Complex formation of MukB, MukE and MukF proteins involved in chromosome partitioning in *Escherichia coli*

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mukF, *mukE* and *mukB* genes are essential for the process of chromosome partitioning in *Escherichia coli*. We have studied protein–protein interactions among MukB, MukE and MukF proteins by co-immunoprecipitation and sucrose gradient sedimentation experiments, using *mukFEB* null cells harboring plasmids carrying the wild-type or mutant-type *mukFEB* operon. MukB forms a complex with MukF and MukE. Analysis of mutant MukB proteins suggested that MukF and MukE bind the C-terminal globular domain of MukB. MukF is indispensable for an interaction between MukB and MukE; however, MukF itself is able to associate with MukB even in the absence of MukE. We have also found that MukF has a Ca²⁺-binding activity. Although purified MukF was able to make a complex either with MukE or MukB, a complex consisting of the three Muk proteins was barely detected *in vitro*. However, increasing the Ca²⁺ or Mg²⁺ concentration in the reaction partially restored complex formation. This suggests that Ca²⁺ or Mg²⁺ may be required for the formation of a complex consisting of the three Muk proteins, and thus may participate in a particular step during chromosome partitioning.

Keywords: Ca²⁺-binding protein/chromosome partitioning/motor protein/MukB–MukE–MukF

Introduction

In eukaryotic cells, replicated chromosomes are inherited by two daughter cells using a specialized mitotic apparatus containing a spindle pole body and spindle microtubules. However, it has been a mystery how prokaryotic cells such as *Escherichia coli* transmit two duplicated chromosomes with high accuracy after chromosomal replication. Until very recently, in contrast to eukaryotes, bacterial cells were regarded as lacking anything like a mitotic apparatus. Nevertheless, on the assumption that bacteria also possess their own partitioning mechanism for the chromosome, even if it might be primitive, we performed

a genetic screening to search for genes whose products are essential in an active way for chromosome partitioning (Hiraga *et al.*, 1989). First, we identified a novel gene *mukB* (Niki *et al.*, 1991). Missense and null *mukB* mutants produce anucleate cells with much higher frequency than wild-type cells (5–15% versus 0.03%). The *mukB* mutants also show temperature-sensitive growth: the mutant cells cannot grow at temperatures >30°C. We also found recently that MukB is involved in cell-cycle-dependent segregation of SeqA–DNA complexes (Hiraga *et al.*, 1998). SeqA itself is involved in controlling initiation of chromosome replication (Lu *et al.*, 1994).

The *mukB* gene encodes a 170 kDa protein (Yamanaka *et al.*, 1994a), one of the largest proteins in *E. coli*. MukB has globular domains at both ends and forms a homodimer by means of an α -helical coiled-coil rod structure in the center of the protein (Niki *et al.*, 1991, 1992). Such a structure reminds us of the motor proteins, such as eukaryotic kinesin and myosin. Biochemical studies further revealed that the N-terminal globular domain has ATP/GTP-binding, ATPase and GTPase activities, while the C-terminal globular domain has a DNA-binding activity (Niki *et al.*, 1992; Saleh *et al.*, 1996; Lockhart and Kendrick-Jones, 1998).

In order to determine how MukB plays a role in the chromosome partitioning process, we have searched specifically for MukB-associated proteins using genetic screening. A linkage group, *smbB*, of suppressors of a *mukB106* mutant was located in the *rneE* gene. All the *smbB* mutations encoded truncated RNaseE proteins lacking the C-terminal half. This was accompanied by an increased level of MukB, probably via an effect on mRNA degradation (Kido *et al.*, 1996). In addition, when the wild-type *cspC* and *cspE* genes were present in elevated copy numbers, the products of which are homologs of a major cold shock protein CspA in *E. coli*, the effects of the *mukB106* point mutation were suppressed (Yamanaka *et al.*, 1994b). However, it is still obscure how overexpression of the genes is involved in chromosome partitioning. All the above suppressors of *mukB* mutations thus may suppress *muk* mutations indirectly.

Through the process of sequencing the region upstream of the *mukB* gene, we found two other novel genes, *mukF* and *mukE*, which are also essential for chromosome partitioning. These two genes compose an operon with *mukB* and *smtA* in the order *smtA*–*mukF*–*mukE*–*mukB* (Yamanaka *et al.*, 1996). The *mukF* gene encodes a 51 kDa protein containing a leucine zipper motif in the middle and an acidic amino acid cluster domain near the C-terminus. The *mukE* gene encodes a 27 kDa protein with as yet no identifiable features from database searches. Overexpression of any one of these three genes does not suppress the phenotypes caused by a defect in any of the other genes, indicating that the three gene products may

constitute an active complex or, alternatively, they may independently play an indispensable role at different stages of chromosome partitioning.

Here we first report the existence of a complex consisting of MukF, MukE and MukB in a wild-type cell lysate, and then describe its conceivable structure. From the results of reconstitution experiments using the purified Muk proteins, we found that formation of the complex is stimulated by Ca^{2+} or Mg^{2+} .

Results

Phenotypes of a *mukFEB* deletion mutant

We have established *mukFEB* deletion strains OT5 and OT7 as described in Materials and methods. These strains carry the disrupted *smtA–mukF–mukE–mukB* genes on the chromosome (see Figure 1B). However, it was previously reported that the *smtA* gene, encoding an *S*-adenosyl-methionine-dependent methyltransferase, is not essential for cell growth and is not involved in chromosome partitioning (Yamanaka *et al.*, 1995). Cells of both OT5 and OT7 form colonies only at a temperature $<25^{\circ}\text{C}$, thus showing temperature-sensitive growth, as observed before for null mutants of individual *mukF*, *E* and *B* genes. Temperature-sensitive growth was complemented with plasmid pAX850 carrying the *mukFEB* genes, but not with plasmids carrying only one or two of these individual *muk* genes. Even at the permissive temperature, 22°C , these *mukFEB* null mutant strains still produced a significant number of anucleate cells, as also reported previously for *mukF*, *mukE* or *mukB* null mutants (Yamanaka *et al.*, 1996). The doubling time of the *mukFEB* null mutants at 22°C in L medium was similar to that of individual *muk* null mutants.

