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Original Article

Effects of forcedly expressed MCM2-7 proteins on nuclear structure in HeLa cells

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ABSTRACT

MCM2-7 hexamer functions as a DNA helicase that unwinds DNA duplex at the DNA replication fork. Here we examined the effects of forcedly expressed MCM (minichromosome maintenance) 2-7 proteins on nuclear structure in HeLa cells. Expressed wild-type MCM3 and MCM5 proteins, which were mainly localized in nuclei, induced the formation of micronuclei-like structure and multi-nuclei, respectively. Mutagenesis at ATP-binding motifs in MCM3 cancelled the abnormal nuclear structure formation but mutagenesis in MCM5 did not. Expression of wild-type MCM2 and MCM7 resulted in the formation of the micronuclei-like structure and/or the multi-nuclei at lesser extents in a ATP-binding site-dependent manner. The results suggest that the presence of extra-amounts of MCM3 and MCM5 exerts strong negative effects on MCM2-7 function. Expressed wild-type MCM2-7 proteins except for MCM6 were largely localized in nuclei but MCM4 and MCM7 mutated at ATP-binding sites were mainly localized in cytoplasm. These results suggest that the ATP-binding activities of MCM4 and MCM7 are required for nuclear localization and/or chromatin binding of MCM2-7 complex.

KEYWORDS: MCM2-7, ATP hydrolysis, nuclear structure, chromatin binding, cellular localization.

INTRODUCTION

DNA replication in eukaryotic cells is driven by a replicative DNA helicase called CMG complex that consists of CDC45, MCM2-7 and GINS [1-4]. All six members of MCM2-7 contain a region involved in ATP binding and hydrolysis in their carboxyl-terminal half. MCM2-7 proteins form a hexameric ring-shaped structure in which the proteins are positioned by the order of 2-6-4-7-3-5. The MCM2-7 complex acts as a molecular motor for the CMG complex translocated on a single-stranded DNA. The MCM2-7 hexameric complex consistently exhibits DNA-dependent ATPase activity. Studies using mutants at ATP-binding sites of MCM2-7 indicate that the sites in MCM4, MCM6 and MCM7 play essential function in the ATPase activity but those in MCM2, MCM3 and MCM5 play regulatory function in the activity [5]. It has been shown that the MCM2-7 complex exhibits weak DNA helicase activity under limited conditions [6], and association of CDC45 and GINS to the MCM2-7 complex is required for exhibiting higher level of DNA helicase activity [7-9]. The analysis of *Drosophila* CMG complex bound to the DNA with fork-like structure indicates that CDC45 mainly interacts with MCM2, while GINS mainly interacts with MCM3 and MCM5, and it also indicates that MCM4, MCM6 and MCM7 proteins, which are neighborly aligned in the MCM2-7 hexamer, directly interact with the single-stranded DNA through specific amino-acids [10]. These results suggest that MCM4, MCM6 and MCM7 are directly involved in MCM2-7 helicase function. Consistent with this notion, human and mouse MCM4, MCM6 and MCM7 proteins exhibit DNA helicase activity by forming a hexameric complex, and mutational analyses of mouse MCM4/6/7 complexes indicate that ATP-binding motifs in MCM7 play essential

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roles in ATPase and DNA helicase activities [11]. However, the notion on the different roles of MCM2-7 proteins in MCM2-7 helicase function remains to be further explored.

Initiation of DNA replication begins by the binding of MCM2-7 complexes onto a replication origin with assistance of two MCM loader proteins of CDC6 and CDT1. After binding of CDC45 and GINS to MCM2-7 complexes on the origin in the presence of CDK, phosphorylation of MCM4 and MCM6 with DDK is required for activation of MCM helicase [12, 13]. Recently it has been reported that ATP-binding and hydrolysis activities of MCM2-7 proteins are required not only for DNA helicase function but for stable binding of MCM2-7 complex to the origin in *S. cerevisiae*, since the mutant MCM2-7 complexes containing MCM2-7 proteins mutated at ATP-binding sites were not retained at origin in reaction mixtures *in vitro* and therefore DNA replication was not initiated [14, 15]. The initiation of DNA replication was arrested at different stages in the reaction mixtures containing the mutant MCM2-7 complexes, depending upon which member of MCM2-7 was mutated at their ATP-binding site. However, these notions remain to be verified in higher eukaryotic cells for generality.

Here we examined the effect of forcedly expressed wild-type and mutant MCM2-7 proteins on nuclear structure in HeLa cells. The results indicate that the expression of wild-type MCM3 and MCM5 resulted in the formation of micronuclei-like structure and multi-nuclei, respectively, suggesting that these MCM proteins exert negative effects on MCM2-7 function. The results also indicate that expression of MCM2 and MCM7 induced the formation of abnormal nuclear structures at lesser extents. ATP-binding activities of the MCM proteins except for MCM5 were required for the negative effect on nuclear structures. Each of the wild-type MCM2-7 proteins except for MCM6 was mainly localized in the nuclei, and MCM2, MCM3 and MCM5 proteins mutated at the ATP-binding site were also localized in the nuclei. However, the mutant MCM4 and MCM7 were localized in the cytoplasm, suggesting that their ATP-binding ability is required for nuclear localization and/or chromatin binding of MCM complexes.

MATERIALS AND METHODS

Site-directed mutagenesis of human MCM2-7 genes

Site-directed mutagenesis was performed according to the manufacturer's protocol (Stratagene). Human MCM2-7 cDNA with nucleotides for flag peptide at amino-terminus were cloned in pTRE-Tight vector (Clontech). For converting DE residues at walker motif B in MCM2 to AA by polymerase chain reaction (DEAA mutant), a forward primer of 5'-GGAGTGTGTCTCATTGCTGCATTTGACAAGATG-3' was used. For construction of MCM3 mutant (DEAA) and MCM5 mutant (DEAA) by polymerase chain reactions, a forward primer of 5'-GCGTGGTTTGCATTGCTGCATTTGACAAAATGTC-3' and a primer of 5'-GTCGTCTGTATTGCCGCGTTTGACAAGATGC-3' were used, respectively. For construction of mutant MCM4 (D574Y) and MCM7 (K387A), a forward primer of 5'-CGGCATCTGCTGTATCTATGAGTTCGACAAG-3' and a primer of 5'-GATCCTGGTGTGGCCGCGTCTCAGCTCCTG-3' were used, respectively. The mutated MCM4 and MCM7 genes were recloned into pVL1392 vector (Pharmingen, BD). To generate recombinant baculovirus for expression of the MCM4 and MCM7 proteins, the DNA purified by Endotoxin-free system (Qiagen) was co-transfected with linealized baculovirus DNA into Sf9 cells according to the procedures described by AB vector (ProEasy, AB Vector) and recombinant baculoviruses were isolated following the manufacturer's protocol. Preparation of recombinant baculoviruses for expression of human wild-type MCM4 and MCM6 protein has been reported [16].

Expression of MCM2-7 in 293T cells

Wild-type and mutant MCM2-7 genes cloned into pTRE-Tight vector was co-transfected with Tet-off plasmid (Clontech) at a ratio of 1:1 into 293T cells and the cells were cultured for 2 days. The cells (2×10^6) were lysed with 100 μl of buffer containing 0.1% Triton X-100, as described previously [16]. After spinning, the supernatant was recovered and used as Triton-soluble fraction (S fraction). The S fraction (100 μl) was loaded onto a linear glycerol gradient ranging from 15%

to 30% and centrifuged at 36000 rpm for 11 h (TLS55, Beckman). Proteins in separated fractions were analyzed by SDS-polyacrylamide gel electrophoresis and detected by western-blotting using anti-flag antibody (Sigma-Aldrich) or anti-myc antibody (sc-40, 9E10, Santa Cruz Bio.) [16]. Anti-MCM6 polyclonal antibody was also used [17].

