods). Control experiments using untagged MukF(1–287), MukE(1–209), or MukB showed that these proteins do not associate independently with the Ni-NTA resin (Supplementary Figure 1). Analysis of the bound and unbound pools in assays using histidine-tagged MukF(1–287) demonstrated binding of the tagged protein to the Ni-NTA resin, and also showed that this

Figure 4 MukF, MukB, and MukE domain interactions. Results of filter-binding assays between different Muk constructs are shown. The Muk fragments and subunits assayed are identified by cartoons: the MukF N-terminal domains are shown as a gray and crosshatched dimer; MukF C-terminal domain fragments are dark gray; MukB (170 kDa) and the MukB ATPase domain (72 kDa) are light gray with coiled squiggles; MukE(1-209) (24 kDa) is white. Representations for the two MukF C-terminal domain constructs can be distinguished by the presence of a small 'tail' that corresponds to the acidic linker region (residues 302-330) between MukF(1-287) and MukF(331-440). Gel lanes are labeled as follows: 'L', lysate; 'U', unbound; 'B', bound. Hexahistidine-tagged fragments are preceded by 'H-'. (A) MukF(1-287) does not bind MukE(1-209). (B) MukF(1-287) does not bind MukB. (C) His-tagged MukF(302-440) binds MukE(1-209). (D) H-F(331-440) does not associate with MukE(1-209). (E) H-F(302-440) pulls down both E(1-209) and MukB. (F) Tagged MukF(331-440) binds MukB. (G) Tagged MukF(331-440) binds the ATPase domain of MukB. (H) Histidine-tagged MukF(302-440) pulls down MukE(1-209) but does not associate with MukF(1-287).

construct is unable to pull down either MukE(1-209) or MukB (Figure 4A and B).

We next assayed the ability of two different MukF C-terminal fragments to bind MukE(1-209). The larger fragment, MukF(302-440), was obtained by deletion mapping using amino-acid homology and contains a conserved acidic region that has been predicted to bind Ca^{2+} ions (Yamazoe *et al*, 1999). The other, MukF(331-440), was identified from limited proteolysis of the full-length protein by N-terminal sequencing and mass spectrometry. As with MukF(1-287), both tagged C-terminal domain constructs of MukF bound the Ni-NTA resin on their own. MukF(302-440) additionally proved capable of pulling down coexpressed MukE(1-209) (Figure 4C). By contrast, tagged MukF(331-

440) showed no stable interaction with untagged MukE(1-209) (Figure 4D).

When assayed for MukB binding, both MukF C-terminal fragments pulled down untagged MukB from crude lysates (Figure 4E and F). The MukF C-terminus also proved capable of associating with a construct of MukB that comprises only the ATPase domains (residues 1-302 and 1180-1486) of the protein (Figure 4G). However, the MukF C-terminal region could not pull down MukF(1-287) (Figure 4H). Since neither untagged MukE(1-209) nor MukB bound appreciably to the nickel resin (Supplementary Figure 1), their interactions with the C-terminal region of MukF appear specific. This observation explains the null phenotype of the *mukF303* mutation (Yamanaka *et al*, 1996), which truncates the MukF protein at

residue 302: since the regions responsible for MukB and MukE binding are missing, the truncated protein is not competent for assembling with a MukBEF complex.

Taken together, these data demonstrate that the last 110 amino acids of MukF are responsible for binding the ATPase domain of MukB, while the acidic linker region between the N- and C-terminal domains of MukF (residues 302–330) is essential for associating with MukE. By contrast, the N-terminal two-thirds of MukF does not interact tightly with MukB, MukE, or the C-terminal domain of MukF. This latter finding is consistent with our structural work showing that the N-terminus of MukF forms a homodimerization module, and that the predicted leucine-zipper region of the protein is actually an integral part of the four-helix coiled-coil segment. Overall, the MukB-binding behavior of the MukF C-terminus is reminiscent of the association of the Scc1 C-terminus with Smc1 (Haering *et al*, 2002, 2004), further supporting a functional role for MukF as a kleisin.