Physical interactions among Muk proteins

As the first test of any physical interactions among MukB, MukF and MukE proteins, the Muk proteins in a cell lysate were immunoprecipitated using polyclonal antibodies raised against each Muk protein. The specificity of the antibodies was confirmed by Western blotting using a lysate of cells overexpressing three Muk proteins and affinity-purified antibodies against each Muk protein (Figure 2A). Each purified antibody recognized only the specific Muk protein but not the other Muk proteins, even when they were overexpressed. Wild-type cells were labeled with [^{35}S]methionine and cysteine *in vivo*, and a cell lysate from the labeled cells was subjected to immunoprecipitation. However, we could not detect any of the Muk proteins in the immunoprecipitate, possibly due to limited amounts of the Muk proteins (data not shown). We therefore overexpressed those proteins by the introduction of pAX850, carrying the whole *mukFEB* operon, into strain OT5 lacking the *mukFEB* genes. From a cell lysate of this strain, following labeling with ^{35}S , we could always immunoprecipitate MukF, MukE and MukB proteins simultaneously with specific polyclonal antibodies against each protein (Figure 2B, top middle), suggesting that the three Muk proteins form a complex. In the absence of all the Muk proteins in the cell lysate, no specific proteins were precipitated by the antibodies (Figure 2B, top left).

We next studied complex formation between different

combinations of only two Muk proteins. For this purpose, we constructed derivative plasmids of pAX850 in which one of the *muk* genes was deleted and then transformed cells of strain OT5 with the new plasmids, pMY1008, pMY1009 and pKX746 (see Figure 1). Proteins in the transformants were radiolabeled and the cell lysate was subjected to immunoprecipitation. Even in the absence of MukB, anti-MukF and anti-MukE antibodies (but not anti-MukB antibodies) co-precipitated MukF and MukE simultaneously (Figure 2B, top right), indicating that those two proteins could form a complex by themselves. In the absence of MukE, anti-MukF antibodies could precipitate MukF and MukB simultaneously. However, anti-MukB antibodies co-precipitated MukF poorly (Figure 2B, bottom left). This could be attributed to the insensitivity of anti-MukB, the decreased expression of MukF and MukB that is observed when MukE is missing (data not shown), and also the inefficient formation of a MukFB complex (see Figure 3H and below). On the other hand, when MukF was absent, MukB was completely unable to form a detectable complex with MukE, indicating that MukF was indispensable for the interaction between MukB and MukE (Figure 2B, bottom middle). It should be noted that in the absence of any particular Muk protein, specific antibodies against this protein precipitated little, if any, of the other Muk proteins, demonstrating again the specificity of the antibodies.

MukF possesses a leucine zipper motif in the center of the protein (Figure 7C). Similarly, there is also a putative leucine zipper motif (1212–1233) in the C-terminal globular domain of MukB (Figure 7B). Such motifs are known to be involved in protein–protein associations. It is likely that this leucine zipper motif plays a critical role in MukF activity because the *mukF233* missense mutation (Leu233 to Pro) located adjacent to this motif (see Figure 7C), which may disrupt the α -helical repeat sequence, fails to suppress temperature-sensitive growth in *mukF* null mutant cells (Yamanaka *et al.*, 1996). We examined a role for the leucine zipper motif of MukF in complex formation using the cell lysate from *mukFEB* null mutant cells, harboring plasmid pMY1010. This plasmid carries the *mukFEB* operon with the *mukF233* mutation. The altered MukF233 protein was still able to form a complex with MukE and MukB (Figure 2B, bottom right), indicating that the leucine zipper motif in MukF may not be required for formation of the MukFEB complex. On the other hand, this motif of MukF could presumably be involved in association with another MukF molecule in the MukFEB complex or other unknown proteins.

Mutational analysis of the MukB protein

The MukB protein has two globular domains at each end that are linked by α -helical coiled-coil structures in the middle (Niki *et al.*, 1991, 1992). It is thus very important to know which globular domain is responsible for interaction with MukF or MukE in order to elucidate a molecular mechanism for how the MukFEB protein complex is involved in the process of active chromosome partitioning. We introduced mutations, including those isolated previously (Saleh *et al.*, 1996), in either the N- or C-terminus of the *mukB* gene in the *mukFEB* operon on pAX850. These mutated derivatives of plasmid pAX850 were introduced into the *mukFEB* null mutant cells of strain OT5.

Fig. 1. Plasmids used in this study. The top bar represents arrangement of genes, restriction enzyme sites and putative promoters (P1, P2 and P3) in the *mukFEB* operon in the *E.coli* chromosome. **(A)** Structures of plasmids. Shaded bars show open reading frames. Solid lines show vector DNA. Restriction enzyme sites with double lines indicate disruption of the corresponding sites, causing a frameshift mutation. Solid bars show positions of non-coding regions downstream of the frameshift mutations. Asterisks represent the position of point mutations. The name of point mutations and the amino acid substitution are in parentheses on the right. Arrowheads in vector DNA show controllable promoters of T5-lac and T7-lac. **(B)** Chromosome of bacterial *mukFEB* null mutant strains, OT5 and OT7. The hatched bar represents the inserted *kan* segment in place of the *XhoI* deletion. B, *Bam*HI; Bg, *Bgl*I; C, *Cla*I; E, *Est*III; K, *Kpn*I; N, *Nco*I; Ns, *Nsi*I; P, *Pst*I; X, *Xho*I.

Fig. 2. Specificity of the purified antibodies against Muk proteins and co-immunoprecipitation of ^{35}S -labeled Muk proteins. (A) Cell lysates were prepared from *mukFEB* null mutant cells (OT5) harboring pAX850 or pACYC184. The cell lysates were separated by SDS–5% PAGE for MukB or SDS–10% PAGE for MukF and MukE. Western blotting was performed using the affinity-purified antibodies against each Muk protein. Positions of MukB, MukF and MukE are shown with arrows. (B) Labeled proteins were extracted from OT5 cells harboring the indicated plasmids and precipitated with specific polyclonal antibodies against MukF, MukE and MukB. The precipitated proteins were examined by SDS–PAGE (10% acrylamide) and autoradiography.