Expression of MCM2-7 in HeLa cells

MCM2-7 genes in pTRE-Tight vector were transfected with Tet-off plasmid into HeLa cells on coverslips in four-well chambers (Nunclon) and the cells were cultured for 2 days. The cells were fixed by incubation with 4% paraformaldehyde in phosphate-buffered saline for 5 min at room temperature, and then permeabilized and blocked, as described previously [16]. Incubation of the cells with anti-flag or anti-myc mouse antibody (2.5 μg/ml) was performed overnight at 4 °C in the blocking solution. Cells were washed with the same solution and then incubated with Cy3-conjugated anti-mouse antibodies (Jackson Immuno-Research) for 1.5 h at 37 °C. Washed cells were stained with 2 μg/ml DAPI for 5 min at room temperature. After washing with PBS, cells were observed using fluorescence microscopy (BZ9000, KEYENCE).

Preparation of MCM4/6/7 complex and DNA helicase activity

High5 cells (2 x 10^7 cells) in a dish of diameter 15 cm were co-infected with three viruses expressing His-MCM4, MCM6 and His-MCM7 proteins (0.7 ml of each viral stock solution) for 2 days. MCM4/6/7 complex was purified from the cells in one dish, and DNA helicase activity was measured essentially as reported previously [17]. The reaction contained 10 mM ATP, 10 mM Mg-acetate, and approximately ~2.5 fmol of DNA with replication fork-like structure. The DNA consists of 21 base pairs of duplex region and poly dT tails of 40 and 60 mers.

RESULTS

Expression of wild-type and mutant MCM3 in human cells

MCM2-7 proteins, which belong to AAA+ family, contains walker A and walker B motifs that are responsible for ATP/Mg binding (Fig. 1A). Wild-type MCM3 cDNA with nucleotide sequences for flag-tag was cloned in pTRE-tight vector and it was transfected into 293T cells. The 293T cells were fractionated into Triton-soluble and -insoluble fractions, and the soluble fraction was fractionated by glycerol gradient centrifugation (Fig. 1B). The MCM3 was recovered in fraction no. 10 and also in fraction no. 6 and 7 where a hexameric MCM2-7 complex sediments, as detected by anti-MCM6 antibody. These results suggest that the MCM3 binds with MCM2-7 proteins to assemble into MCM complexes containing the MCM2-7 hexamer in the cells. The flag-MCM3 was expressed in HeLa cells on coverslips and it was detected by staining with anti-flag antibody and with DAPI (Fig. 1C). The MCM3 was detected in nucleus, and the results are consistent with the notion that signals for nuclear localization are present in carboxyl-terminal half region of MCM3 [18]. We focused on nuclear structures of flag-positive cells, and their nuclear structures were classified into five groups; cells with normal nuclear structure (normal), two cells that are adjacently localized (neighboring two cells), cells with more than two nuclei (multi-nuclei), cells with fragmented or small nuclei (micronuclei-like structure) and cells without round-shaped nuclei (not round-shaped) (Supplementary Fig. 1). In the cells expressing wild-type MCM3, the nucleus was fragmented into small pieces in about 34% of the flag-positive cells (Fig. 1C and 1E). They resemble the cells with micronuclei, and we call these as the cells with micronuclei-like structure. The micronuclei are produced by DNA breakages [19], which can be generated by prolonged stalling of the DNA replication fork. Also the cells with two nuclei in a cell were detected at about 8% of the flag-positive cells. We call these cells as the cells with multi-nuclei that can be produced by incompletion of DNA replication in S phase and also by failure in nuclear division in M phase. It is assumed that the amounts of the forcedly expressed wild-type MCM3 are comparable or superior to that of endogenous MCM3 protein in the flag-positive cells. Thus it is suggested that the presence of extra-amounts of MCM3 in HeLa cells is hazardous for DNA replication and/or for cell division, as they most probably disturb MCM2-7 helicase function.

Walker motif B of MCM3 contains amino acid residues of DE that play critical function for binding with ATP/Mg. DE were changed to AA to generate a mutant DEAA MCM3 (Fig. 1A). The

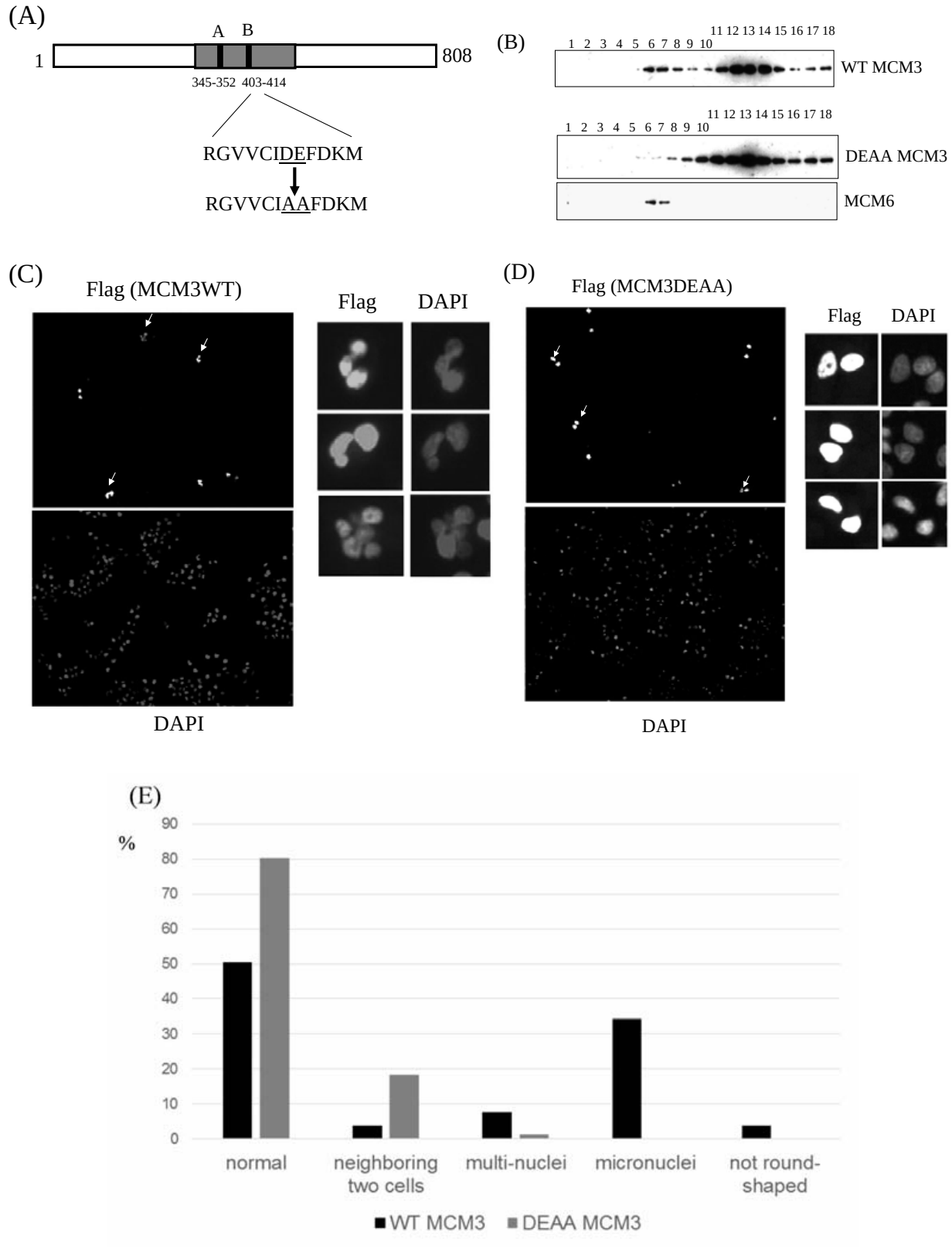
(A)
A B
1
808
345-352 403-414
RGVVCIDEFDKM
RGVVCIAAFDKM
(B)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
WT MCM3
DEAA MCM3
MCM6
(C)
Flag (MCM3WT)
Flag
DAPI
DAPI
(D)
Flag (MCM3DEAA)
Flag
DAPI
DAPI
(E)
%
90
80
70
60
50
40
30
20
10
0
normal
neighboring two cells
multi-nuclei
micronuclei
not round-shaped
WT MCM3
DEAA MCM3

