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Interaction of human MCM2-7 proteins with TIM, TIPIN and Rb

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Interactions of human MCM2-7 proteins with the proteins of TIM, TIPIN, an amino-terminal fragment of Rb, and p27 were examined by co-immunoprecipitation experiment using cell lysates of co-expressed insect cells. TIM and TIPIN, both of which are involved in regulation of DNA replication fork progression, mainly interacted with MCM3-7 proteins. The amino-terminal fragment of Rb, which inhibits DNA replication in *Xenopus* egg extracts, was able to bind with MCM3 and MCM6 proteins in addition to MCM7 protein. In contrast, p27 was not able to bind any MCM2-7 proteins under the comparable conditions. These results indicate that the proteins, which are known to interact with MCM proteins, bind with MCM2-7 proteins with different affinities and specificities.

Keywords: association/enzymes/helicase/protein/replication/synthesis.

Abbreviations: MCM, minichromosome maintenance; Rb, retinoblastoma; TIM, timeless; TIPIN, timeless-interacting protein.

MCM2-7 proteins play an essential role in the initiation and elongation of eukaryotic DNA replication (*1–4*). It is probable that they function as a replicative DNA helicase that unwinds DNA duplex at the replication forks. Consistent with this notion, several complexes, including the MCM4/6/7 hexamer, exhibit DNA helicase activity *in vitro* (*5–9*). The DNA helicase activity of the MCM2-7 heterohexameric complex was hardly detected under the standard reaction conditions but it has been reported that this complex exhibits the activity in the presence of acetate or glutamate anions (*10*). It has been shown that a CMG complex consisting of CDC45, MCM2-7 and GINS complex exhibits DNA helicase activity *in vitro* (*11*). *In vivo*, it appears that CDC45, MCM2-7 and GINS complex migrate on the genome with the replication forks in *Saccharomyces cerevisiae* (*12*,*13*) and they also migrate on plasmid DNA with the forks in a DNA replication system using *Xenopus* egg extracts (*14*). CDC45 protein, which is required for DNA replication, can directly interact with MCM proteins (*15*). GINS complex is required for the association of CDC45 with MCM complex in replisome progression complexes (*16*). Thus, it is probable that the proteins including CDC45 and GINS complex are required for activation of the helicase activity of MCM2-7 complex, although it remains to be determined what form of MCM complex functions as a DNA helicase *in vivo*.

It has been suggested that a number of proteins interact with MCM proteins to regulate cellular DNA replication. Both TIM and TIPIN proteins, which form a hetero-dimer, are involved in the activation of CHK1 by ATR/ATRIR in the DNA replication checkpoint system (*17*,*18*). It is also suggested that they are required for inhibition of the replication fork movement at the time when DNA synthesis is inhibited (*17–20*). The findings that both TIM and TIPIN are co-immuno-precipitated with MCM proteins in cell lysates suggest a possibility that they directly interact with MCM proteins to regulate the progression of the replication forks (*18*, *19*). Retinoblastoma (Rb) gene product is one of the major regulators that control the cell cycle transition from G1 to S phase. The amino-terminal fragment (1–400) of Rb (Rb-N) interacts with MCM7 to inhibit the DNA replication in *Xenopus* egg extracts (*21*,*22*). p27, which negatively regulates CDK/cyclin activity at G1 phase, also interacts with MCM7 to inhibit DNA replication in human cells (*23*).

In order to understand the mechanism by which the MCM2-7 helicase activity is regulated with these proteins, we examined the direct interaction of MCM2-7 proteins with these proteins by immuno-precipitation experiment using the co-expressed insect cell lysate. The results indicated that TIM, TIPIN and the Rb-N fragment interacted with MCM2-7 proteins with different affinities and specificities, but p27 protein did not interact with any of MCM2-7 proteins under comparable conditions. Thus, these proteins may regulate DNA replication by distinct mechanisms.