Discussion

MukF function within the MukBEF complex

The relationship of MukF to kleisins provides an important clue to its function. Several studies have shown that SMC subunits can bring together distant DNA segments (Hirano and Hirano, 1998, 2002, 2004; Yoshimura *et al*, 2002; Volkov *et al*, 2003). The subunits that associate with SMCs appear to regulate and direct this function to help different SMC complexes manifest distinct biochemical activities. For example, in the Muk system, MukB can bind DNA on its own, but is converted into an active condensin only when functional MukF and MukE subunits are associated (Niki *et al*, 1992; Saleh *et al*, 1996; Yamanaka *et al*, 1996; Yamazoe *et al*, 1999). In eukaryotes, distinct regulatory subunits associate with different SMC dimers to create complexes that act specifically in chromosome segregation and condensation, DNA repair, and gene regulation (Kimura and Hirano, 2000; Hirano, 2002; Hagstrom and Meyer, 2003; Ono *et al*, 2003; Haering *et al*, 2004).

What physical purpose might MukF serve? Our structural studies demonstrate that the N-terminal two-thirds of this protein forms a stable, domain-swapped dimerization element. Our work further shows that it is the extreme C-terminus of MukF that is responsible for binding the ATPase domains of MukB, and that this domain likely forms an HTH or WH fold related to the C-terminal, SMC-binding domain of kleisins (Figures 3 and 4). Given that the C-terminus of Scc1 binds specifically to the ATP-binding domains of Smc1 (Haering *et al*, 2002, 2004; Gruber *et al*, 2003), we anticipate that the C-terminus of MukF interacts in a similar manner with the ABC cassette of MukB. Observations from Yamazoe *et al* (1999) support this idea, showing that deletion of the extreme MukB C-terminus (residues 1372–1486) destroys its ability to associate with full-length MukF. Two point mutations in this region of MukB, L1403P and Q1429R, also abrogate this interaction (Saleh *et al*, 1996).

These relationships indicate that a dimer of MukF is most likely capable of associating with two MukB subunits (Figure 5), and suggest that at least part of MukF's purpose is to link together the SMC components of *E. coli* condensin. This action is entirely analogous to that proposed for known

Figure 5 Organizational model for the MukBEF assembly. Left panel: MukF is a dimer with a pair of MukB interaction domains that likely binds two MukB and MukE subunits. Colors are as follows: MukB ATPase domains and coiled coils, orange; MukE, gray; MukF N-terminal WHD, red; MukF domain II, blue; MukF C-terminal domain, green; MukF N- and C-terminal domain linker, black line. The MukF C-terminal domain is proposed to interact with the SMC ATPase domains of MukB and the MukF linker with MukE (see text). Right panel: Proposed organization for cohesin, in which the C-terminal WHD of Scc1 (green) binds to Smc1 (orange) and the N-terminal HTH domain of Scc1 (red) binds to Smc3 (yellow) (Haering *et al*, 2002; Gruber *et al*, 2003). The two Scc1 terminal domains are linked together by an intervening amino-acid sequence of unknown structure (gray). Scc3 (gray) binds to the C-terminus of Scc1 in the complex.

kleisins such as Scc1/Mcd1/Rad21 and ScpA (Haering *et al*, 2002; Schleiffer *et al*, 2003; Volkov *et al*, 2003; Dervyn *et al*, 2004; Hirano and Hirano, 2004). In addition, it is interesting to note that for the bacterial Smc/Scp system, ScpA has been proposed to act as a scaffold that anchors ScpB protomers to the Smc subunits (Volkov *et al*, 2003; Dervyn *et al*, 2004; Hirano and Hirano, 2004). MukF, specifically its C-terminal region downstream of the N-terminal dimerization module, likewise is required to bind MukE and bring it to MukB (Yamazoe *et al*, 1999; Figure 4).