Fig. 1

DEAA mutant of MCM3 was expressed in 293T cells and Triton-soluble fraction was fractionated by glycerol gradient centrifugation (Fig. 1B). The mutant MCM3 was recovered in fraction no. 10, suggesting that it binds with endogenous MCM2-7 proteins to assemble into MCM complexes but the complexes are smaller than MCM2-7 hexamer in the cells (Fig. 1B). Since the mutant MCM3 was not detected in fraction no. 6-7, the mutation may affect formation of MCM2-7 hexamer. The mutant MCM3 expressed in HeLa cells was detected in the nucleus but micronuclei-like structure detected by the expression of wild-type MCM3 was not observed (Fig. 1D & 1E). It was observed that two flag-positive cells are adjacently localized in a significant proportion (18%). These cells may be derived from those arrested in M phase and also those with mitotic chromosomal bridge in which two replicated nuclei are connected by unreplicated chromosomal region. These results suggest that the hazardous effect of expressed wild-type MCM3 on DNA replication basically requires its ATP-binding ability.

Expression of wild-type and mutant MCM5 in human cells

Wild-type and the mutant MCM5 (DEAA) where DE residues in walker motif B are converted to AA were expressed in 293T cells (Fig. 2A). The results on glycerol gradient centrifugation indicate that both wild-type and the mutant MCM5 were recovered at fraction no. 12, suggesting that they interact with endogenous MCM2-7 proteins to form MCM complexes smaller than MCM2-7 hexamer (Fig. 2B). Both wild-type and the mutant MCM5 expressed in HeLa cells were mainly detected in the nucleus but also detected in the cytoplasm (Fig. 2C & 2D). MCM5 that does not retain canonical nuclear localization signal may bind to MCM3 to enter nucleus. The cells with multi-nuclei (two nuclei) were frequently detected in cells expressing wild-type MCM5 (about 36%) and they were also detected in the cells expressing the mutant MCM5 (about 24%) (Fig. 2E). The cells without round-shaped nuclear structures were detected in the presence of wild-type and the mutant MCM5 in a small proportion. They can be generated by disturbing the DNA replication, as reported previously [16]. These results suggest that the presence of expressed wild-type and the mutant MCM5 is inhibitory for DNA replication in S phase and/or for nuclear division in M phase. The expression of both wild-type and the mutant MCM5 results in the generation of abnormal nuclear structures but ATP-binding ability of MCM5 is not required for the negative effects on nuclear structures; the results are in contrast with those for the expression of MCM3.

Expression of wild-type and mutant MCM2 in human cells

Wild-type MCM2 and the mutant MCM2 (DEAA) where DE residues in walker motif B are converted to AA were expressed in 293T cells (Fig. 3A). The Triton-soluble fraction was fractionated by glycerol gradient centrifugation (Fig. 3B). Wild-type MCM2 and the mutant MCM2 were mainly recovered in fraction no. 13-14 and no. 11-13, respectively. These results suggest that expressed flag-MCM2 binds to endogenous MCM3-7 proteins to form MCM

Legend to Fig. 1. Expression of wild-type and mutant MCM3 in human cells.
(A) MCM-box in the central domain where the Walker A and B motif are located is indicated in MCM3. Amino acid sequences of the Walker B motif for wild-type and mutant MCM3 are indicated. (B) Flag-tagged wild-type MCM3 and the mutant MCM3 mutated at walker motif B (DEAA) were expressed in 293T cells, and Triton-soluble fraction was fractionated by glycerol gradient centrifugation into 18 fractions. After SDS-gel electrophoresis of proteins in the fractions, western-blotting was performed using anti-flag antibody to detect wild-type MCM3 and anti-MCM6 antibody to detect endogenous MCM6. (C) Flag-tagged MCM3 proteins of wild-type were expressed in HeLa cells and they were detected by staining with anti-flag antibody. Pictures of staining with anti-flag antibody and with DAPI are shown on the left side. On the right side, enlarged pictures of selected cells (micronuclei) indicated by arrows in left are shown. (D) Flag-tagged mutant MCM3 (DEAA) expressed in HeLa cells was detected. On the right side, enlarged pictures of selected cells (normal) indicated by arrows in left are shown. (E) Percentages of the cells with normal nuclear structure and those with abnormal structures such as micronuclei-like structure, multi-nuclei (two nuclei), and no round-shaped nuclei, are shown. Two cells that are closely located each other are also identified as neighboring two cells. In total, 105 flag-positive cells for wild-type MCM3 and 76 cells for the mutant MCM3 were examined.