Materials and Methods

Cloning of human TIM, TIPIN, Rb, p27 and RAD17 genes

Total cDNA was synthesized from HeLa mRNA by reverse transcriptase using oligo dT or ramdom hexamer as a primer (Invitrogen, Carlsbad, CA, USA). Human TIM cDNA was amplified from the cDNA using gene-specific primers with nucleotide sequence for a Flag-tag (DYKDDDDK) at the amino-terminus. Flag-TIM gene was cloned in pVL1392 DNA, and nucleotide sequence of the cloned gene was determined by DNA sequencing in OpenGene system (Veritas, Tokyo, Japan). In comparison to the known sequence (AB015597) in NCBI, changes in several nucleotides were identified. Among them, T at no. 1363 was converted to A, which results in an amino acid change from L to I. The recombinant baculovirus expressing the recombinant Flag-TIM protein was prepared as the manufacturer's protocol (Pharmingen, BD, San Jose, CA, USA). Human TIPIN cDNA was amplified from the cDNA using gene-specific primers with nucleotide sequence for a His-tag at the amino-terminus. $(His)_6$-TIPIN gene was cloned in pVL1392

DNA and the recombinant baculovirus expressing the recombinant $(His)_6$-TIPIN protein was prepared. The nucleotide sequence of the cloned TIPIN gene was identical to the reported one (NM_017858). Human p27 cDNA was amplified from the cDNA using gene-specific primers with nucleotide sequence for a Flag-tag at the amino-terminus. Flag-p27 cDNA was cloned in pVL1392 DNA and the recombinant baculovirus expressing the recombinant Flag-p27 protein was prepared. The nucleotide sequence of cloned p27 gene was identical to the reported one (NM_004064). cDNA of the amino-terminal region (amino acid no. 1–400) of human Rb protein was amplified using the cloned full-size human Rb gene (kindly provided by Kitagawa, M.) as a template using primers with a His-tag at the amino-terminus and a Flag-tag at the carboxyl-terminus, and it was cloned into pVL1392 DNA. The recombinant baculovirus expressing the Rb fragment with a $(His)_6$ tag and a Flag tag was prepared. The nucleotide sequence of the cloned Rb gene was different from the reported one (M15400) at four sites of 810 (A to G), 997 (C to G), 998 (G to C) and 1102 (A to G). Resultant amino acids changes were T to G (810), R to A (997 and 998) and I to V (1102), respectively. In addition, a nucleotide sequence from 350 to 372 was changed from ACTGAGCTACAGA AAAACATAC (LLSYRKYYE in amino acids) to TACTGAGCT ACAGAAAAACATA (FTELQKNIE). Human RAD17 cDNA was amplified from the cDNA using gene-specific primers with nucleotide sequence for a His-tag at the amino-terminus. The $(His)_6$-RAD17 gene was cloned in pVL1392 DNA and the recombinant baculovirus expressing the recombinant $(His)_6$-RAD17 protein was prepared. In comparison to the known sequence (NM_133344) in NCBI, changes in two nucleotides were identified. Among these two, A at no. 713 was converted to G, which results in an amino acid change from I to V.

Co-expression of proteins and immuno-precipitation

High5 cells were co-infected with the recombinant baculoviruses for 2 days. Typically, 0.5 ml of each viral stock solution was added to 7×10^6 cells. The infected cells were suspended in lysis buffer consisting of 10 mM Tris–HCl (pH 7.5), 130 mM NaCl, 1% Triton X-100, 10 mM NaF, 10 mM Na-phosphate buffer, 10 mM $Na_4P_2O_7$ and protease inhibitors (Pharmingen, BD). After incubation for 40 min on ice, insoluble material was removed by centrifugation at 40,000 r.p.m. (TLS55, Beckman, Fullerton, CA, USA) for 40 min at 4°C. To 200 µl of the clarified lysate, 4 µg of anti-Flag-antibody (Sigma, St Louis, MO, USA), 2 µg of anti-RAD17 antibody or 200 µl of culture supernatant of 13B4 hybridoma cells producing anti-TIPIN antibody was added, and the mixture was incubated for 1 h at 4°C on a rocking platform. Protein G-Sepharose (20 µl) was added to the mixture and the solution was incubated at 4°C overnight. The beads were then collected by centrifugation and washed 10–12 times with 10-fold volumes of phosphate-buffered saline (PBS). The proteins bound to the beads were eluted by adding an equal volume of 0.1 M glycine, pH 2.5 and 0.15 M NaCl. The elution of proteins was repeated two more times and these eluates were neutralized by adding 1/10 vol of 2 M Tris–HCl, pH 8.0. In case of precipitation of Rb-N fragment and p27 (Fig. 6, Supplementary Figs 5 and 6), anti-Flag agarose beads (Sigma) were used. Proteins bound to the beads were eluted by incubating the beads with a Tris-buffered saline (TBS) containing 50 µg/ml of 3× Flag peptides (Sigma).

