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Effect of an MCM4 mutation that causes tumours in mouse on human MCM4/6/7 complex formation

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It has been reported that a point mutation of minichromosome maintenance (MCM)4 causes mammary carcinoma, and it deregulates DNA replication to produce abnormal chromosome structures. To understand the effect of this mutation at level of MCM2–7 interaction, we examined the effect of the same mutation of human MCM4 on the complex formation with MCM6 and MCM7 in insect cells. Human MCM4/6/7 complexes containing the mutated MCM4 were formed, but the hexameric complex formation was not evident in comparison with those containing wild-type MCM4. In binary expression of MCM4 and MCM6, decreased levels of MCM6 were recovered with the mutated MCM4, compared with wild-type MCM4. These results suggest that this mutation of MCM4 perturbs proper interaction with MCM6 to affect complex formation of MCM4/6/7 that is a core structure of MCM2–7 complex. Consistent with this notion, nuclear localization and MCM complex formation of forcedly expressed MCM4 in human cells are affected by this mutation. Thus, the defect of this mutant MCM4 in interacting with MCM6 may generate a decreased level of chromatin binding of MCM2–7 complex.

Keywords: MCM2–7/DNA helicase/DNA replication/MCM2/4/6/7/nuclear localization.

In addition to activation of the oncogenes and inactivation of the tumour suppressor genes, aberration of chromosome structures is involved in malignant transformation of human cells (*1*). DNA replication of human genome requires concerted actions by a number of proteins (*2–4*), and problems during DNA replication are one of the major causes that produce the chromosome aberrations. Generation of unreplicated or overreplicated DNA regions during S phase leads to segregation of altered chromosome structures to daughter cells at M phase. DNA replication is mainly regulated by following two systems during S phase: one is to prevent overreplication in a single S phase and another is the DNA replication checkpoint system that mainly deals with problems during elongation of DNA replication. A number of origins of DNA replication are present in a single chromosome, but they fire once in a cell cycle. In this system called by the DNA replication licensing, several proteins including minichromosome maintenance (MCM)2–7 are involved. With the assistance of CDC6 and CDT1, MCM2–7 complexes load onto the origins where the origin replication complex has been bound. At the initiation of DNA replication, an activated MCM DNA helicase, which is generated from MCM2–7 complex with the assistance of CDC45 protein and GINS complex, unwinds origin DNA to initiate DNA synthesis (*5–7*). During S phase, the loading of MCM2–7 proteins onto DNA is prevented by inactivation of CDC6 and CDT1 that is mainly performed with cyclin-dependent kinases (*8*). Overreplication in a single S phase is at least in part prevented by this regulation.

A large body of evidence indicates that MCM proteins are one of the most reliable markers for cancer diagnosis (*9*), which is in part supported by the fact that MCM proteins are overexpressed in cancer cells (*10*, *11*). It has been reported that induced overexpression of MCM7 facilitates transformation of human cells (*12*). These findings suggest that deregulation of MCM function plays causal roles in the cell transformation. Recently, Shima *et al*. (13) found that a point mutation of MCM4 in mouse causes mammary carcinoma. They selected progeny mice harboring nuclear fragmentation of erythrocytes, after random mutagenesis of male mouse. It has been determined that the point mutation causing the mammary carcinoma is F345I near the Zn finger domain of MCM4. They also reported that the same mutation causes minichromosome loss phenotype in *Saccharomyces cerevisiae*, and the abnormal chromosome structures, which may be generated by fragility of chromosomes, are produced on exposure of mouse embryonic fibroblast from the mutant mouse with aphidicolin, an inhibitor of DNA polymerases. These results suggest that this mutation of MCM4 deregulates DNA replication to produce abnormal chromosome structures.

To understand the effect of this mutation at the level of MCM2–7 interaction, we expressed human MCM4 with the same mutation (F346I) in insect cells and in human cells to examine the effect of this mutation on the interaction with MCM6 and MCM7 and its nuclear localization. It has been shown that this mutation of MCM4 affects the binding with MCM6 in the coexpression experiment in insect cells, and it affects its nuclear localization in human cells. These results raise the possibility that the formation of a subcomplex of MCM2/4/6/7, which may be imported to nucleus by

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nuclear localization signals (NLSs) in MCM2, is affected by the MCM4 mutation.

Materials and Methods

Site-directed mutagenesis (F346I) of human MCM4 gene

Site-directed mutagenesis was performed according to the manufacturer's protocol (QuickChange XL Site-Directed Mutagenesis Kit, Stratagene, LaJolla, CA, USA). Human MCM4 cDNA cloned at EcoRI site in pAcUW31 as a template, a forward primer (CACAAC CGCTCCCTCATCTCTGACAAGCAG) and a reverse primer (CT GCTTGTCAGAGATGAGGGAGCGGTTGTG) were used for polymerase chain reaction. The nucleotide sequencing of the cloned MCM4 gene, which was determined by OpenGene system (Veritas, Tokyo, Japan), revealed that the sequence was identical to the banked sequence (BC031061) except for the mutated site. To generate recombinant baculovirus for expression of the MCM4 protein, BaculoGold *Autographa californica* nuclear polyhedrosis virus DNA (BaculoGold AcNPV; Pharmingen, BD, San Jose, CA, USA) and the cloned MCM4 gene were cotransfected into Sf9 cells, and recombinant baculoviruses were isolated according to the manufacturer's protocol. Recombinant baculoviruses for expression of human MCM6 were prepared from the cDNA cloned at BamH1 site of pAcUW31. Preparation of recombinant baculoviruses for expression of human MCM7 (*14*) and MCM-BP (*15*) protein has been reported.