Despite these similarities, there are differences between MukF and some of the kleisins. For example, the N-terminal domain of Scc1 has been reported to bind to the ATPase domain of Smc3 (Gruber *et al*, 2003), yet we find no equivalent interaction between the N-terminal domain of MukF and MukB. However, there are distinguishing features between different kleisin subfamilies as well: as one illustration, Scc1 is thought to bind to two SMC subunits as a monomer while two ScpA subunits appear to bind two sets of prokaryotic Smc and ScpB (Haering *et al*, 2002; Volkov *et al*, 2003; Dervyn *et al*, 2004; Hirano and Hirano, 2004). These differences may arise in part because the MukB and prokaryotic Smc-binding partners of MukF and ScpA exist as homodimers, and complexes between these partners presumably form through identical sets of kleisin/SMC contacts. By contrast, eukaryotic SMC proteins form heterodimers, and the total number and types of their associated factors are quite diverse. These relationships suggest that gene rearrangements or domain duplications may have occurred among kleisin proteins during evolution, perhaps coincident with the expansion of their repertoire of SMC paralogs. The close structural similarity between the N-terminal MukF and C-terminal Scc1 WHDs (Figure 1D) may be evidence that such shuffling has occurred in the past. Which WHD functions and arrangements arose first among the kleisins remains to be determined; however, given prevalence of ScpA in archaea and in bacteria outside of the γ -proteobacter family, as well as the protein's small size and relative simplicity, we suspect that ScpA may most closely reflect the ancestral protein.

Since ScpA shows a tendency to dimerize on its own (Volkov *et al*, 2003; SD Gradia and JM Berger, unpublished), it is possible that the architecture we see for MukF(1–287) first arose, and was then modified, from the ScpA N-terminal domains.

The potential for a MukF dimer to act as a kleisin and bind to the ATPase domains of two MukB subunits has certain implications for its function. For example, the tightly entwined structure of its N-terminal region may help stabilize the fully assembled MukBEF complex against some of the forces required to compact DNA in the cell. It could also prevent MukB dimers from binding overlapping segments of DNA, a mode of action that, if conserved with other kleisin proteins, may explain why isolated SMC proteins form large, irregular protein-DNA aggregates *in vitro* (Hirano and Hirano, 1998, 2002; Yoshimura *et al*, 2002; Stray and Lindsley, 2003; Volkov *et al*, 2003). Such a role also would be consistent with observations that Smc2 and Smc4 of *Schizosaccharomyces pombe* condensin form large clusters on DNA *in vitro* but distribute themselves discretely on DNA in the presence of their respective non-SMC subunits (Yoshimura *et al*, 2002). Similarly, the *B. subtilis* Smc protein localizes properly on chromosomes *in vivo* and binds DNA stably *in vitro* only when ScpA and ScpB are present (Volkov *et al*, 2003; Hirano and Hirano, 2004).

Overall, our data are consistent with proposed mechanisms in which MukF and other related klesins serve to link together a pair of SMC subunits (Figure 5). Kleisin-mediated linkages could, in principle, occur between two different condensin complexes or within a complex. One set of models has proposed that multiple condensin complexes can associate with each other on DNA and act cooperatively to organize a superhelical structure (Kimura *et al*, 1999; Stray and Lindsley, 2003; Swedlow and Hirano, 2003; Strick *et al*, 2004). For condensin at least, a kleisin link between the SMC subunits within a single condensin assembly would not only stabilize the complex internally, but may also permit multiple complexes to associate cooperatively through interactions mediated by the SMC components and ATP. Future work on the intact MukBEF complex will be needed to resolve these issues further and help tease apart the manner by which the regulatory factors of different SMC systems direct their respective functions in the cell.