Immuno-blotting

Proteins were separated by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis. After the proteins in the gel were transferred to Immobilon-P transfer membrane (Millipore, Bedford, MA, USA), the membrane was incubated for 1 h at room temperature with a blocking buffer (EzBlock, ATTO, Tokyo, Japan) diluted 3-fold with TBS plus 0.1% Triton X-100; it was then incubated overnight at 4°C with 0.5–1 µg/ml of 1st 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 2nd antibody conjugated with horseradish peroxidase (BioRad, Hercules, CA, USA). After washing, the membrane was incubated with SuperSignal West Pico or Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL, USA) and chemiluminescent signals were detected by Light-Capture (ATTO, Tokyo, Japan). The intensity of signals was quantified using CS analyzer 2.0. Anti-MCM2 antibodies (serum) were prepared by immunizing full-size human MCM2 to rabbits. Anti-MCM3, -MCM4 and -MCM5 antibodies were prepared as reported (*24*,*25*). Anti-MCM6 (Santa Cruz, Biotechnology, Santa Cruz, CA, sc-9843), anti-MCM7 (Santa Cruz Bio, sc-9966), anti-RAD17 (MBL, Nagoya, Japan) and anti-p27 (Santa Cruz Bio, sc-1641) antibodies were purchased.

Monoclonal antibody production

Synthetic peptides (Sigma-Genosys) of 20 amino acids from the amino-terminus and the carboxyl-terminus of human TIPIN were coupled to keyhole limpet hemocyanin and used to immunize mice (*26*). After generating hybridomas, clones were screened by enzyme-linked immunosorbent assay (ELISA).

Purification of MCM complexes

Human His-MCM4/MCM6/His-MCM7 complex, His-MCM2/His-MCM4/MCM6/His-MCM7 complex and MCM3/His-MCM5 complex were purified from co-expressed High5 cells as reported (*27*,*28*). These proteins were used as a standard to quantify the immuno-precipitated MCM proteins.

Results

Interaction of MCM7 with MCM2-6 proteins

To confirm the applicability of our assay system to examine protein–protein interactions, we performed the following experiments. After human Flag-MCM7 protein was co-expressed with one of MCM2-6 proteins in insect cells, MCM7 in the lysate was immuno-precipitated with anti-Flag antibody, and the presence of the co-expressed MCM2-6 proteins in the precipitate was examined. After the precipitate was extensively washed with PBS, the MCM proteins in the precipitate were electrophoresed in an SDS-polyacrylamide gel and they were detected with anti-MCM-specific antibodies. In Fig. 1A, MCM7 was co-expressed with MCM2. Proteins in Triton-soluble (S) and -insoluble (I), those not bound with the antibody (U), those in supernatant after the final wash (W) and those eluted from the precipitate (P) were all electrophoresed. MCM7 protein, which migrated at the position of 90 kDa protein in SDS-gel, was detected in the S, I and U fractions. The finding that almost comparable amounts of MCM7 were detected between the S and U fractions suggests that the anti-Flag antibody used are limiting under these conditions. MCM7 protein was detected in the precipitate (P) fractions, which is shown in Fig. 1A. However, MCM2 was hardly detected in the precipitate fractions. When MCM7 was co-expressed with MCM3, MCM4, MCM5 or MCM6 protein, each of MCM3-7 proteins was co-precipitated with Flag-MCM7 (Fig. 1B–E). In the absence of co-expressed Flag-MCM7, almost no MCM2-7 proteins were precipitated (Supplementary Fig. 1), indicating the precipitation of MCM3-6 proteins in the above experiment is dependent upon the presence of co-expressed MCM7 protein. The amounts of the precipitated MCM3-6 proteins in the complete experiments were determined by comparing the staining intensity of the precipitated MCM proteins with those of purified MCM proteins in the gel (Fig. 1F). The results show that the highest amount was detected for MCM3 and then the amounts decreased by the order of MCM4, MCM6 and MCM5. The band intensity of precipitated Flag-MCM7 was compared to the intensity in the S fraction in the co-expression of Flag-MCM7 and