Protein expression and purification of MCM4/6/7 complex

High5 cells (2×10^7 cells) were coinfected with the three viruses expressing the His-MCM4, MCM6 and His-MCM7 proteins (0.7 ml of each viral solution) for 2 days. The MCM4/6/7 complex was purified from 1.2×10^8 infected cells in total (*14*, *16*). The cells were suspended in a 6 ml of lysis buffer consisting of 10 mM Tris–HCl (pH 7.5), 130 mM NaCl, 1% Triton X-100, 10 mM NaF, 10 mM sodium phosphate buffer, 10 mM $Na_4P_2O_7$, protease inhibitors (Pharmingen) and 10 mM imidazole. The mixture was incubated for 40 min on ice, and insoluble components were separated by centrifugation at 40,000 rpm (TLS55; Beckman, Fullerton, CA, USA) for 40 min at 4°C. To 1 volume of the clarified lysate, 1/10 volume of Co-beads (Talon Metal Affinity Resin, Clontech, Mountain View, CA, USA) that had been washed with the lysis buffer was added, and the mixture was incubated for 1.5 h at 4°C on a rocking platform. The beads were then collected by centrifugation and washed six times with buffer A (50 mM Na phosphate buffer, pH 6.0, 300 mM NaCl and 10% glycerol) containing 10 mM imidazole six times. Next, the beads were incubated for 10 min at 4°C with equal volumes of buffer B (50 mM Na phosphate buffer, pH 8.0, 300 mM NaCl and 10% glycerol) containing 50, 100, 200, 300 and 400 mM of imidazole stepwise, and eluted proteins were recovered after centrifugation. Proteins were analyzed by SDS-PAGE and stained with silver (ATTO, Tokyo, Japan). The pooled protein fractions (200–400 mM imidazole) were loaded onto a MonoQ column in a Smart system (GE Healthcare, Piscataway, NJ, USA), and the bound proteins were eluted by a linear gradient from 0.1 to 0.6 M NaCl. The MCM4, 6 and 7 proteins were coeluted at near 0.4 M NaCl, and pooled fractions were concentrated with Microcon-30 or Ultra4 (Amicon, Millipore, Bedford, MA, USA) after dilution. Then, it was loaded onto 15–30% linear glycerol gradient and centrifuged at 36,000 rpm (TLS55; Beckman) for 14 h (*17*). His-MCM2 was purified from overexpressed insect cells as described earlier. Flag-tagged human MCM-BP was purified from overexpressed insect cells using anti-Flag agarose beads (Sigma, St Louis, MO, USA). MCM-BP eluted with 3XFLAG peptide (Sigma) was further purified to homogeneity by MonoQ column chromatography as described earlier.

The proteins were separated by SDS–PAGE. After the proteins in the gel were transferred to Immobilon-P transfer membrane (Millipore), the membrane was incubated for 1 h at room temperature with a blocking buffer (EzBlock; ATTO) diluted by 3-fold with Tris-buffered saline (TBS) plus 0.1% Triton X-100; it was then incubated overnight at 4°C with 0.5 μg/ml of first antibodies in the diluted blocking buffer. After washing the membrane with TBS containing Triton X-100, it was incubated for 2 h at 27°C with second antibody conjugated with horseradish peroxidase (BioRad, Hercules, CA, USA). After washing, the membrane was incubated with SuperSignal West Pico Maximum Sensitivity Substrate (Pierce, Rockford, IL, USA), and chemiluminescent signals were detected by Light-Capture (ATTO). Anti-MCM6 (sc-9843, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-MCM7 (sc-9966, Santa Cruz Biotechnology) antibodies were purchased. Anti-MCM4 polyclonal antibodies (*18*) and anti-MCM-BP monoclonal antibody (*15*) were prepared as described.

Interaction of MCM4 with MCM6, MCM7 and MCM-BP

In the experiments to examine the interaction of His-MCM4 with MCM6, Flag-MCM7 or Flag-MCM-BP, 0.3 ml of each viral stock solution was added to High5 cells (7×10^6). The infected cells were lysed with the lysis buffer containing 20 mM imidazole (500 μl), and cleared lysate (400 μl) after centrifugation was mixed with 1/10 volume of Ni-NTA agarose (Qiagen, Hilden, Germany). After mixing for 1.5 h at 4°C, the agarose was washed 5–7 times with buffer A containing 20 mM imidazole, and proteins were eluted from agarose two times by incubation of beads with equal volumes of buffer B containing 300 mM imidazole. The final eluates were pooled and electrophoresed.