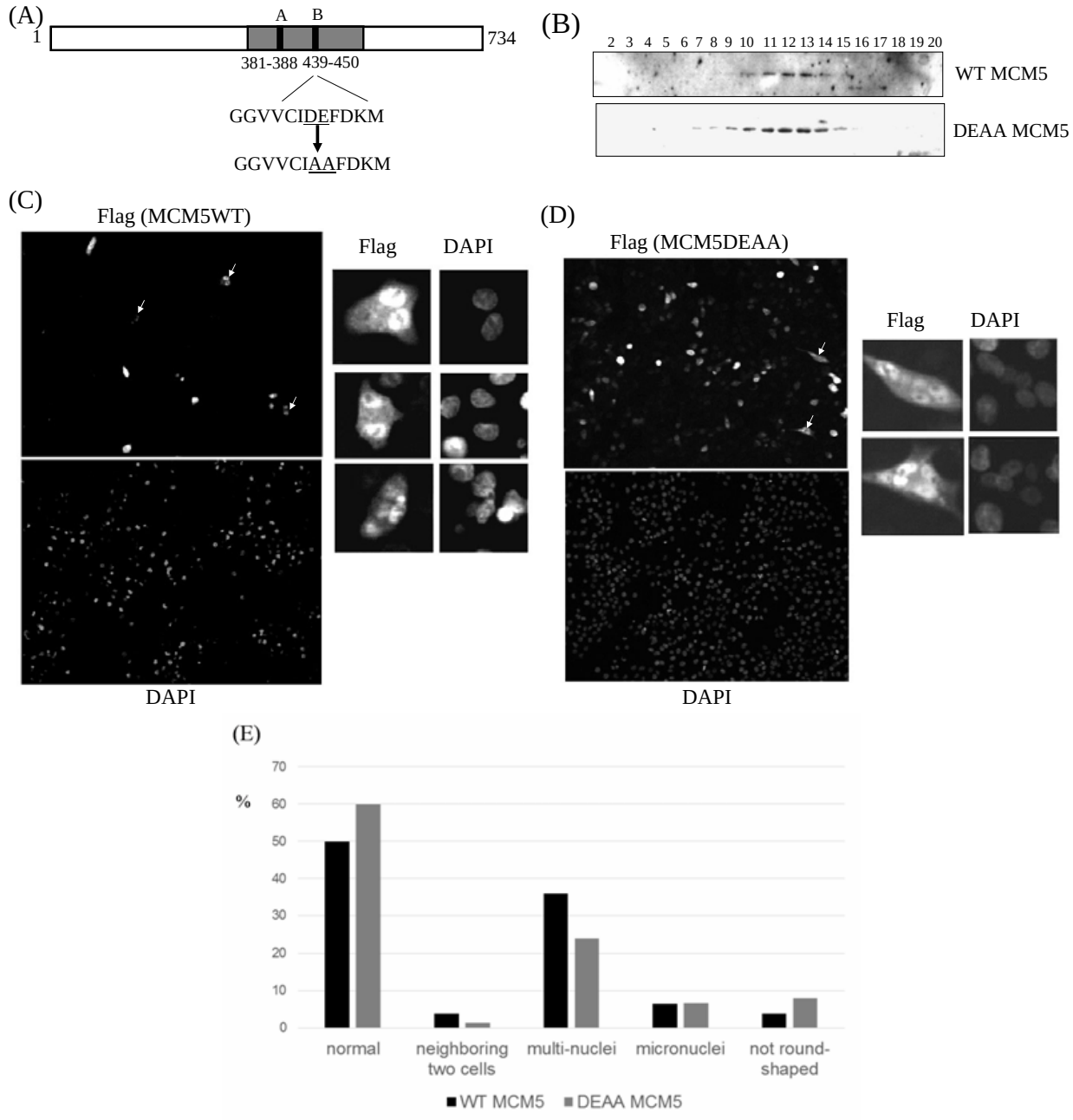

Fig. 2. Expression of wild-type and mutant MCM5 in human cells.
(A) Walker A and B motif in MCM-box of MCM5 are indicated and amino-acid sequences of Walker B motif for wild-type and mutant (DEAA) MCM5 are indicated. (B) Flag-tagged wild-type MCM5 and the mutant MCM5 mutated at walker motif B were expressed in 293T cells, and Triton-soluble fraction was fractionated by glycerol gradient centrifugation into 20 fractions. After SDS-gel electrophoresis of proteins in the fractions (no. 2-20), western-blotting was performed using anti-flag antibody. (C) Flag-tagged wild-type MCM5 protein was expressed in HeLa cells and they were detected with anti-flag antibody. Pictures of staining with anti-flag antibody and with DAPI are shown on the left side. On the right side, enlarged pictures of selected cells (multi-nuclei) indicated by arrows in left are shown. (D) Flag-tagged mutant MCM5 protein expressed in HeLa cells were detected with anti-flag antibody. On the right side, enlarged pictures of selected cells (multi-nuclei) indicated by arrows in left are shown. (E) Percentages of the cells with normal nuclear structure and those with abnormal nuclear structures are shown. In total, 78 flag-positive cells for wild-type MCM5 and 75 cells for the mutant MCM5 were examined.

complexes smaller than MCM2-7 hexamer in the cells. Wild-type flag-MCM2 was expressed in HeLa cells on coverslip and it was detected by staining with anti-flag antibody and with DAPI (Fig. 3C). The wild-type flag-MCM2 was detected in the nucleus. These results are consistent with the notion that nuclear localization signals are present in the amino-terminal region of MCM2 [18]. The cells with micronuclei-like structure, multi-nuclei and no round-shaped nuclei were significantly detected, although their proportion was smaller than those observed by the expression of MCM3 or MCM5 (Fig. 3E). These results suggest that the presence of expressed wild-type MCM2 is hazardous for DNA replication and/or for cell division, as they most probably disturb MCM2-7 helicase function. The mutant MCM2 expressed in HeLa cells was detected in nuclei, similar to wild-type MCM2, but the cells with micronuclei-like structure, multi-nuclei and no round-shaped nuclei were almost undetected (Fig. 3D & 3E). Instead, the two cells which are localized in neighboring positions were detected. Thus the ability of expressed MCM2 to induce the formation of abnormal nuclear structures appears to be dependent on its ATP-binding activity.

Expression of wild-type and mutant MCM4 in human cells

Wild-type MCM4 gene was cloned into pTRE-tight vector for expression in human cells (Fig. 4A). As a mutant at ATP-binding site, the D574Y MCM4 where D residue in walker motif B is converted to Y was constructed. The D574Y MCM4 had been detected in human cancer cells including stomach cancer, which was obtained from Catalogue of Somatic Mutations in Cancer data base of Sanger Institute. These MCM4 proteins expressed in 293T cells were mainly recovered in fraction no. 11-13, suggesting that wild-type and the mutant MCM4 can bind with endogenous MCM2-7 proteins to assemble into MCM complexes smaller than MCM2-7 hexamer (Fig. 4B). Wild-type MCM4 expressed in HeLa cells was largely detected in the nucleus, and the cells containing micronuclei-like structure were detected in 7.3% of flag-positive cells (Fig. 4C & 4E). MCM4 that does not retain nuclear localization signals may bind to MCM2-7 proteins to form MCM complexes containing MCM2/4/6/7 tetramer to enter nucleus. Wild-type MCM4 was also detected in the cytoplasm when the expression level is high. In contrast, the mutant D574Y MCM4 was not concentrated in the nucleus but it was mainly detected in the cytoplasm, regardless of the expression level (Fig. 4D). These results suggest that translocation of MCM complex containing the mutant MCM4 into the nucleus and/or chromatin binding of the MCM complex is perturbed by the MCM4 mutation.

Wild-type MCM4/6/7 hexamer consisting of two molecules of each member exhibits DNA helicase activity (Supplementary Fig. 2). The mutant MCM4/6/7 complex containing D574Y MCM4 did not exhibit any DNA helicase activity; the results are consistent with the published data indicating that the ATP-binding sites in mouse MCM4 are required for DNA helicase activity [11].

Expression of wild-type and mutant MCM7 in human cells

Wild-type MCM7 protein was transiently expressed in 293T cells (Fig. 5A). As a mutant at the ATP-binding site, the K387A MCM7 where K residue in walker motif A is converted to A was constructed. Fractionation of cell extracts by glycerol gradient centrifugation indicates that wild-type MCM7 was recovered in fraction no. 6-12 and the mutant MCM7 was recovered in fraction no. 9-14. These data suggest that wild-type MCM7 binds with endogenous MCM2-6 proteins to form MCM complexes containing MCM2-7 hexamer but the mutant MCM7 forms smaller complexes than the complexes containing wild-type MCM7 (Fig. 5B). Thus the mutation may partially affect MCM2-7 hexamer formation. Wild-type MCM7 expressed in HeLa cells was largely detected in the nucleus (Fig. 5C). MCM7 that does not retain nuclear localization signal may form MCM2/4/6/7 tetramer to enter the nucleus. At a small proportion (13.2%), the cells with two nuclei were detected, suggesting that expression of MCM7 affects DNA replication. The result may be consistent with the finding that over-expression of wild-type MCM7 inhibits entry of S phase [20]. The cells with multi-nuclei detected in the presence of wild-type MCM7 were also detected in the presence of the mutant MCM7 but its proportion was decreased (Fig. 5D & 5E). Thus expression of the K387A mutant MCM7 did not significantly generate the cells with abnormal nuclear structures. Since the K387A MCM7 was not detected in the nucleus but detected in the