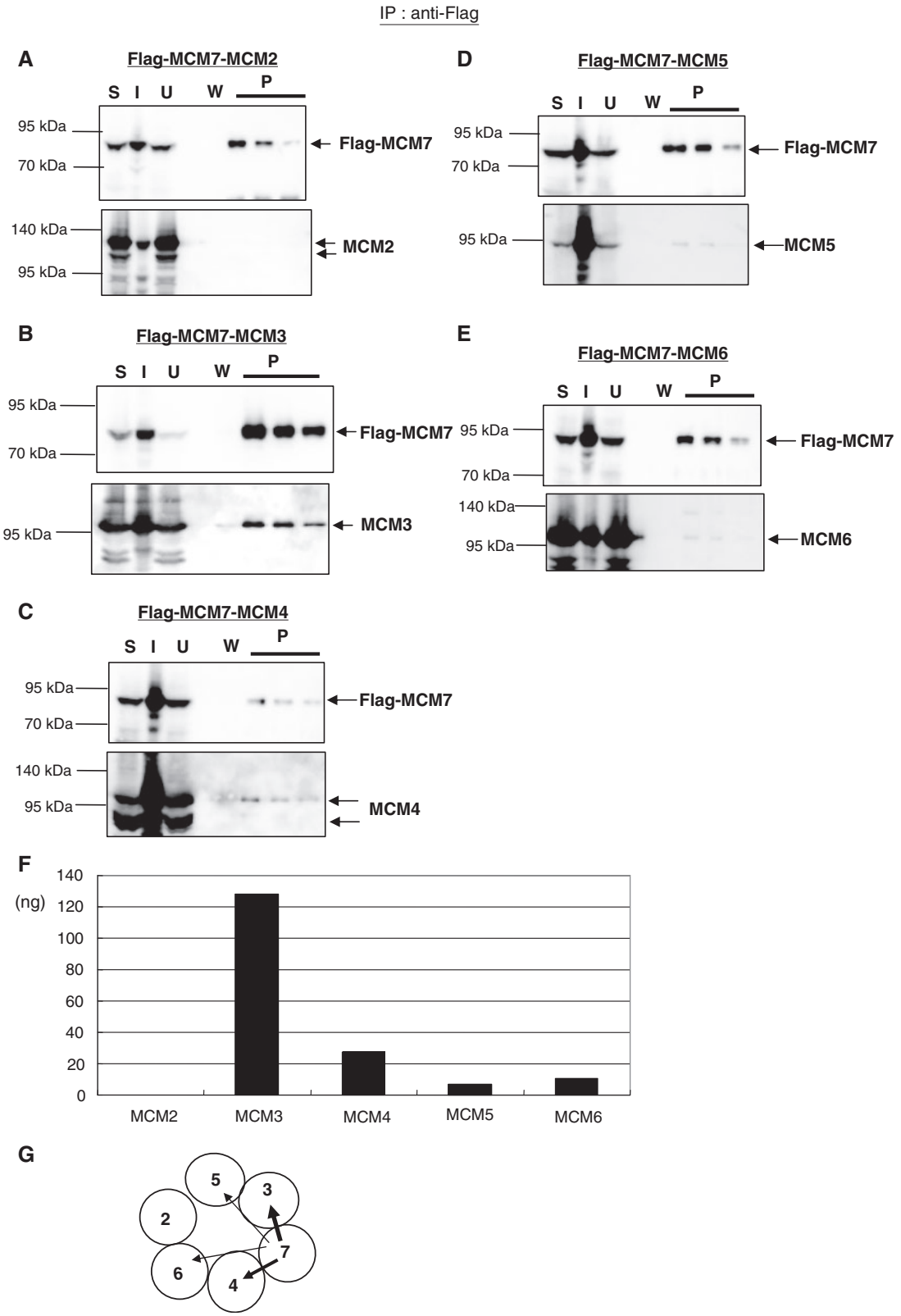

Fig. 1 Interaction of MCM7 with MCM2-6 proteins. Flag-MCM7 protein was co-expressed with proteins of MCM2 (A), MCM3 (B), His-MCM4 (C), His-MCM5 (D) or MCM6 (E) in insect cells using recombinant baculoviruses. The insect cells were lysed with buffer containing Triton to obtain Triton-soluble (S) and -insoluble (I) proteins. Flag-MCM7 in the S fraction was incubated with anti-Flag antibody and then with protein G-Sepharose beads. After spin, proteins that did not bind to the beads were recovered (U). The beads were extensively washed and supernatant after the final washing was recovered (W). Proteins bound to the beads were eluted three times (P). Proteins in S, I, W and P fractions were electrophoresed and transferred to a filter. The filter was probed with anti-Flag antibody and anti-MCM-specific antibodies. Precipitated Flag-MCM7 was shown in (A–E). Co-precipitated MCM proteins in (A–E) were quantified and presented in (F). Bands migrate faster than intact MCM2 and MCM4 proteins are degradation products. In (G), the interaction between MCM7 and MCM2-6 proteins are depicted in a model of arrangement of MCM2-7 proteins in MCM2-7 hexamer (*29*). The difference in the levels of the interaction was indicated by the thickness of arrows.

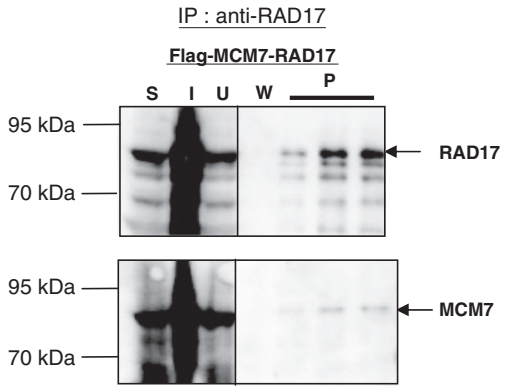

Fig. 2 Interaction of MCM7 with RAD17 protein. Flag-MCM7 protein was co-expressed with RAD17 protein in insect cells. The insect cells were lysed to obtain Triton-soluble (S) and -insoluble (I) proteins. RAD17 in the S fraction was incubated with anti-RAD17 antibody and then with protein G-Sepharose beads. Unbound (U) and wash (W) fractions were obtained and proteins bound to the beads were eluted (P). Proteins in S, I, W and P fractions were electophoresed and they were probed with anti-RAD17 and anti-MCM7 antibodies.