Materials and methods

Expression plasmids

The pBADBEF01 plasmid (gift of N Cozzarelli, University of California, Berkeley) contains the entire *muk* operon and was used as a PCR template to clone the *mukF* and *mukE* genes into pET28b (Novagen). The resulting construct, pRF13, overexpresses full-length MukF with a thrombin-cleavable hexahistidine tag and full-length MukE. An overexpression vector for MukF(1–287), pRF14, was generated from pRF13 using ExSite (Stratagene) mutagenesis. A construct expressing untagged MukF(1–287), pRF30, was made by subcloning the *NdeI/XhoI* fragment from pRF14 into pET24b. Open reading frames encoding MukF(302–440), MukF(331–440), and MukE(1–209) were amplified by PCR from pRF13 and placed into pET28b to create pRF65, pRF75, and pRF70. A plasmid expressing just the MukB ATPase domain, pRF301, was made by creating a construct that fuses together amino acids 1–320 and 1180–1486 of MukB with a short SGGSGGS linker sequence, and cloning this into pET3a.

During these studies, N-terminal sequencing and mass spectrometry revealed that MukE, when expressed from the *mukFE*

operon, begins one methionine codon upstream of that previously reported from sequence analyses (Yamanaka *et al*, 1996). As a consequence, the *E. coli mukE* reading frame overlaps with the 3' end of the *mukF* gene, and appends the sequence MSSTNIEQV to the protein's N-terminus. This segment is present in other MukE homologs. The updated sequence has been deposited in GenBank (accession #: AY626237).

We also found that full-length MukE expressed relatively poorly and inconsistently, a finding that complicated our initial control pull-down experiments. To overcome this problem, we made a variant of MukE that lacks several poorly conserved, predominately hydrophilic residues at its C-terminus. This construct, MukE(1–209), expresses significantly higher levels of protein than full-length MukE. MukE(1–209) also behaves identically to full-length MukE in its purification behavior and ability to bind MukF (not shown).

Expression, purification, and crystallization of MukF(1–287)

MukF(1–287) was expressed in BL21 Codon Plus *E. coli* cells (Novagen) at 37°C with 1 mM IPTG for 3–4 h. Harvested cells were resuspended in buffer EFi20 (20 mM HEPES (pH 7.5), 20 mM imidazole (pH 7.5), 400 mM KCl, 10% glycerol, 1 mM 2-mercaptoethanol, and protease inhibitors), and frozen in liquid nitrogen. After thawing, sonication, and centrifugation, the supernatant was loaded onto a Ni-NTA column. The column was washed with EFi20, exchanged into cleavage buffer (20 mM HEPES (pH 7.5), 20 mM imidazole (pH 7.5), 100 mM KCl, 10% glycerol, and 1 mM 2-mercaptoethanol), and then incubated in 1 column volume of cleavage buffer supplemented with 250 U bovine thrombin (Sigma) overnight at 4°C. Cleaved protein was eluted with protease-free cleavage buffer and passed over a HiTrap Q column (Amersham). Fractions containing MukF(1–287) were pooled, concentrated, and run on a GSW4000 (Toso BioSep) size-exclusion column; peak fractions from this step were pooled and concentrated with a Centrprep-YM10 (Millipore) to 30–50 mg/ml as assayed by UV absorbance (Edelhoch, 1967). Selenomethionine-derivatized protein was expressed according to Van Duyne *et al* (1991) and purified similarly.

MukF(1–287) was dialyzed into 150 mM KCl, 10 mM HEPES (pH 7.5), and 2 mM TCEP for crystallization trials. Crystals of MukF(1–287) were grown by hanging-drop vapor diffusion by mixing protein and reservoir solution (100 mM (NH₄)₂HPO₄, 34–39% PEG 2K MME, 10 mM CaCl₂, and 100 mM bis-tris-propane (pH 8.5) at a 1:2 ratio. Crystals (hexagonal cones) grew in 5–10 days at 19°C. For harvesting, crystals were transferred briefly to 25 mM (NH₄)₂HPO₄, 34–39% PEG 2K MME, 10 mM CaCl₂, 100 mM bis-tris-propane (pH 8.5), and 15% xylitol, and then looped and flash-frozen in liquid nitrogen.