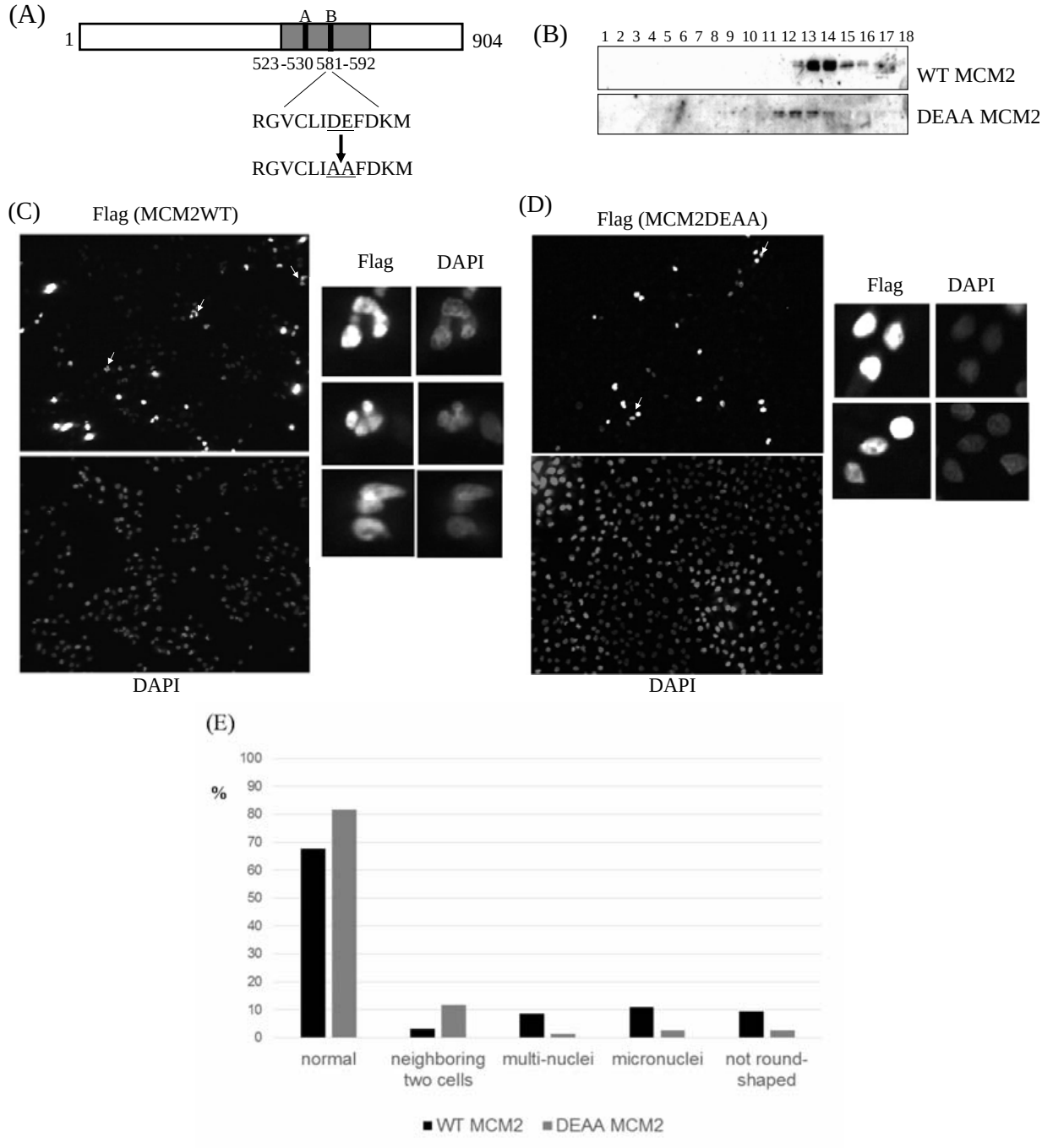

Fig. 3. Expression of wild-type and mutant MCM2 in human cells.
(A) Walker A and B motif in MCM-box of MCM2 are indicated, and amino-acid sequences of Walker B motif for wild-type and mutant (DEAA) MCM2 are indicated. (B) Flag-tagged wild-type MCM2 and the mutant MCM2 mutated at walker motif B (DEAA) were expressed in 293T cells, and Triton-soluble fraction was fractionated by glycerol gradient centrifugation into 18 fractions. Western-blotting was performed using anti-flag antibody. (C) Flag-tagged wild-type MCM2 protein expressed in HeLa cells was detected by staining with anti-flag antibody. On the right side, enlarged pictures of selected cells (multi-nuclei) indicated by arrows in left are shown. (D) Flag-tagged mutant MCM2 (DEAA) in HeLa cells was detected. On the right side, enlarged pictures of selected cells (normal) indicated by arrows in left are shown. (E) Percentages of the cells with normal nuclear structure and those with abnormal nuclear structures are shown. In total, 127 flag-positive cells for wild-type MCM2 and 76 cells for the mutant MCM2 were examined.

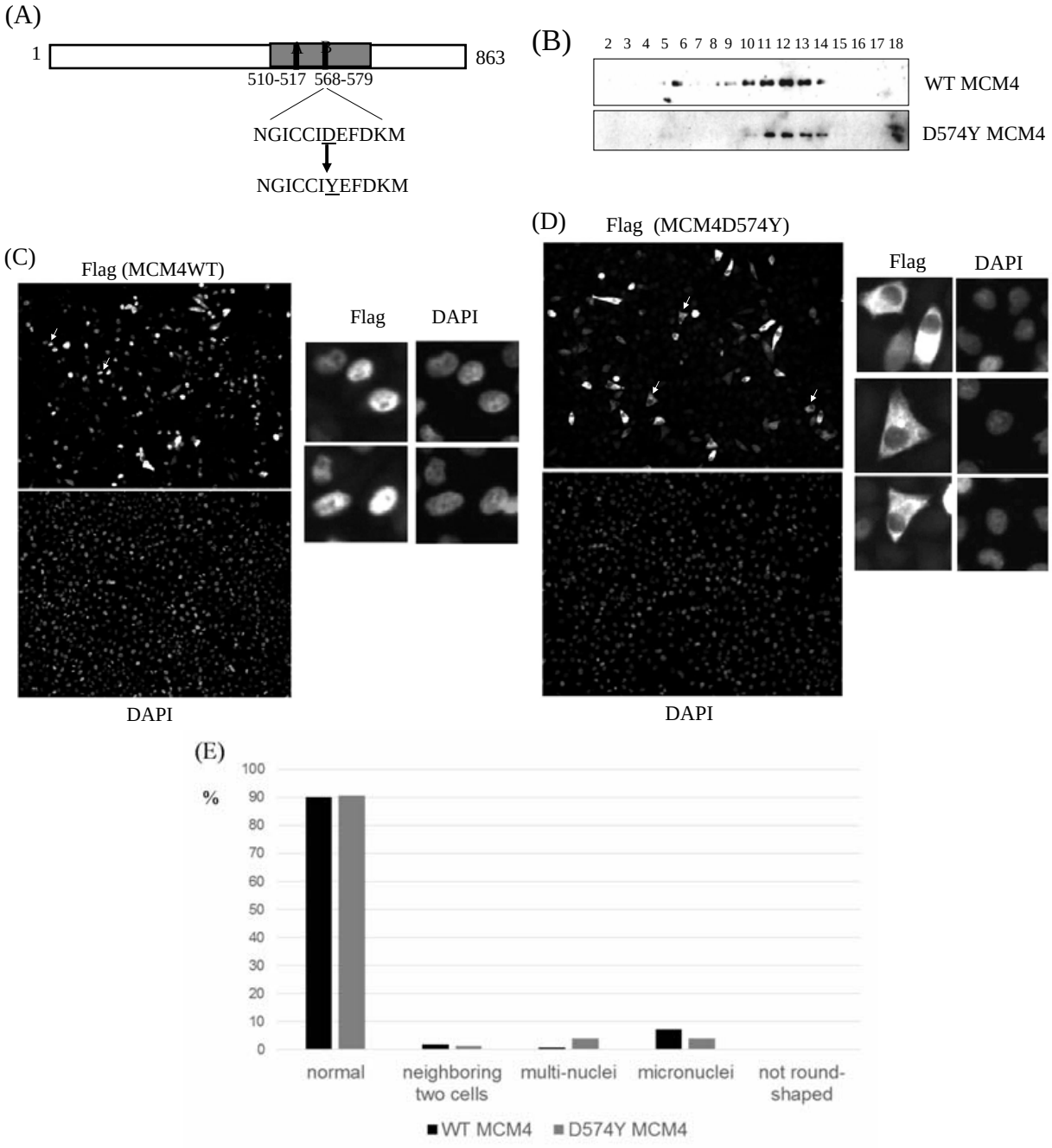

Fig. 4. Expression of wild-type and mutant MCM4 in human cells.
(A) Walker A and B motif in MCM-box of MCM4 are indicated, and amino-acid sequences of Walker B motif for wild-type and mutant MCM4 (D574Y) are indicated. (B) Flag-tagged wild-type MCM4 and the mutant MCM4 mutated at walker motif B were expressed in 293T cells, and Triton-soluble fraction was fractionated by glycerol gradient centrifugation into 18 fractions. After SDS-gel electrophoresis of proteins in the fractions (no. 2-18), western-blotting was performed using anti-flag antibody. (C) Flag-tagged wild-type MCM4 protein expressed in HeLa cells was detected by anti-flag antibody. On the right side, enlarged pictures of selected cells (normal) indicated by arrows in left are shown. (D) Flag-tagged mutant MCM4 expressed in HeLa cells was detected. On the right side, enlarged pictures of selected cells (normal) indicated by arrows in left are shown. (E) Percentages of the cells with normal nuclear structure and those with abnormal nuclear structures are shown. In total, 110 flag-positive cells for wild-type MCM4 and 148 cells for the mutant MCM4 were examined.