Table I summarizes the results of co-immunoprecipitation experiments studying interactions between the mutant MukB proteins and MukF–MukE. The mutant MukB1–1371 protein, lacking 115 amino acids from the C-terminal region, abolished any detectable interaction with both MukF and MukE, in addition to the DNA-binding activity of MukB. In contrast, the mutant MukB255–1486 protein, lacking the N-terminal 254 amino acids, retained the ability to interact with MukF and MukE. We next introduced point mutations into the N- or C-terminal globular domain of MukB and examined complex formation of these mutants with MukF and MukE. Two mutant proteins, MukB1008 (Gln1429 to Arg) and MukB1009 (Leu1403 to Pro), which have a point mutation in the C-terminal globular domain, could not associate with either MukF or MukE. However, these mutants retained the DNA-binding activity of MukB. On the other hand, another point mutation in the C-terminal domain, MukB1013 (Val1381 to Leu), did not affect complex formation but impaired DNA-binding activity. From these results, we conclude that MukF and MukE proteins associate with the C-terminal globular domain of MukB. Interestingly, a mutation in the nucleotide-binding site of the Walker motif A (MukB1004, Lys40 to Ile) (Walker *et al.*, 1982) retained the ability to form a complex, indicating that nucleotide binding or nucleotide hydrolysis of MukB is not necessary either for interactions with MukF and MukE or for the DNA-binding activity of MukB.

Sucrose gradient sedimentation of Muk complexes

To confirm a physical interaction among the three Muk proteins and to examine the Muk complex in a semi-quantitative way, cell lysates were subjected to sedimentation through a 10–60% sucrose gradient. The presence of the three Muk proteins in each fraction was examined by Western blotting using specific antibodies.

All three Muk proteins showed the same sedimentation coefficient of ~11.5S in wild-type cells (Figure 3A) as well as in cells overexpressing the three Muk proteins from plasmid pAX850 (Figure 3B). It should be noted that free Muk proteins are hardly detected, suggesting that almost all molecules of each Muk protein in the cell are recruited into the complex. On the other hand, MukB,

MukF and MukE proteins sedimented differently at 8.6S, 5.3S and 2.7S, respectively, when the other two Muk proteins were absent (Figure 3C, D and E). These results indicate that the three Muk proteins form a large complex, which can be detected in cell lysates. To measure the Stokes' radii of each Muk protein and the MukFEB complex, gel filtration was performed using the same lysate. The Stokes' radii of MukB, MukF, MukE and MukFEB were measured as 9.2, 4.7, 3.1 and 12.3 nm, respectively (Table II). Each purified Muk protein showed similar values of the sedimentation coefficient and Stokes' radius as in the cell lysates (data not shown). After combining the two parameters (Siegel and Monty, 1966), the native molecular weight was estimated as 330 kDa for MukB, 110 kDa for MukF, 35 kDa for MukE and 600 kDa for the MukFEB complex. It is thus reasonable to suppose that MukB and MukF form a dimer, MukE is present as a monomer by itself, and the three Muk proteins make a large complex as big as 600 kDa. In addition, MukB1008 was sedimented at 8.6S, separating from a 7.3S complex consisting of MukF and MukE (Figure 3F and 3G), consistent with the co-immunoprecipitation experiment.

In the absence of MukB, the MukF and MukE proteins were apparently tightly bound together, generating a 7.3S complex (Figure 3G). In the absence of MukE, MukB showed sedimentation coefficients of 25S and 8.6S, while MukF sedimented at 25S and 5.3S (Figure 3H). Presumably, the 25S material corresponds to a MukFB complex and the smaller 8.6S and 5.3S complexes may correspond to a MukB dimer (340 kDa) and a MukF dimer (102 kDa), respectively. It is notable that the MukFB complex gave a larger sedimentation coefficient (25S) than the MukFEB complex (11.5S; see Figure 3H and B). Therefore without MukE, MukB and MukF seem to form an aberrantly large complex in which multiple MukF molecules may bind to a MukB dimer. The amount of this complex is lower than that of free MukB and MukF; therefore, this may result in the inefficient co-immunoprecipitation of MukB and MukF, shown in Figure 2B. In the absence of MukF, MukB and MukE sedimented separately (Figure 3I) at the positions of a MukB dimer (8.6S) and a MukE monomer

Fig. 3. Sedimentation analysis of Muk proteins of a cell lysate by sucrose gradient centrifugation. A cell extract from wild-type cells (PB103) or *mukFEB* null mutant cells (OT5), harboring the indicated plasmids, was subjected to sedimentation through a 10–60% sucrose gradient. Proteins in each fraction were analyzed by SDS–5% PAGE for MukB and SDS–PAGE (10% acrylamide) for MukF and MukE, and were detected by Western blotting using specific antibodies. Positions of MukB, MukF and MukE are shown with arrows. Marker proteins, thyroglobulin (19.2S), ferritin (17.6S), catalase (11.3S), aldolase (7.3S), bovine serum albumin (4.3S), ovalbumin (3.5S) and chymotrypsinogen (2.4S), were also subjected separately to the same sedimentation. Note that errors of collected volume in each fraction are corrected by the actual volume in the fractions.

(2.7S), respectively. This indicated that MukB cannot bind MukE in the absence of MukF. We therefore concluded that MukF links MukB and MukE in the MukFEB complex.

Oligomerization of MukF and MukE proteins

As described above, in the absence of MukB, MukF and MukE were able to form a complex by themselves, and MukF can make a homodimer *per se*. We next asked how MukE molecules exist in the MukFE complex. We transformed *mukFEB* null mutant cells with plasmid pMY134 DNA, which can express MukE tagged with 11 amino acids (T7 tag) at the C-terminus. To the resultant transformant, we introduced a compatible plasmid encoding the authentic *mukF*, *mukE* or *mukB* genes in various combinations. Proteins in the transformants were labeled

with ^{35}S and a cell lysate was prepared for immunoprecipitation. When anti-T7 monoclonal antibody was used for immunoprecipitation, MukE was co-immunoprecipitated with MukE-T7 only when MukF was co-expressed (Figure 4, compare lanes 3 and 4). In contrast, MukB did not affect the association between MukE-T7 and MukE (Figure 4, lane 5). This result showed that more than two MukE molecules were able to associate only in the presence of MukF, probably through dimerization of MukF.