Data collection and structure determination

Diffraction data were collected at Beamline 8.3.1 at the Advanced Light Source of Lawrence Berkeley National Laboratory and processed using DENZO and SCALEPACK (Otwinowski and Minor, 1997). The positions of eight selenium sites were identified from MAD data using SOLVE (Terwilliger and Berendzen, 1999). Initial maps were made using DM (Cowtan and Main, 1998) with two-fold NCS averaging, and improved maps were generated with SHARP (Fortelle and Bricogne, 1997). Manual rebuilding was carried out with O (Jones *et al*, 1991), and REFMAC5 with TLS restraints (Murshudov *et al*, 1997; Winn *et al*, 2001) was used for model refinement. The refined model includes residues 8–91 and 100–281 of monomer A and residues 5–91, 100–194, 201–239, and 243–281 of monomer B. The model has good geometry with no residues in disallowed regions of Ramachandran space (Table I).

Ni-NTA pull-down assays

Expression of individual Muk subunits and domains was carried out in BL21 Codon Plus cells transformed with the appropriate plasmids. Cultures were induced with 1 mM IPTG and shifted to 25°C for 5 h before harvesting by centrifugation. Cell culture (1 l) was pelleted, and cells were resuspended in 5 ml lysis buffer (20 mM HEPES (pH 7.5), 20 mM imidazole (pH 7.5), 400 mM KCl, 10% glycerol, 3 mM CaCl₂, 3 mM MgCl₂, and 1 mM 2-mercaptoethanol) prior to freezing at –80°C in 1–2 ml aliquots.

For pull-down assays, aliquots were thawed and the cells lysed by treating with 50 µg/ml hen egg white lysozyme (Sigma) for 5 min at 20°C, followed by sonication. Lysates were clarified by centrifugation. Pull-downs were performed by mixing lysates

containing hexahistidine-tagged proteins (bait) with those containing untagged factors (prey). Control experiments assayed only tagged or untagged proteins. Expression levels were prechecked by SDS-PAGE to ensure that comparable or excess amounts of prey were incubated with bait during the assay. Because very little MukE(1–209) coexpressed with MukF(331–440), we performed a similar pull-down experiment using a lysate supplemented with a second lysate expressing only MukE(1–209). After 1 h incubation, lysates were mixed with 50 µl Ni-NTA Superflow resin (Qiagen) equilibrated in lysis buffer, supplemented with 1 ml binding buffer (10 mM HEPES (pH 7.5), 10 mM imidazole (pH 7.5), 200 mM KCl, 5% glycerol, 1.5 mM CaCl₂, 1.5 mM MgCl₂, 0.5 mM 2-mercaptoethanol, and 0.5% Tween 20), and incubated with rocking at 4°C for 1 h. Resin was pelleted by centrifugation, the supernatant removed, and the resin washed five times with 1 ml of 2 × binding buffer. After the last wash, buffer was removed and 2 × SDS loading dye containing 300 mM imidazole and 1 mM DTT was added to the pelleted resin. All fractions were assayed by SDS-PAGE.

Analytical ultracentrifugation

Analytical ultracentrifugation of purified MukF(1–287) was performed on samples dialyzed into 20 mM HEPES (pH 7.5) and 200 mM KCl at 4°C using a Beckman Optima XL-I analytical ultracentrifuge. Sedimentation equilibrium experiments were performed at protein concentrations of 416, 249, and 167 µg/ml. Data were collected after spinning for 18 h at speeds of 10 000 and 12 000 r.p.m. For both speeds, additional data sets were acquired 3 h after the first. The lack of variation in the overlay of UV traces from

successive scans confirmed that the species were at equilibrium. The partial specific volume was calculated from the residue-weighted average of the amino-acid sequence. Solvent density was calculated from solvent composition (Laue *et al*, 1992). Data fitting to a single species model using the Beckman XL-A/XL-I Origin Data Analysis package (v 6.03) calculated a mass of 69.1 kDa. Theoretical models for the monomeric, dimeric, and trimeric species of the 32.7 kDa MukF(1–287) protein were calculated using the same software.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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