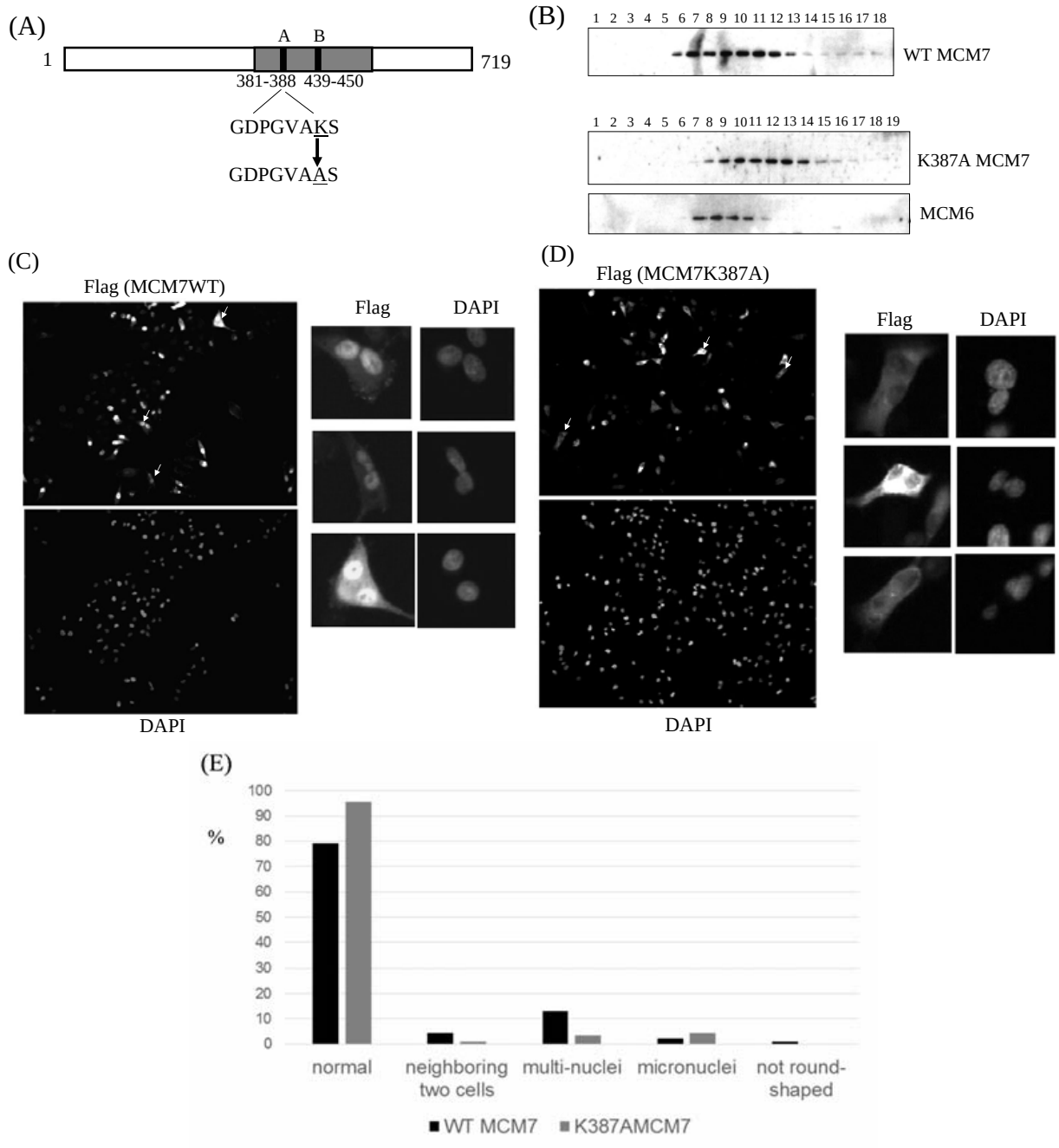

Fig. 5. Expression of wild-type and mutant MCM7 in human cells.
(A) Walker A and B motif in MCM-box of MCM7 are indicated, and amino-acid sequences of Walker A motif for wild-type and mutant (K387A) MCM7 are indicated. (B) Flag-tagged wild-type MCM7 and the mutant MCM7 mutated at walker motif A were expressed in 293T cells, and Triton-soluble fraction was fractionated by glycerol gradient centrifugation into 18 fractions for wild-type and 19 fractions for the mutant MCM7. Western-blotting was performed using anti-flag and anti-MCM6 antibodies. (C) Flag-tagged wild-type MCM7 protein expressed in HeLa cells was detected with anti-flag antibody. On the right side, enlarged pictures of selected cells (multi-nuclei) indicated by arrows in left are shown. (D) Flag-tagged mutant MCM7 protein expressed in HeLa cells was detected. On the right side, enlarged pictures of selected cells (multi-nuclei) indicated by arrows in left are shown. (E) Percentages of the cells with normal nuclear structure and those with abnormal nuclear structures are shown. In total, 91 flag-positive cells for wild-type MCM7 and 115 cells for the mutant MCM7 were examined.