Ca^{2+} -binding activity of the Muk proteins

MukF protein has another characteristic domain: an acidic amino acid cluster in the C-terminal region. Mutational analysis of the *mukF* gene revealed that this region is also essential for MukF function *in vivo* (Yamanaka *et al.*,

Table I. Complex formation and DNA-binding activity of various MukB mutant proteins

A				
	MukB mutation	Substitution	DNA binding*	Complex formation with MukF & MukE**
①	MukB 106	S33F	NT	+
②	MukB 1004	K40I	+	+
③	MukB 33	D1201N	NT	+
④	MukB 1013	V1381L	—	+
⑤	MukB 1009	L1403P	+	—
⑥	MukB 1016	F1404L	NT	+
⑦	MukB 1014	D1406N	+	+
⑧	MukB 1015	M1428L	NT	+
⑨	MukB 1008	Q1429R	+	—
⑩	MukB (1-1371)	deletion	—	—
⑪	MukB (255-1486)	deletion	NT	+

(A) Summary of the DNA-binding activity and complex-forming activity of MukB mutants.

*DNA-binding activity was examined using purified mutant MukB proteins and 32 P-labeled *Hpa*II DNA fragments of pUC118 (Saleh *et al.*, 1996; our unpublished data).

**Complex formation was examined using a cell lysate containing the mutant MukB proteins (this study).

NT, not tested.

(B) A schematic representation of positions of the *mukB* mutations used in this study. Closed bars represent N- and C-terminal globular domains. Open and hatched bars represent coiled-coil regions.

Table II. Hydrodynamic properties of Muk proteins

Muk protein or Muk complex	Stokes' radius (nm)	$S_{20,w}$ (S)	Calculated mol. wt (kDa)
MukF	4.7	5.3	110
MukE	3.1	2.7	35
MukB	9.2	8.6	330
MukFEB	12.3	11.5	600

Stokes' radii and sedimentation coefficients were determined experimentally as described. The native molecular weight of the proteins was calculated from these values (Siegel and Monty, 1966) using a partial specific volume of $0.73 \text{ cm}^3/\text{g}$.

1996). By analogy with eukaryotic calreticulin, in which an acidic domain is responsible for its low-affinity Ca^{2+} -binding activity, we examined whether MukF also possesses a Ca^{2+} -binding activity. Using purified His₆-tagged MukF, MukE and MukB proteins, we carried out [$^{45}\text{Ca}^{2+}$]

Fig. 4. Association of MukE molecules in a MukFE complex. Cellular proteins in the *mukFEB* null mutant strain of OT5 (λ DE3) harboring two compatible plasmids were labeled with [35 S]methionine and cysteine. Cell lysates were prepared and subjected to immunoprecipitation using polyclonal antibodies against MukE (upper autoradiograph) or monoclonal antibodies against the T7 tag (lower autoradiograph). Cells harboring the plasmids indicated below were used to determine different interactions. pET21a Δ R1 and pKX766 (lane 1), pMY134 and pACYC184 (lane 2), pMY134 and pKX766 (lane 3), pMY134 and pKX748 (lane 4), pMY134 and pMY1008 (lane 5).

overlay experiments in the presence of excess Mg^{2+} , as described in Materials and methods. As anticipated, His₆-tagged MukF showed a distinct Ca^{2+} -binding activity compared with bovine serum albumin (Figure 5A) or His₆-tagged MukB (data not shown). His₆-tagged MukE also displayed a weak Ca^{2+} -binding activity. Interestingly, FtsZ, as predicted by Löwe and Amos (1998) from crystallography, demonstrated a slightly higher Ca^{2+} -binding activity than His₆-tagged MukF (Figure 5C; Discussion).

Reconstruction of the Muk complex using purified Muk proteins *in vitro*

We purified independently the three Muk proteins, each with six histidine residues (His₆ tag) or with 11 amino acid residues followed by His₆ (T7-His₆ tag), using metal-affinity chromatography. With these purified proteins, we attempted to reconstruct a complex consisting of the three proteins *in vitro*. After mixing MukF-His₆ with either MukB-His₆ or MukE-His₆, MukF-His₆ was immunoprecipitated using affinity-purified anti-MukF antibodies. MukE-His₆ (Figure 6A, lane 4) or MukB-His₆ (Figure 6A, lane 5) was co-immunoprecipitated with MukF-His₆, demonstrating that we had succeeded in making MukFE and MukFB complexes *in vitro*. In the presence of the three Muk-His₆ proteins in the reaction mixture, both MukE-His₆ and MukB-His₆ were co-immunoprecipitated with MukF-His₆ (Figure 6A, lane 6), suggesting the existence of both MukFE and MukFB complexes.

Next we performed the following experiments. MukF-His₆, MukB-His₆ and MukE-T7-His₆ were mixed and immunoprecipitated with monoclonal antibody against the T7 tag. Under the standard conditions, MukF-His₆ was co-immunoprecipitated with MukE-T7-His₆ efficiently; however, only a small amount of MukB-His₆ was co-immunoprecipitated (Figure 6B, lane 1). This indicated that the MukFE complex was dominant in the reaction,

Fig. 5. Ca^{2+} -binding activity of Muk proteins by $[^{45}\text{Ca}^{2+}]$ overlay. Purified FtsZ, His₆-tagged MukE and MukF, or bovine serum albumin were electrophoresed in SDS-PAGE (10% acrylamide) and blotted on a nitrocellulose membrane. The membrane was incubated in a solution containing $[^{45}\text{Ca}^{2+}]$, autoradiographed (A) and finally stained with 0.1% Amido-Black solution (B). Reading from the left, samples contained 2.5, 5.0 or 10.0 μg of each purified protein. The numbers on the left indicate the molecular size (kilodaltons) of standards used. The density of the autoradiography was measured with MacBAS (Fuji film) and plotted against the amount of the proteins applied (C).

in contrast to the studies with crude cell lysates above, and that the MukFEB complex was only a very minor constituent under these conditions. This may be due to the loss of some unknown factor(s) during purification, essential for formation of the MukFEB complex, or alternatively due to a requirement for an intermediate complex before the final complex is generated. To test the

Fig. 6. Examination of interactions among the purified Muk proteins by immunoprecipitation. (A) Samples of 0.5 μg of purified His₆-tagged Muk proteins were examined by SDS-PAGE (10% acrylamide). Lane 1, MukF; lane 2, MukE; lane 3, MukB. Reaction mixtures containing 0.6 μg of the His₆-tagged Muk proteins were incubated at 37°C for 30 min and subjected to immunoprecipitation using antibody against MukF. The precipitate was analyzed by SDS-PAGE, followed by staining in Coomassie Blue solution. Lane 4, MukF and MukE; lane 5, MukF and MukB; lane 6, MukF, MukE and MukB. (B) Reaction mixtures containing 0.67 μg of His₆-tagged MukF were pre-incubated with the indicated concentration of CaCl_2 (lanes 2–6) or MgCl_2 (lanes 7–11) at 37°C for 15 min. Then 0.40 μg of T7-His₆-tagged MukE and 1.12 μg of His₆-tagged MukB were added, followed by further incubation at 37°C for 30 min. The samples were precipitated with monoclonal antibody against the T7 tag. Positions of MukB, MukF, MukE-T7 and immunoglobulin heavy and light chains are shown with arrows. The numbers in the left lane indicate the molecular size of standards used in parallel with samples (kilodaltons).

former possibility, the cell lysate from *mukFEB* null mutant cells was added to the mixture of the three purified Muk proteins; to test the latter possibility, two of the three proteins were pre-incubated in advance and then the third one was added to the reaction. However, neither of these strategies improved the formation of the complex of the three Muk proteins (data not shown).