DNA helicase activity of purified MCM4/6/7 complexes

The DNA helicase substrates were prepared as reported (*17*), except that 5′-labelled 17-mer oligonucleotide (5′-GTTTTCCCAGTCACG AC-3′) annealed to M13 mp18 single-stranded DNA were purified by centrifuging four times through a spin column (G-50; Roche, Mannheim, Germany), instead of being purified via sucrose gradient centrifugation. DNA helicase activity was measured essentially as reported previously. The reaction contained 10 mM ATP, 10 mM $Mg(OAc)_2$ and 1–2.5 fmol of 17-mer oligonucleotide annealed to M13 mp18 DNA.

Expression of MCM4 protein in human cells

Human MCM4 cDNA cloned into pAcUW31 were recloned into pFLAG-CMV-6c (Sigma) at EcoRI site. The plasmid DNA purified using Endo-free purification system (Qiagen) was tranfected into HeLa cells on coverslip in four-well chambers (Nunclon, Denmark) in the presence of Lipofect-amine 2000 (Invitrogen, Carlsbad, CA, USA) and incubated for 2 days. After transfection, cells were fixed by incubation with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 5 min at room temperature and then permeabilized and blocked by incubation with 0.1% Triton X-100, 0.02% SDS and 2% non-fat dried milk in PBS for 1 h at room temperature (*16*). Incubation of the cells with anti-Flag antibody (2.5 μg/ml) (Sigma) was performed overnight at 4°C in the aforementioned blocking solution. Cells were washed with the same solution and then incubated with Cy3-conjugated anti-mouse antibodies (Jackson ImmunoResearch, West Grove, PA, USA) for 1.5 h at 37°C in the blocking solution. Washed cells were stained with 2 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) for 15 min at room temperature. After washing with PBS, cells were mounted in 90% glycerol and 10% PBS solution containing 1,4-diazabicyclo[2,2,2]-octane (DABCO, 2.3%; Sigma) and observed using fluorescence microscopy (BZ9000; Keyence, Japan).

For the immunoprecipitation experiment, the plasmid DNA containing human MCM4 cDNA was transfected into 293T cells in the presence of polyethyleneimine (*19*) and incubated for 2 days. The cells (9×10^6) were lysed with 450 μl of buffer (100 mM piperazine-1,4-*bis* (2-ethanesulfonic acid) (PIPES), pH 6.8, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.1% Triton X-100 and 100 mM ATP) containing protease inhibitor cocktail (Pharmingen). After centrifugation, precipitated materials, named as P, and supernatant fraction, named as I, were obtained. The supernatant (400 μl) was mixed overnight at 4°C with protein G-Sepharose-antibody beads (30 μl) (Amersham Biosciences, Piscataway, NJ, USA) that had been incubated with anti-Flag antibody (10 μg) overnight at 4°C. After centrifugation, proteins unbound to the Sepharose beads were recovered (U). The beads were washed eight times with 300 μl of PBS containing 0.01% Triton X-100. The proteins bound to the beads were eluted twice with 30 μl of elution buffer (0.1 M glycine, pH 2.5 and 0.15 M NaCl). These eluates, named as B, were neutralized by adding 1/10 volume of 2 M Tris–HCl, pH 8.0.

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Results

MCM4/6/7 complex formation with mutant MCM4

The CMG complex that is constituted by CDC45, MCM2–7 and GINS may function as the replicative DNA helicase that unwinds DNA duplex at the replication forks (*5–7*). The finding that the mutations at the ATP-binding motif of MCM2–7 proteins abolishes DNA helicase activity of CMG complex indicates that the activity is intrinsic to MCM2–7 proteins (*20*). However, it appears that the effects of the mutations of MCM2–7 proteins on DNA helicase activity do not correlate to the effects of the mutations of MCM2–7 *in vivo* in yeasts (*21*, *22*). The mutation study in yeast emphasizes the importance of the ATP-binding motifs of MCM4, MCM6 and MCM7. Biochemically, MCM4/6/7 trimer forms a stable complex (*18*), and a dimer of the MCM4/6/7 trimer exhibits distinct DNA helicase activity *in vitro* (*17*, *23–25*), suggesting that the MCM4/6/7 trimer constitutes a core structure of MCM2–7 hexamer.

To understand the effect of the MCM4 mutation (F346I) on MCM complex formation, MCM4/6/7 hexameric complex was prepared from baculovirus-infected insect cells where human MCM6 and His-MCM7 were coexpressed with wild-type or mutant (F346I) His-MCM4. After purification using Co-beads and then with ion-exchange column chromatography, MCM complexes were fractionated by glycerol gradient centrifugation (Fig. 1A and B). Wild-type MCM4 was mainly cosedimented with MCM6 and 7 proteins at fraction nos 3–5 (Fig. 1A). It has been reported that these three MCM proteins form a hexameric MCM4/6/7 complex (*17*). When the mutant MCM4 (MCM4m) was coexpressed with MCM6 and MCM7 and purified MCM complexes were fractionated by glycerol gradient centrifugation, MCM4, MCM6 and MCM7 proteins were cosedimented but broadly distributed in fraction nos 1–7 (Fig. 1B). These findings indicate that MCM complexes were formed with the mutant MCM4, MCM6 and MCM7, but they were heterogeneous in size, suggesting that MCM4m does not properly interact with MCM6 and MCM7 to form the MCM4/6/7 hexamer.