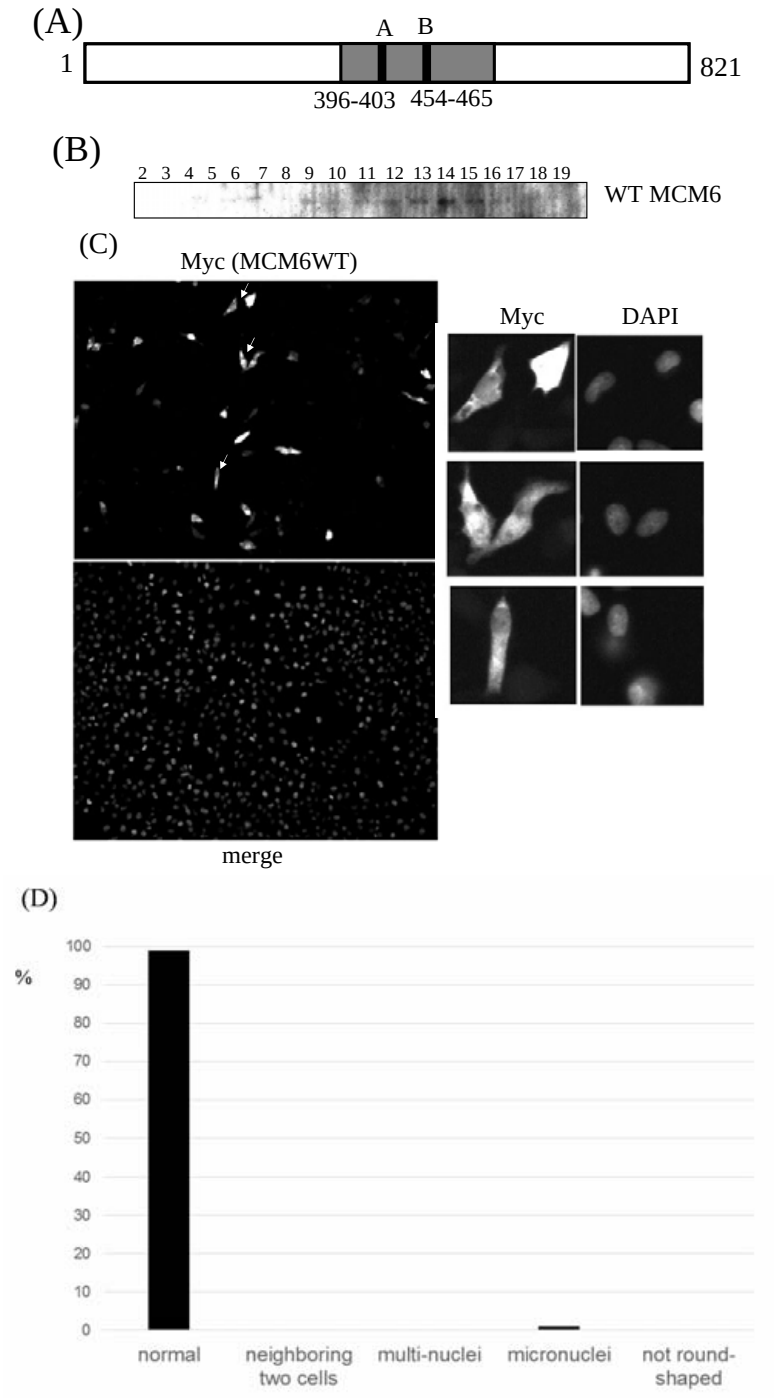

Fig. 6. Expression of wild-type MCM6 in human cells.
(A) Walker A and B motif in MCM-box of MCM6 are indicated. (B) Myc-tagged wild-type MCM6 was expressed in 293T cells, and Triton-soluble fraction was fractionated by glycerol gradient centrifugation into 19 fractions. After SDS-gel electrophoresis of proteins in the fractions (no. 2-19), western-blotting was performed using anti-myc antibody. (C) Myc-tagged wild-type MCM6 protein expressed in HeLa cells was detected with anti-myc antibody. On the right side, enlarged pictures of selected cells (normal) indicated by arrows in left are shown. (D) Percentages of the cells with normal nuclear structure and those with abnormal nuclear structures are shown. In total, 96 flag-positive cells were examined.

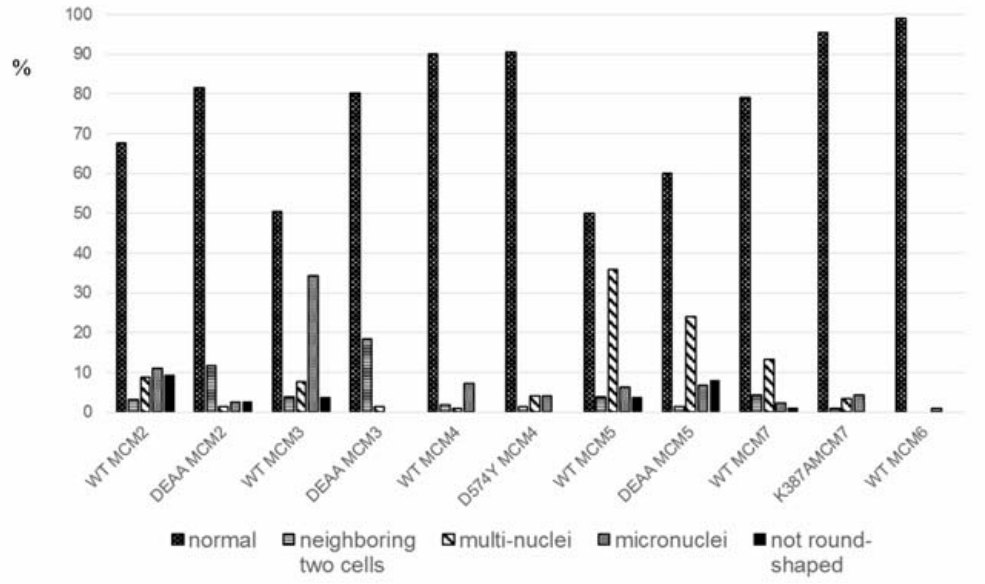

Fig. 7. Effect of expression of MCM2-7 on nuclear structure.
Percentages of the cells with normal nuclear structure and those with abnormal nuclear structures in the presence of forcedly expressed wild-type and mutant MCM2-7 are summarized.

cytoplasm, the mutation may affect translocation of MCM complex containing the mutant MCM7 into the nucleus and/or chromatin binding of the MCM complex.

The mutant MCM4/6/7 hexamer containing the K387A MCM7 was prepared and examined for DNA helicase activity (Supplementary Fig. 3). The results show that the mutant MCM4/6/7 complex did not exhibit DNA helicase activity. The result is consistent with the published data showing that the ATP-binding sites in MCM7 are required for ATPase and DNA helicase activities of MCM4/6/7 complex [11].

Expression of wild-type MCM6 in human cells

Wild-type MCM6 expressed in 293T cells was recovered in fraction no. 14 after glycerol gradient centrifugation (Fig. 6A & 6B). The myc-MCM6 was detected in the nucleus but it was mainly detected in the cytoplasm in HeLa cells (Fig. 6C). The cells with abnormal nuclear structures were almost undetected in the presence of expressed wild-type MCM6 (Fig. 6D).

DISCUSSION

We showed that forced expression of wild-type MCM3 and MCM5 resulted in the generation of micronuclei-like structure and multi-nuclei, respectively. Expression of wild-type MCM2 and MCM7 also resulted in the formation of these abnormal nuclear structures at lower frequency (Fig. 7). Mutation at walker motif B in MCM2, MCM3 and MCM7 cancelled these negative effects on nuclear structure but the mutation in MCM5 did not. The micronuclei are produced by the DNA strand brakes [19], which can be generated by prolonged arrest of DNA replication fork in S phase. The presence of unreplicated DNA regions left in S phase leads to failure in segregation of replicated sister chromosomes in M phase and thereby multi-nuclei formation. The cells with more than two nuclei detected in the presence of wild-type and mutant MCM5 in addition to wild-type MCM7 may also be generated by the presence of unreplicated DNA in S phase and by failure in nuclear division at M phase. Thus it is suggested that the presence of forcedly expressed wild-type MCM2, MCM3, MCM5 and MCM7 disturbs the function of MCM2-7 helicase activity and thereby inhibits the DNA replication, and ATP-binding abilities of MCM2, MCM3 and MCM7 are required for the negative effects on DNA replication.

All the members of MCM2-7 are required for the progression of S phase in *S. cerevisiae* [21].

MCM2-7 hexamer exhibits DNA-dependent ATPase activity. Studies using mutants at ATP-binding sites show that MCM4, MCM6 and MCM7 play critical roles in the ATPase activity and in contrast, MCM2, MCM3 and MCM5 play regulatory roles, since double mutations at the sites in MCM4, MCM6 and MCM7 abolished the ATPase activity but those in MCM2, MCM3 and MCM5 did not [5]. Recent structural studies on CMG complex bound to DNA with fork-like structure indicate that MCM4, MCM6 and MCM7 contacts directly with the single-stranded DNA through the central domains [10]. In addition, it has been shown that conserved positively charged amino-acids in the amino-terminal region of MCM4, MCM6 and MCM7 are required for DNA helicase activity by interacting with single-stranded DNA [22]. Thus, it is suggested that MCM2-7 proteins are functionally divided into two groups, namely catalytic subunits of MCM4, MCM6 and MCM7 and regulatory subunits of MCM2, MCM3 and MCM5, and ATP-binding activities of MCM2-7 may be required for these two functions. These notions on the two different functions of MCM2-7 may be consistent with the present data showing that forced expression of MCM3 and MCM5 proteins exerts strong negative effects on nuclear structures and that of MCM2 does the effects albeit at lesser extent. These MCM proteins may disturb progression of DNA replication forks by interacting with MCM2-7 complex at the DNA replication forks to interfere the MCM2-7 helicase function. The present finding that the ATP-binding ability in MCM3 is required for the generation of micronuclei-like structure but that in MCM5 is not required for inducing multi-nuclei formation suggests the different roles of their ATP-binding sites in MCM2-7 function.