Since we had demonstrated that MukF is a Ca^{2+} -binding protein, we investigated whether the addition of Ca^{2+} in the reaction mixture would promote complex formation with the three proteins effectively. In fact, increasing the concentration of Ca^{2+} did result in increasing amounts of MukB-His₆ co-immunoprecipitated with

MukE-T7-His₆ and MukF-His₆ using the anti-T7 monoclonal antibody (Figure 6B, lanes 2–6). This effect of Ca²⁺ was maximal with 10–30 mM CaCl₂. A further increase in the Ca²⁺ concentration had a negative effect on formation of the complex and finally completely inhibited its formation at 100 mM CaCl₂. Importantly, at any concentration of Ca²⁺, none of the Muk proteins were precipitated with control rabbit IgG (data not shown). This indicated that the Muk proteins were not precipitated in a non-specific way, e.g. resulting from aggregation by increasing the concentration of Ca²⁺.

It is known that Mg²⁺ can compete with Ca²⁺ for the binding site of proteins in some cases. A similar experiment was therefore performed using MgCl₂ instead of CaCl₂ in the reaction mixture (Figure 6B, lanes 7–11). Increasing the concentration of Mg²⁺ resulted in co-immunoprecipitation of MukB-His₆, but was slightly less effective than with Ca²⁺. Mg²⁺, however, had an additive effect on formation of the MukFEB complex in the presence of Ca²⁺ (data not shown).

Interestingly, MukF-His₆ was detected as a doublet through SDS-PAGE (see Figure 6A and B), suggesting degradation or some modification of MukF-His₆ such as phosphorylation or another post-translational modification. Amino acid sequence analysis of the two bands of the doublet revealed that both of the protein bands were in fact MukF-His₆, with identical N-termini.

Discussion

We have shown here for the first time that MukF and MukE associate *in vitro* with MukB, all of which are essential for chromosome partitioning in *E. coli*. It is conceivable that the three Muk proteins may also act as a complex *in vivo*, since none of the three proteins are dispensable for proper chromosomal DNA partitioning. To facilitate the analysis of interactions among mutant Muk proteins, we introduced mutations into *mukB* or *mukF* genes in the context of the *mukFEB* operon on a plasmid. From the co-immunoprecipitation experiments using various mutant MukB proteins, mutants that have an amino acid substitution in the C-terminal domain can be classified into three groups. The first group contains MukB1013 and MukB1015, which retain the ability to associate with MukF and MukE. Overexpression of these mutant MukB proteins can suppress temperature-sensitive growth of the *mukFEB* null mutant OT5, when both the wild-type MukF and MukE proteins are also overexpressed (our unpublished data), but not when wild-type MukF and MukE are at the wild-type level (Saleh *et al.*, 1996). This is one piece of *in vivo* genetic evidence suggesting an association between MukB, MukF and MukE. The second group of mutants contains MukB33 and MukB1014, which also retain the ability to associate with MukF and MukE, although in this case overexpression of these MukB mutants with the wild-type MukF and MukE proteins does not suppress the temperature-sensitive growth of OT5 cells. Thus, MukB mutant proteins in this class can associate with MukF and MukE to make a complex; however, the resultant complex is not active because of some other deficiency. This might involve the failure to associate with proteins other than MukF and MukE, or a deficiency in an unknown function residing in the

C-terminal domain of MukB. The third group contains MukB1008, MukB1009 and the C-terminal truncated MukB1–1369 protein. These proteins are unable to make a detectable complex with MukF and MukE, and are thus unable to suppress the temperature-sensitive growth of OT5 cells, even with an excess of MukF and MukE. The properties of this third group of MukB proteins suggest that complex formation of the three Muk proteins is essential for the Muk proteins to exert their activities.

Mutational analysis using truncated MukB or missense MukB mutants shows that the C-terminal globular domain is responsible both for complex formation with MukF and MukE and for the DNA-binding activity. However, it should be noted that the capacities for DNA binding and for complex formation can be separated. MukB1013 is deficient in DNA binding but is capable of forming a complex, confirming that the three Muk proteins associate together in the absence of DNA. On the contrary, MukB1008 and MukB1009 retain the DNA-binding activity but are deficient in complex formation, indicating that MukF and MukE are unnecessary for the DNA-binding activity of MukB. MukB1004 and MukB106, which have an amino acid substitution in or close to the Walker motif A, respectively, retain the ability to associate with MukF and MukE. These MukB mutants are likely to have a defect in the ATP/GTP-binding or ATPase activity, suggesting that nucleotide binding or hydrolysis is not necessary for complex formation.

It is still unclear how many molecules constitute the MukFEB complex because of the large size of MukB molecules compared with MukF and MukE. However, we have some suggestive results, as follows. From sucrose gradient sedimentation experiments, when MukB is absent (Figure 3G) or the mutant MukB1008 protein is unable to associate with MukF or MukE (Figure 3F), MukF and MukE can still form a complex, which has a sedimentation coefficient of ~7.3S. This value corresponds to ~150 kDa in the case of globular proteins. MukF can form a homodimer by itself and MukE forms oligomers dependent on the MukF dimer (Figure 4), suggesting that two MukF molecules and two MukE molecules are brought together into the MukFE complex, resulting in a heterotetramer (155 kDa). MukB can associate with MukF in the absence of MukE, but not with MukE in the absence of MukF (Figures 2B, 3H and I). Mutational analysis of the MukB protein showed that the C-terminal globular domain of MukB is important for MukFEB complex formation. The evidence above suggests that the MukFE complex associates with a MukB homodimer and presumably the MukF subunit, but not MukE, binds directly to the C-terminal globular domain of MukB. By combining two hydrodynamic parameters, the MukFEB complex is predicted to be ~600 kDa (Table II). After subtracting 340 kDa for a MukB dimer from 600 kDa, a mass of 260 kDa remains. Considering that the MukB molecules form a homodimer in an antiparallel arrangement (Melby *et al.*, 1998), the 260 kDa may correspond to two sets of the MukFE complex.