The DNA helicase activity of the mutant MCM4m/6/7 complex was measured. Fractions no. 3–6 were pooled and concentrated for the mutant MCM4m/6/7 complexes, as well as for the wild-type MCM4/6/7 complex. Almost similar protein concentration and purity were obtained. The results indicate that the mutant MCM4m/6/7 complex exhibits DNA helicase activity (Fig. 2). It should be noted that increased levels of the input DNA helicase substrate were detected in the reactions containing the MCM4m/6/7 complex. However, it is difficult to conclude that the mutant MCM4m/6/7 complex retains reduced DNA helicase activity, as the proportion of the input DNA helicase substrate remained is low.

Interaction of the mutant MCM4 with MCM6

Interactions between MCM4–MCM6 and MCM4–MCM7 have been identified among MCM4, MCM6 and MCM7 proteins, but the interaction between MCM6 and MCM7 is not evident (*26*). To understand the reason why the hexameric MCM4/6/7 complex is not dominantly formed with the mutant MCM4 in Fig. 1, the interaction with MCM6 was compared between wild-type and mutant MCM4. After coexpression of His-MCM4 and MCM6 proteins in insect cells, His-MCM4 was isolated from cell lysate using Ni-NTA agarose after washing with buffer containing 0.3 M NaCl (Fig. 3). Decreased levels (~25%) of MCM6 were recovered with MCM4m in comparison with wild-type MCM4. However, similar levels of Flag-MCM7 (125%) and Flag-minichromosome maintenance complex-binding protein (MCM-BP) were recovered with MCM4m in comparison with wild-type MCM4 in the binary expression and pull-down experiment. MCM-BP that is a new MCM2–7 member but is largely recovered in Triton-soluble fraction may regulate the function of MCM2–7 helicase (*27*, *28*). To further examine the interaction between MCM4m with MCM6, recovered MCM4m and MCM6 were quantified (Fig. 4A and B). The data indicate that stoichiometrical amounts of MCM6 were recovered with wild-type MCM4 but decreased levels (only ~30%) of MCM6 to the mutant MCM4 were

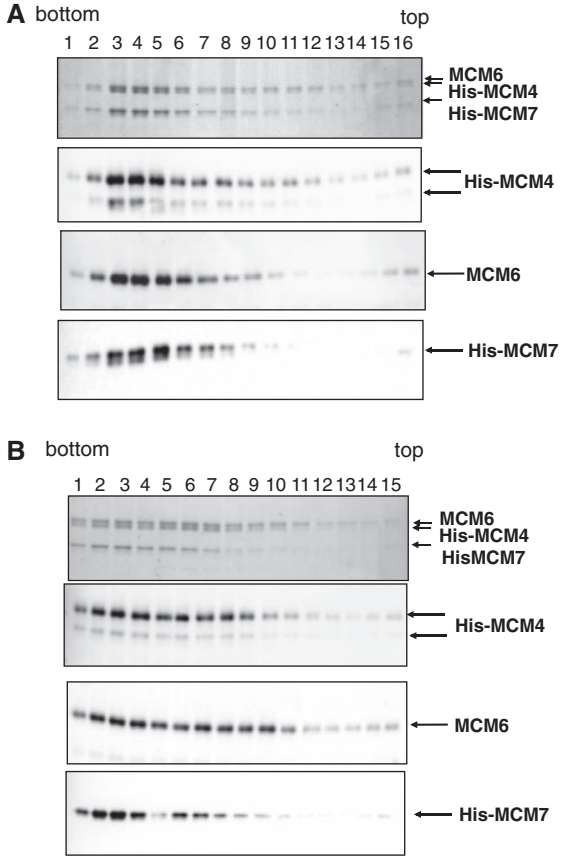

Fig. 1 Glycerol gradient centrifugation of MCM4/6/7 complex. His-MCM4 of wild-type (**A**) or mutant (**B**) was coexpressed with MCM6 and His-MCM7 in insect cells. MCM protein complexes purified using Ni-NTA agarose and then with ion-exchange column chromatography were fractionated by glycerol gradient centrifugation. Proteins in the fractions were electrophoresed. They were stained with silver (top), and MCM4, MCM6 and MCM7 proteins were detected by western blotting as indicated (bottom).