Recently it has been shown that the ATP-binding and hydrolysis activities of MCM2-7 proteins are required for stable binding of MCM2-7 complex onto the chromatin [14, 15]. One plausible explanation of the findings is that structural changes of MCM2-7 complex accompanied with ATP binding and hydrolysis are required for the stable binding of the complex to the chromatin. In this study, the findings that the ATP-binding-site mutants of MCM4 (D574Y) and MCM7 (K387A) are not localized in the nucleus may be consistent with the above findings [14, 15]. That is the forcedly expressed mutant MCM4 and MCM7, which may bind to endogenous MCM2-7, cannot stably bind to DNA due to their inability to bind to ATP. The findings that expression of wild-type MCM2 and MCM3 induced the formation of abnormal nuclear structures but their mutants did not show the negative activity suggest that ATP-binding sites of MCM2 and MCM3 are required for interaction with other MCM members and/or for the stable binding of MCM complex to chromatin.

The mutation at K574Y of MCM4 was found in several cancer cells including stomach cancer. The mutant MCM4 in HeLa cells may bind to endogenous MCM2-7 proteins to form non-functional MCM2-7 complexes. As a result, functional MCM2-7 on genome may become limited, which inhibits the completion of DNA replication in S phase. Such negative effects on DNA replication of expressed mutant MCM4 (G486D) from cancer cells has been observed [16]. The presence of these mutant MCMs in human cells may contribute to cancer progression by inducing DNA breakages in S phase.

CONCLUSION

The results on the negative effects of forced expression of wild-type MCM2-7 proteins on nuclear structure in HeLa cells differentiate them into two groups, namely highly effective (MCM2, MCM3 and MCM5) and lowly effective proteins (MCM4, MCM6 and MCM7). The findings are consistent with the notion that MCM4, MCM6 and MCM7 play catalytic roles and MCM2, MCM3 and MCM5 play regulatory roles in DNA helicase function of MCM2-7 complex. These negative effects are dependent on the ATP-binding activities of MCM2-7 proteins except for MCM5. Cellular localization of expressed mutant MCM2-7 proteins suggests that ATP-binding activities of MCM4 and MCM7 are required for nuclear localization and/or stable binding of the MCM2-7 complex to chromatin.

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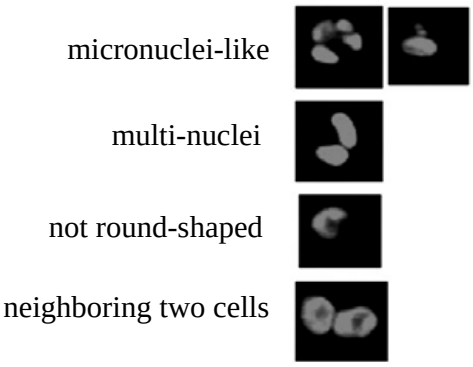
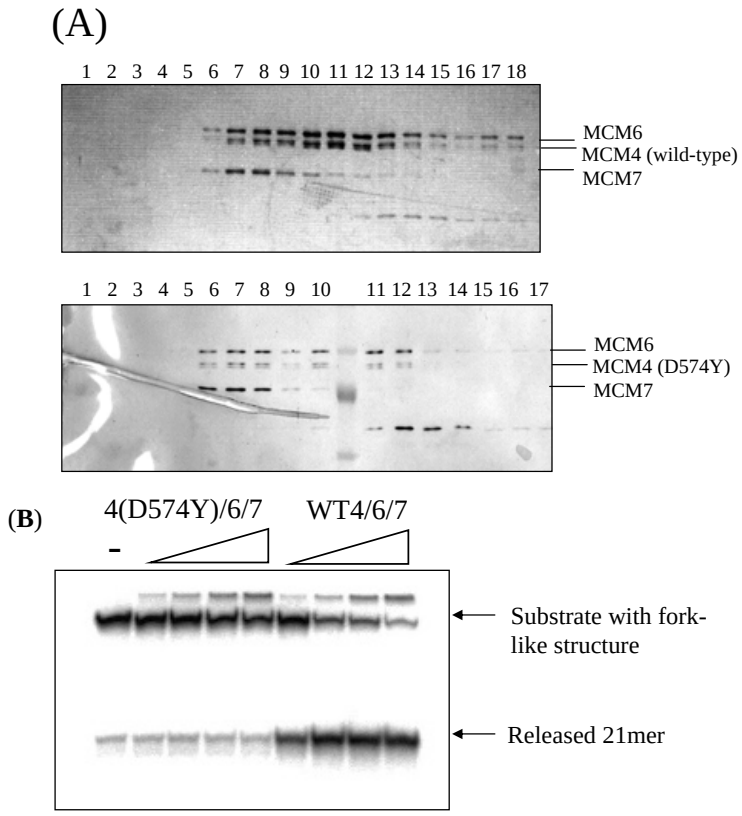
CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

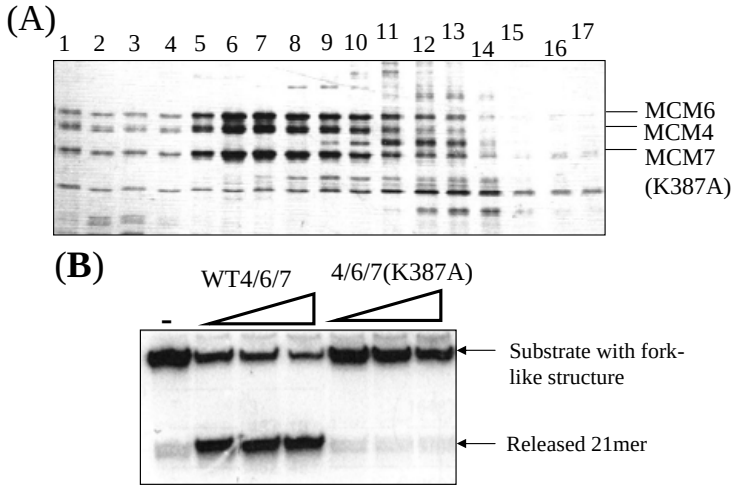
SUPPLEMENTARY MATERIAL



Supplementary Fig. 1. Classification of abnormal nuclear structures.
Abnormal nuclear structures from flag-positive cells observed in the presence of wild-type MCM3 are classified into micronuclei-like structure, multi-nuclei, not round-shaped and neighboring two cells. Representatives of these structures are shown.



Supplementary Fig. 2. DNA helicase activity of MCM4/6/7 hexamer containing mutant MCM4.
(A) MCM4/6/7 containing wild-type MCM4 or mutant MCM4 (D574Y) was fractionated by glycerol gradient centrifugation. Proteins separated by SDS-gel electrophoresis were stained with silver. Positions of MCM4, 6 and 7 proteins are indicated. (B) DNA helicase activity was measured in the presence of increasing amounts (0, 10, 20, 40 and 60 ng) of purified MCM4/6/7 complexes containing wild-type MCM4 and mutant MCM4.



Supplementary Fig. 3. DNA helicase activity of MCM4/6/7 hexamer containing mutant MCM7.
(A) MCM4/6/7 containing mutant MCM7 (K387AY) was fractionated by glycerol gradient centrifugation. Proteins separated by SDS-gel electrophoresis were stained with silver. Positions of MCM4, 6 and 7 proteins are indicated. (B) DNA helicase activity was measured in the presence of increasing amounts (0, 20, 40 and 80 ng) of purified MCM4/6/7 complexes containing wild-type MCM7 and the mutant MCM7.

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