Previously, we predicted that regulation of MukF activity may involve Ca²⁺ binding (Yamanaka *et al.*, 1996), by analogy with the sequence of calreticulin in eukaryotic cells. Calreticulin is a ubiquitous major Ca²⁺-binding protein localized in the endoplasmic reticulum (Michalak

Norris *et al.* (1988) have previously proposed that fluctuations in the intracellular level of free Ca^{2+} could provide a mechanism for the initiation of DNA replication, chromosome partitioning and cell division. Although there is circumstantial evidence that Ca^{2+} is implicated in cell cycle events, any regulatory role for Ca^{2+} in *E. coli* cells *in vivo* is still obscure. To date, there have been some reports on the presence of prokaryotic calmodulin-like proteins (Laoudj *et al.*, 1994; Smith, 1995). However, detailed analysis of bacterial calmodulin-like proteins and, importantly, their corresponding genes has not yet been described. In this study, we demonstrated that the gene product of *mukF* is a Ca^{2+} -binding protein. The optimum concentration of Ca^{2+} necessary for MukFEB complex formation *in vitro* is substantially higher than the values reported for the average concentration of free Ca^{2+} (0.1 μM) in *E. coli* cells *in vivo* (Gangola and Rosen, 1987). The high concentration required for the formation of the MukFEB complex *in vitro* may be placed in better perspective by the fact that one genome equivalent of *E. coli* can bind 7.8×10^5 Ca^{2+} ions and that the nucleoid may act as a Ca^{2+} -chelating buffer (Smith, 1995). From this value, the Ca^{2+} concentration in the nucleoid is ~3–4 mM. We also found that FtsZ, a tubulin homolog in *E. coli* that forms a constriction ring at the division site, shows a higher level of Ca^{2+} -binding activity compared with MukF. However, it is controversial whether Ca^{2+} plays a physiological role in the organization of the FtsZ ring *in vivo* (Yu and Margolin, 1997; Mukherjee and Lutkenhaus, 1999).

The significance of the association of MukF and MukE with MukB still remains to be assessed. Eukaryotic calmodulin binds cytoplasmic dynein from sea urchin in a calcium-dependent manner and the dynein ATPase activity is stimulated by calmodulin (Hisanaga and Pratt, 1984). In addition, Ca^{2+} regulates myosin activity by binding to the regulatory light chain directly or through the activation of myosin light chain kinase by calmodulin. Since the overproduction of MukF, but not MukE or MukB, in wild-type cells results in the production of anucleate cells, mimicking the deletion of any of the *muk* genes (Yamanaka *et al.*, 1996), it is feasible that Ca^{2+} -dependent MukF, in association with MukE, which may also bind Ca^{2+} weakly, modulates the biochemical activities of MukB, such as the DNA-binding and ATPase activity.

MukF, MukE and MukB have been found so far only in *E. coli*, *Haemophilus influenzae* (Fleischmann *et al.*, 1995), *Vibrio cholerae* (Melby *et al.*, 1998), *Haemophilus actinomycetemcomitans*, *Yersinia pestis* (H.P. Erickson, personal communication and <http://note.cellbio.duke.edu/Faculty/7EErickson/7ENucleus>) and *Salmonella typhi* (sequence data were obtained from the Sanger Center via the website at http://www.sanger.ac.uk/Projects/S_typhi/blast_server.shtml). Among these bacterial species, amino acid sequences are well conserved in MukB, particularly in N- and C-terminal globular domains (Figure 7A and B), MukF (Figure 7C) and MukE (our unpublished data). It should be noted that point mutations in MukB used in this study affect well-conserved amino acids among different bacterial species.

Materials and methods

Bacterial strains and media

All strains used are derivatives of *E. coli* K-12. MC1061 (*araD139* Δ [*ara*, *leu*]*7697* Δ *lacX74* *galU* *galK* *hsr* *hsm*⁺ *strA*) (Casadaban and Cohen,

1980) and its derivative MS8 (MC1061 Δ *rnhA::cat*) (Ohmori *et al.*, 1995). XL1-blue (*recA1* *endA1* *gyrA96* *thi-1* *hsdR17* *supE44* *recA1* *lac* [*F'* *proAB* *lacI*^q *lacZ* Δ M15 Tn10]) was used as a host for both general cloning and protein purification. PB103 (*dadR* *trpE61* *trpA62* *tna-5*) (de Boer *et al.*, 1989), A33 (PB103 *mukF::kan*), A34 (PB103 *mukE::kan*) (laboratory strains) and GC7528 (PB103 Δ *mukB::kan*) (Niki *et al.*, 1991) were used. The site-specific integration of prophage λ DE3 into the chromosome of a host cell was performed using the λ DE3 Lysogenization Kit (Novagen). L medium (1% Bacto-tryptone, 0.5% Bacto-yeast extract and 0.5% NaCl, pH 7.4) and M9 medium supplemented with 0.5% glucose, 1 mM MgSO_4 and required L-form amino acids (50 $\mu\text{g}/\text{ml}$ each) were used for cell growth. When necessary, antibiotics were added to media as follows if not specified: ampicillin, 100 $\mu\text{g}/\text{ml}$; tetracycline, 15 $\mu\text{g}/\text{ml}$; kanamycin, 30 $\mu\text{g}/\text{ml}$; chloramphenicol, 30 $\mu\text{g}/\text{ml}$; streptomycin, 120 $\mu\text{g}/\text{ml}$.