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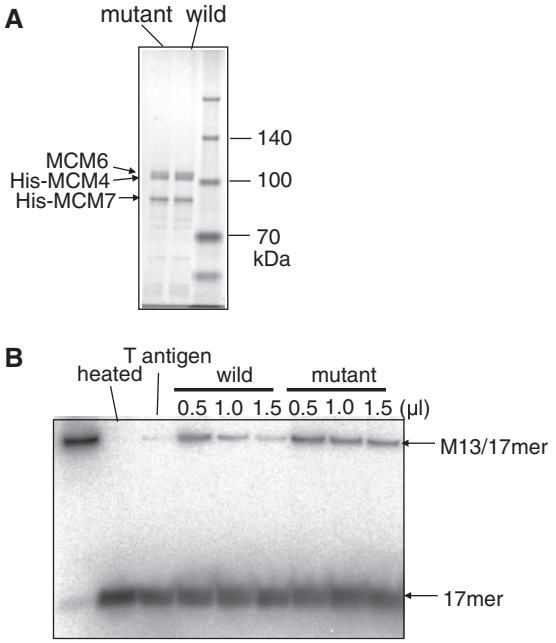

Fig. 2 DNA helicase activity of MCM4m/6/7. (**A**) MCM4/6/7 hexameric complexes containing wild-type MCM4 and the mutant MCM4 were purified. Proteins in these complexes were electrophoresed in SDS-containing polyacrylamide gel and stained with silver. (**B**) Increasing volumes of purified samples were added to DNA helicase reaction where 17-mer oligonucleotides annealed with M13 single-stranded DNA was used as a substrate.

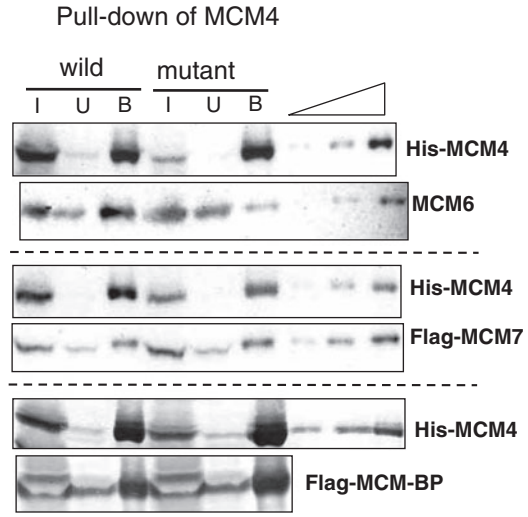

Fig. 3 Binding of wild-type and mutant MCM4 with MCM6, MCM7 and MCM-BP. Wild or mutant His-MCM4 was coexpressed with MCM6, Flag-MCM7 or Flag-MCM-BP in insect cells. MCM complexes containing His-MCM4 recovered using Ni-NTA agarose were electrophoresed with purified MCM proteins. Input material of pull-down experiment (I, 8 μl), proteins unbound to the agarose (U, 8 μl) and those bound to the beads (B, 2 μl) were electrophoresed with increasing amounts (5, 10 and 20 ng) of purified MCM proteins, and MCM4, MCM6 and MCM-BP proteins were detected by western blotting.

recovered. The volume of the virus solution at infection for expression of mutant MCM4 was increased. With increased virus solution (600 and 900 μl), the levels of the mutant MCM4 recovered in Triton-soluble fraction were slightly increased (Supplementary Fig. S1), but unexpectedly, the level

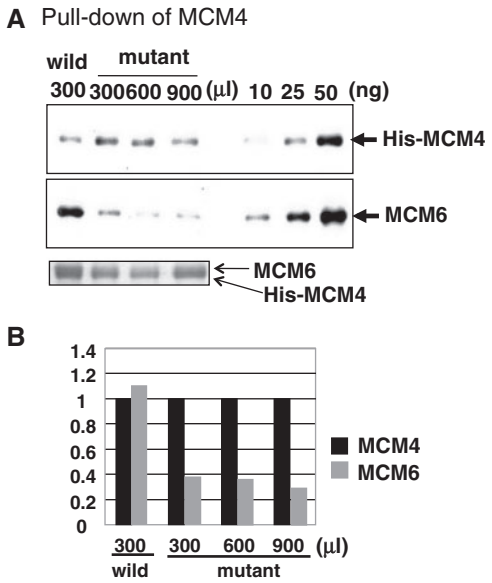

Fig. 4 Binding of wild-type and mutant MCM4 with MCM6. (**A**) Wild or mutant His-MCM4 was coexpressed with MCM6 in insect cells. Increasing volumes (300, 600 and 900 μl) of viral solution was used for the expression of the mutant MCM4. MCM complexes containing His-MCM4 recovered using Ni-NTA agarose were electrophoresed with purified MCM4/6 proteins. MCM4 and MCM6 proteins were detected by western blotting. In the bottom, the precipitated MCM4 and MCM6 were stained with silver. (**B**) The amounts of the recovered MCM4 and MCM6 proteins were quantified using the purified MCM proteins as standards. Relative amounts of MCM6 to MCM4 were determined in each experiment. Averages of two experiments are shown.

of recovered mutant MCM4 was slightly decreased (Fig. 4B and Supplementary Fig. S1). The low level of MCM6 recovery was not changed under these conditions (Fig. 4B and Supplementary Fig. S1). These results indicate that the mutant MCM4 cannot properly interact with MCM6.