Plasmids

A summary of plasmids used in this study is depicted in Figure 1. Plasmid pAX850 carries the 8.5 kb *Bam*HI fragment containing the *mukFEB* operon (Yamanaka *et al.*, 1996). Plasmids pMY1001, pMY1002, pMY1004, pMY1006, pMY1003 and pMY1005 were constructed by replacing the 4.6 kb *Hind*III fragment in pAX850 with the corresponding fragments in pCX13, pCX9, pCX14, pCX8 (Saleh *et al.*, 1996), pCX16 and pCX15, respectively (our unpublished data). Plasmids pKX733 and pKX732 carry mutations *mukB33* and *mukB106* in the *mukFEB* operon on pACYC184, respectively (Yamanaka *et al.*, 1994a). Elimination of the *Kpn*I site in *mukB* gave rise to C-terminal truncation of MukB in the *mukFEB* operon (pMY1007). Introduction of a frameshift at the *Cl*aI site in *mukF*, the *N*siI site in *mukE* and the *B*stEII site in the *mukB* gene on pAX850 resulted in elimination of each gene (pMY1008, pMY1009 and pKX746, respectively). Plasmid pMY1010 carries the *mukFEB* operon with the *mukF233* mutation, which was transferred from plasmid pKX305 (Yamanaka *et al.*, 1996). Plasmids pKX748, pKX766, pKX768, pKX750 and pAX804 were described previously (Yamanaka *et al.*, 1996).

mukF and *mukE* genes were amplified by PCR and cloned into an expression vector pQE60 (Qiagen), yielding plasmids pKX789 and pMY105, respectively. Both the N- and C-terminal regions in the *mukB* gene were amplified by PCR and ligated into plasmid pQE60, together with the 3.6 kb *Xho*I–*Kpn*I fragment containing the middle portion of *mukB* from pAX804 (Niki *et al.*, 1991). The resultant plasmid was named pMY171. Addition of isopropyl- β -D-thiogalactopyranoside (IPTG) induces production of MukF, MukE and MukB tagged with His₆ at their C-terminus, encoded by pKX789, pMY105 and pMY171, respectively. Plasmids pMY155 and pMY156 are derivatives of pQE60, which can express wild-type MukB and mutant MukB1004 (Lys-40 to Ile) proteins without any tags, respectively. A pET21a DNA (Novagen) was digested with *Eco*RI, filled in with Klenow enzyme and recircularized with ligase, resulting in pET21a Δ RI. PCR-amplified *mukE* gene was ligated with the *Nco*I–*Nde*I-digested pET28b DNA, yielding pMY119. Expression plasmid pMY119 expresses MukE protein tagged with T7-His₆ at its C-terminus. An *Xba*I–*Bam*HI fragment from pMY119 was ligated with *Xba*I–*Bam*HI-digested pET21a Δ RI DNA, resulting in pMY134. Plasmid pMY134 expresses MukE tagged with the T7 peptide at its C-terminus. A 4.2 kb *Av*aII–*Bam*HI fragment from pAX850 was filled in with Klenow enzyme and ligated with pRSETB DNA (Invitrogen), which was digested with *Sac*I and filled in. The resulting plasmid pMY121 expresses the N-terminal truncated MukB (MukB255–1486), tagged with His₆ at its N-terminus. All of the tagged wild-type Muk proteins function normally *in vivo* because expression of the plasmid-encoded *muk* gene suppressed temperature-sensitive growth of the corresponding null mutant cell.

Construction of a *mukFEB* deletion mutant

The kanamycin-resistance-conferring gene cassette from pACYC177 (Chang and Cohen, 1978) (1.4 kb *Hae*II fragment) was replaced with a 2.9 kb *Xho*I fragment on pAX850. The *muk* gene fragment with the kanamycin-resistance-conferring gene was excised and inserted into a *Bam*HI site in plasmid pKH5002 (Ohmori *et al.*, 1995). The resulting construct was introduced into strain MC1061 at 22°C and kanamycin-resistant (20 $\mu\text{g}/\text{ml}$), streptomycin-resistant (120 $\mu\text{g}/\text{ml}$) and ampicillin-sensitive (50 $\mu\text{g}/\text{ml}$) transformants, which carry the deleted *mukFEB* genes in the chromosome, were confirmed by Southern hybridization. The resulting strain was named OT5. The disrupted *mukFEB* genes in strain OT5 could be transduced with phage P1vir into the wild-type strain PB103 only below 25°C in the presence of kanamycin. One of the transductants, OT7, was used for further analysis.

Purification of His₆-tagged Muk proteins

MukF-His₆, MukE-His₆ and MukB-His₆ were overexpressed in *E. coli* strain YM198 (XL1-blue[pKX789]), YM171 (XL1-blue[pMY105]) and YM944 (XL1-blue[pMY171]), respectively. MukE-T7-His₆ was overexpressed in YM334 (XL1-blue[λDE3, pMY119]). Cells were grown at 30°C in 1 l of L medium containing tetracycline and 1% glucose and supplemented with ampicillin (YM198, YM171, YM944) or kanamycin (YM334). When the turbidity of the cells reached 50 Klett units (Klett–Summerson colorimeter with a No. 54 green filter), 1 mM IPTG was added. The cells were grown at 30°C for a further 2 h before harvesting. The cell pellets were resuspended with 20 ml of sonication buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10% glycerol, pH 8.0) with 0.5 mg/ml lysozyme and incubated on ice for 30 min. The cells were lysed by three cycles of freeze–thawing and sonication. Cell lysates were cleared by centrifugation at 75 600 g at 4°C for 30 min, followed by passage through a 0.45 µm filter. Chelating Sepharose FF (Pharmacia) was immobilized with Ni²⁺ and equilibrated with sonication buffer. Four milliliters of a Sepharose suspension for MukF or MukE and 8 ml for MukB were used for purifying each His₆-tagged protein. The supernatant was loaded onto a Chelating Sepharose FF column at a flow rate of 0.4 ml/min. The column was washed with 15 vols of the sonication buffer and then 20 vols of the sonication buffer with 20 mM imidazole. Bound proteins were eluted with a 20–500 mM linear gradient of imidazole in the sonication buffer. Fractions containing the His₆-tagged proteins were pooled and precipitated with 35% ammonium sulfate. The precipitated proteins were dissolved with a small volume of buffer A [25 mM HEPES–KOH, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 25 mM NaCl, 10% glycerol, pH 7.6] and dialyzed extensively against buffer A. The MukB-His₆ protein was applied to a Fractogel EMD DEAE column (4 ml, from Merck). Bound proteins were eluted with a 25 mM–1 M linear gradient of NaCl in buffer A. The peak fractions were pooled, dialyzed with buffer B (25 mM HEPES–KOH, 0.1 mM EDTA, 2 mM DTT, 25 mM KCl, 10% glycerol, pH 7.6) and stored at –80°C. The MukF-His₆ protein was applied to a Q Sepharose HP column (3 ml, from Pharmacia). Bound proteins were eluted with a 25 mM–1 M linear gradient of NaCl in buffer A. The peak fractions were pooled, concentrated with Centricon 10 (Millipore) and loaded onto a Superdex 200 HR10/30 column (Pharmacia) equilibrated with buffer C (25 mM HEPES–KOH, 0.1 mM EDTA, 1 mM DTT, 0.2 M NaCl, 10% glycerol, pH 7.6). Fractions containing MukF-His₆ were pooled, dialyzed against buffer B, concentrated with Centricon 10 and stored at –80°C. The MukE-His₆ or MukE-T7-His₆ proteins were purified further in a Superdex 200 HR10/30 column equilibrated with buffer C. Fractions containing MukE were pooled, dialyzed against buffer B, concentrated with Centricon 10, and stored at –80°C. All the His₆-tagged proteins had high purity (>95% as judged by SDS–polyacrylamide gels).

Antibodies

Purified His₆-tagged MukF, MukE and MukB proteins were fractionated further by SDS–PAGE. After staining the gels with 0.3 M CuCl₂ (Lee *et al.*, 1987), the corresponding bands were trimmed and sent to Panapharm Laboratories Co., Ltd for raising antibodies in rabbits. Sera were affinity purified using immobilized proteins on a nitrocellulose membrane (Pringle *et al.*, 1991). The purified antibodies did not cross-react with each other, proving the specificity of the antibodies. Anti-T7-tag monoclonal antibody directed against the polypeptide of 11 amino acids (MASMTGGQMQG) was purchased from Novagen.

In vivo labeling of proteins

Cells having an indicated plasmid were grown at 22°C in M9 medium supplemented with 0.5% glucose, 1 mM MgSO₄ and L-form amino acids (50 µg/ml each), except for methionine and cysteine, and containing appropriate antibiotics. When the turbidity reached 80 Klett units, 1.2 ml of cells was removed, labeled with 4 µl of redivue Pro-mix L-[³⁵S] *in vitro* cell labeling mix (37 TBq/mmol; Amersham) at 37°C for 15 min and chased with 0.2 mg/ml unlabeled methionine and cysteine at 37°C for 2 min with agitation. The labeled cells were harvested by centrifugation and washed three times with saline. After the final wash, the cell pellet was suspended with 0.1 ml of buffer YA (25 mM HEPES–KOH, 100 mM NaCl, 5% glycerol, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, pH 7.6) supplemented with 1.0 mg/ml of lysozyme, incubated on ice for 30 min and subjected to three cycles of freeze–thawing. The cleared lysate was prepared by centrifugation at 15 000 g at 4°C for 20 min, followed by addition of NP-40 at 0.2%.

Immunoprecipitation

Reaction mixture in buffer YA was mixed with 1 µg of affinity-purified polyclonal antibodies or T7 monoclonal antibody. After 1 h on ice, a

50 µl slurry of 30% protein A–Sepharose 4FF (Pharmacia) pre-equilibrated with buffer YA was added. The samples were mixed at 4°C with gentle mixing for 1 h and centrifuged at 8000 g for 1 min. The precipitated Sepharose was washed six times with 1 ml of ice-cold buffer YA, suspended with 25 µl of SDS–PAGE loading buffer, boiled for 5 min and centrifuged briefly at room temperature. The supernatant was analyzed by SDS–10% PAGE. Where non-labeled proteins were used, the gel was stained with Coomassie Brilliant Blue R250. Where radioisotope-labeled proteins were used, dried gel was autoradiographed using a BAS2000 image analyzer (Fuji film).

Sucrose gradient sedimentation and gel filtration

Cells were grown in 20 ml of L medium supplemented with appropriate antibiotics where necessary. When the turbidity of the culture reached 80 Klett units, cells were harvested by centrifugation, washed with 10 ml of 25 mM HEPES–KOH pH 7.6 and finally suspended with 200 µl of buffer B containing 0.5 mg/ml lysozyme. The cell suspension was incubated on ice for 30 min and treated with three cycles of freeze–thawing. Then 3 µl of 1 M MgCl₂ and 1 µl of 20 mg/ml DNase were added to the cell suspension. After incubation at 25°C for 1 h, the supernatant was recovered by centrifugation for 30 min at 4°C. A 2 ml sucrose gradient (10–60%, w/w) was prepared by layering 0.36 ml each of 10, 17.5, 25, 32.5 and 40% sucrose onto 0.20 ml of 60% (w/w) sucrose (in 25 mM HEPES–KOH pH 7.6, 25 mM KCl, 0.1 mM EDTA, 2 mM DTT, 10% glycerol) in a polyallomer centrifuge tube (Beckman; 11 × 34 mm). The tubes with the sucrose solution were incubated at 37°C for 1 h and then at 4°C for 1 h before use. The cell lysate in 70 µl was loaded onto the gradient and centrifuged at 55 000 r.p.m. at 4°C for 13 h in a Beckman TLS-55 rotor. Approximately 100 µl of fractions were collected from the bottom of the tube after centrifugation.

The same lysates were subjected to gel filtration column chromatography using a Superose 6 HR10/30 column (Pharmacia; 1.0 × 30 cm). The standard proteins (Stokes' radius) used were as follows: thyroglobulin (8.50 nm), ferritin (6.10 nm), catalase (5.22 nm), aldolase (4.81 nm), bovine serum albumin (3.55 nm), ovalbumin (3.05 nm) and chymotrypsinogen A (2.09 nm).

[⁴⁵Ca²⁺] overlay experiment

The [⁴⁵Ca²⁺] overlay experiment was performed according to the method of Maruyama *et al.* (1984). Purified proteins were subjected to SDS–PAGE and transferred to a nitrocellulose membrane. The membrane was washed in a solution containing 60 mM KCl, 5 mM MgCl₂ and 10 mM imidazole–HCl pH 6.8 three times in an hour. Then the membrane was incubated in the same solution containing 40 kBq/ml of radioactive calcium ions [⁴⁵Ca²⁺] (0.185–1.85 GBq/mg Ca; Amersham), washed in distilled water for 10 min and dried at room temperature for 3 h. Autoradiography was performed by a BAS2000 image analyzer and MacBAS program (Fuji Film). After autoradiography, proteins on the membrane were stained in an Amido-Black solution.

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