Nuclear localization and MCM complex formation of the mutant MCM4

The findings that the mutant MCM4 cannot properly interact with MCM6 suggest that the assembly of MCM proteins into MCM2–7 is perturbed by the MCM4 mutation. To examine this possibility, the following experiments were performed. The Flag-tagged wild-type MCM4 was expressed in HeLa cells for 2 days, and cellular localization of the forcedly expressed MCM4 was detected (Fig. 5A). Approximately 20–30% of the cells exhibited expression of the wild-type MCM4, which was detected using anti-Flag antibodies. A similar level of expression was detected for the mutant MCM4. The wild-type and mutant Flag-MCM4 expressed at high level were detected in both nucleus and cytoplasm (data not shown). It is possible that MCM4 detected in cytoplasm mainly forms protein aggregates. In contrast, the wild-type MCM4 expressed at a lower level is mainly localized in nucleus, but the mutant MCM4 is mainly localized in cytoplasm (Fig. 5A). In the cells that expressed wild-type MCM4 at a lower level, the localization of the wild-type MCM4 was grouped into three types:

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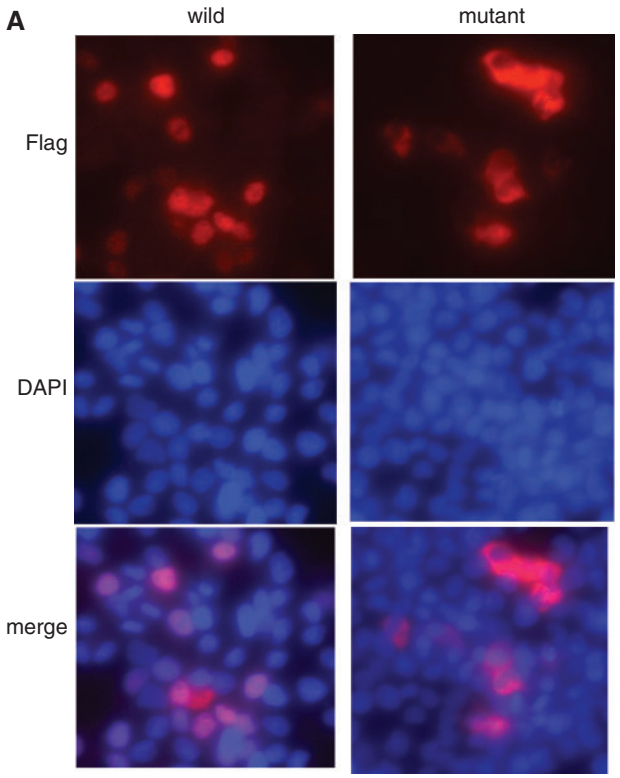

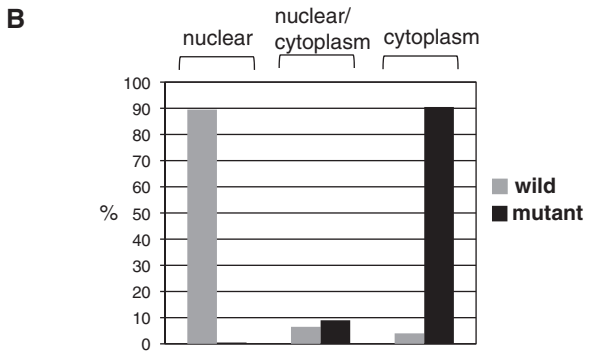

Fig. 5 Nuclear localization of wild-type and mutant MCM4 in HeLa cells. (**A**) Flag-tagged wild-type (left) or mutant (right) MCM4 was expressed in HeLa cells, and they were detected with anti-Flag antibody (top). Total cell nuclei were detected by staining with DAPI (middle) and merged views are shown (bottom). The cells expressing Flag-tagged MCM4 at lower level were presented. (**B**) Cellular localization of the wild-type (grey) or the mutant (black) Flag-tagged MCM4 expressed at lower level was divided into three groups: mainly localized in nucleus, evenly localized in nucleus and cytoplasm and mainly present in cytoplasm, as indicated. Results are presented as percentages in two hundred cells and averages of two experiments are shown.

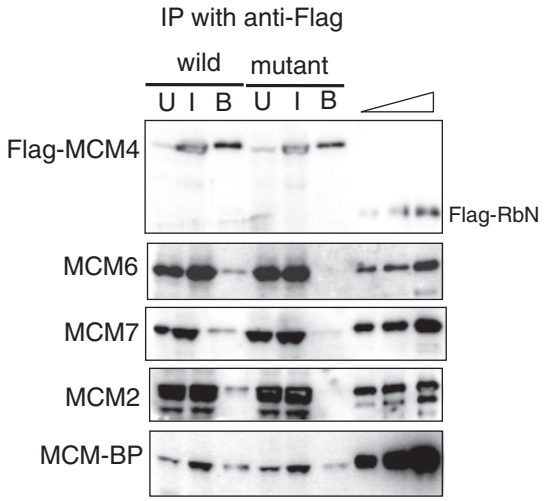

Fig. 6 Complex formation of the mutant MCM4 with MCM proteins in 293T cells. Flag-tagged wild-type and mutant MCM4 was forcedly expressed in 293T cells. MCM complexes containing Flag-MCM4 were precipitated with anti-Flag antibody. Input material of immuno-precipitation experiment (I, 10 µl), proteins unprecipitated (U, 10 µl) and those precipitated (B, 10 µl) were electrophoresed with increasing amounts (5, 10 and 20 ng) of purified MCM proteins and Flag-tagged amino-terminal fragment of human Rb (*15*). Coprecipitated MCM2, MCM4, MCM6 and MCM-BP proteins, as well as precipitated Flag-MCM4, were detected by western blotting.

mainly nuclear localization, evenly present in nucleus and cytoplasm and mainly present in cytoplasm (Fig. 5B). Almost all of the wild-type MCM4 expressed at a lower level was present in nucleus. In contrast, the mutant MCM4 was mainly localized in cytoplasm of the cells. These results indicate that the mutant MCM4 is difficult to be localized in nucleus.

To examine the interaction of the forcedly expressed mutant MCM4 with MCM2–7 proteins, the levels of MCM2, MCM6 and MCM7 coprecipitated with Flag-tagged mutant MCM4 were compared with those coprecipitated with wild-type MCM4 in 293T cells (Fig. 6). Similar levels of wild-type and the mutant MCM4 were precipitated with anti-Flag antibody. MCM6 coprecipitated with Flag-tagged wild-type MCM4 was detected but that coprecipitated with Flag-tagged mutant MCM4 was almost undetectable. Decreased levels of MCM2 and MCM7 were recovered with the mutant MCM4. Quantification shows that only 30% of MCM7 and 14 % of MCM2 were precipitated with the mutant MCM4 in comparison with wild-type MCM4. Slightly decreased amounts of MCM-BP (68%) were precipitated with the mutant MCM4, compared with wild-type MCM4. These results indicated that the interaction of MCM4 with MCM2, MCM6 and MCM7 was perturbed by the MCM4 mutation in human cells.

Discussion

We presented data indicating that the human MCM4 mutation (F346I), which causes tumours in mice, affects the complex formation with MCM6 in insect cells. MCM2–7 proteins in the hexamer are arranged by the order of MCM2–MCM5–MCM3–MCM7–MCM4–MCM6 clockwise. MCM2/4/6/7, MCM4/6/7 and MCM3/5 form relatively stable subcomplexes. MCM4 most strongly interacts with MCM6 and MCM7, and it has been shown in this study that the interaction of MCM4 and MCM6 is affected by the MCM4 mutation in insect cells (Figs 3 and 4). However, in the experiment of immunoprecipitation of MCM4 in the insect cell lysate using an anti-MCM4 antibody where the precipitated materials were washed with PBS–0.01% Triton before elution,

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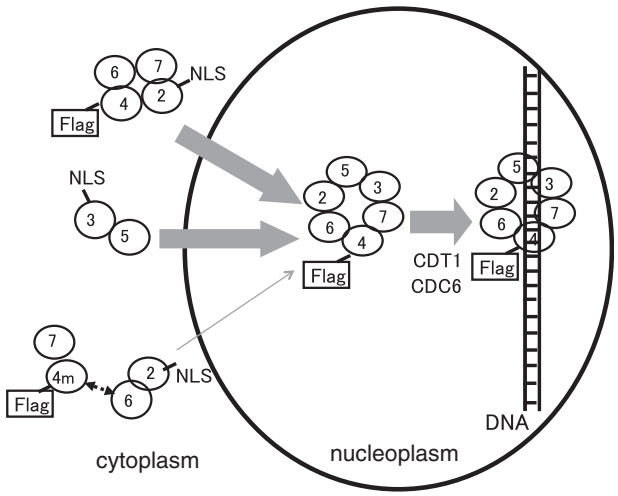

Fig. 7 Model for effect of the MCM4 mutation on MCM2–7 complex formation and on nuclear translocation. Newly synthesized MCM2, 4, 6 and 7 proteins and MCM3 and MCM5 proteins form an MCM2/4/6/7 complex and an MCM3/5 complex in cytoplasm, respectively, and they are translocated into nucleus by the NLS in MCM2 and MCM3. The MCM2–7 complex assembled by these two MCM complexes binds chromatin in the presence of CDT1 and CDC6 to form a pre-replication MCM2–7 complex. The presented data raise a possibility that formation of MCM2/4/6/7 complex, which can be translocated into nucleus, is perturbed by the MCM4 mutation due to inefficient interaction with MCM6.

amounts of MCM6 precipitated with the mutant MCM4 did not decrease in comparison with wild-type MCM4 (data not presented). Pull-down experiment with Ni-NTA beads used a buffer containing 300 mM NaCl for washing (Fig. 3 and 4). It is possible that the fragile interaction of the mutant MCM4 with MCM6 was perturbed during the treatment.

The forcedly expressed human MCM4 with this mutation hardly enters nucleus and cannot stably interact with MCM2, MCM6 and MCM7 in human cells. We consider the following model to explain these findings (Fig. 7). Among MCM2–7 proteins, only MCM2 and MCM3 have NLS (*29–31*). It is possible that newly synthesized MCM2, 4, 6 and 7 proteins and MCM3 and MCM5 proteins assemble into the MCM2/4/6/7 tetramer and MCM3/5 dimer, respectively, in cytoplasm, and they are then translocated into nucleus to assemble into an MCM2–7 hexamer or that the MCM2–7 complex assembled by these two complexes in cytoplasm is translocated to nucleus. It has been reported that such a proper complex formation of MCM proteins is required for nuclear retention of MCM proteins (*30*). It is probable that the forcedly expressed wild-type MCM4 is assembled into MCM2/4/6/7 and MCM2–7 complexes, but the mutant MCM4 is hardly assembled into these complexes due to inefficient interaction with MCM6. Such a defect of the mutant MCM4 would result in difficulty of being translocated into nucleus. This may lead to decreased amounts of MCM2–7 complexes bound to chromatin. The data show that only the Flag-tagged wild-type MCM4, which are expressed at lower level, is localized to nucleus. The MCM4 expressed at higher level may form aggregates in cytoplasm and thus cannot properly interact with endogenous MCM2–7 members. Recently, it has been reported that mouse cells with this MCM4 mutation have reduced amounts of the MCM2–7 proteins on chromatin, resulting in a reduced number of dormant replication origins (*32*).

Human MCM4 consists of 863 amino acids. The amino-terminal region contains a number of phospho-acceptor sites and zinc finger domain (amino acids no. 306–331), and the central and carboxyl-terminal regions contain domains that are essential for ATP binding and hydrolysis. The mutation site (amino acids no. 346) of human MCM4 is located near the zinc finger domain. Archia MCM consists of ~700 amino acids and forms a homogeneous hexamer. Biochemical analysis indicates that the amino-terminal region (1–500) of archia MCM is mainly responsible for hexameric complex formation (*33*). Structural data suggests that the site of the MCM4 mutation, which is conserved from Archia to human, appears to interact with an adjacent MCM subunit in the hexamer. Thus, it is possible that the MCM4 mutation affects the interaction with neighboring MCM6 subunit in MCM2–7 hexamer.

MCM2–7 complex bound to DNA has to be activated to exhibit DNA helicase activity to function as a replicative DNA helicase. Although the structure of the activated MCM helicase remains to be determined, it is possible that MCM2–7 hexamer is converted to the active form. It is also possible that MCM4/6/7 hexamer that exhibits distinct DNA helicase activity *in vitro* is generated on the activation. Recently, it has been reported that other MCM complex consisting of MCM4 and MCM7 also exhibits DNA helicase activity (*34*). As the ATP-binding site in MCM7 plays a major role in exhibiting ATPase activity, it is probable that MCM complexes containing MCM7 are capable of hydrolyzing ATP and exhibit DNA helicase activity (*35*). The MCM4m/6/7 complexes containing the mutated MCM4 exhibited DNA helicase activity that is almost comparable with the complex containing wild-type MCM4. However, the data suggest that the formation of MCM4m/6/7 hexamer with the mutant MCM4 is less stable than that with wild-type MCM4 (Fig. 1). This is probably due to the changes in the interaction of MCM4 with MCM6. Recently, it has been shown that Drosophila CMG complex containing MCM4 with the same mutation exhibits DNA helicase activity, but it is unstable in comparison with the complex containing wild-type MCM4 (*32*).

Based on the presented data, we consider the following model to explain why mouse cells with this mutation are transformed. As it is suggested from this study that the formation of the MCM2/4/6/7 subcomplex or MCM2–7, which is assembled from newly synthesized MCM proteins, is limited in mouse cells with the MCM4 mutation, enough of the MCM2–7 complex is not formed on DNA. Initiation of DNA replication is triggered by the action of MCM helicase that is activated from the MCM2–7 complex. Although the number of the MCM2–7 complex may be excess to the number of the replication origin in the cells containing

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wild-type MCM4, the decrease in the MCM2–7 complexes bound to origin DNA in the cells containing the mutant MCM4 would result in generation of unreplicated DNA region during S phase that leads to segregation of abnormal chromosome structures to daughter cells at M phase. Consistent with this notion, it has been shown that the inactivation of MCM proteins generates abnormal chromosomes in *S. pombe* (*36*, *37*). Recently, it has been shown that elevated levels of DNA damages are observed in mouse cells with this mutation (*32*). It can be speculated that the formation of the aberrant chromosome structures finally generates the malignant transformation of mouse cells with the mutated MCM4. Shima *et al.* (*13*) reported that the amounts of MCM4 and MCM7 decrease in mouse embryonic fibroblasts with this mutation of MCM4. This may be caused by degradation of MCM proteins that are not assembled into proper MCM complexes. This will also contribute to the decrease in MCM2–7 complexes bound to DNA. However, forced expression of MCM4m in 293T cells did not lead to a decrease in levels of MCM7 protein in Triton-soluble and -insoluble fractions (Supplementary Fig. S2). Further analysis is required to address this point.

Supplementary Data

Supplementary Data are available at *JB* Online.

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Conflict of interest

None declared.

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