MCM3. It should be noted that such the relative intensity of the precipitated Flag-MCM7 band in this case was higher than those in other cases, suggesting that the amounts of MCM3 co-precipitated with Flag-MCM7 are over-estimated. This result of Fig. 1 can be compared with the proposed arrangement of MCM2-7 proteins in the MCM2-7 hexamer (Fig. 1G). This arrangement is proposed based upon the results on the interaction of two MCM proteins in two-hybrid experiment and those on protein–protein cross-linking experiment of isolated MCM2-7 hexamer (*29*). The present result is consistent with the proposed arrangement of MCM2-7 proteins in that MCM7 mainly interacts with MCM3 and MCM4.

It has been reported that MCM7 directly interacts with RAD17 protein which functions as one of the sensor proteins that recognize the single-stranded DNA region in the arrested DNA replication forks (*30*). Both Flag-MCM7 and RAD17 proteins were co-expressed, and RAD17 in the lysate was precipitated with anti-RAD17 antibodies (Fig. 2). MCM7 was co-precipitated with RAD17, indicating that these two proteins directly interact. These results indicate that this assay system is applicable to examine protein–protein interactions.

Interaction of TIM and TIPIN with MCM2-7 proteins

TIM and TIPIN, which form a hetero-dimer, play a role in the DNA replication checkpoint system by facilitating the phosphorylation of CHK1 with ATR/ATRIP and also by protecting the arrested replication fork structure (*17–20*). The movement on the genome of the proteins involved in the fork progression proceeds even in the absence of DNA synthesis without Tof1, a TIM homologue, in *S. cerevisiae*. This finding suggests that Tof1 functions to inhibit the progression of the replication fork when DNA synthesis is inhibited (*12*). Tof1 and Csm3, which is a homologue of TIPIN, have been originally identified as proteins that are required for protecting the replication fork structures (*31*). Interaction of TIM or TIPIN with several MCM members in cells has been reported (*18–20*) but the experiments to examine systematically the direct interaction of these proteins have not been carried out. Flag-TIM and one of MCM2, MCM3, His-MCM4, His-MCM5, MCM6 and His-MCM7 proteins were co-expressed in insect cells, and the Flag-TIM in the lysate was immuno-precipitated with anti-Flag antibody (Fig. 3A–F). The precipitated TIM was detected by using anti-Flag antibody, which is shown in Fig. 3A–F. All the co-expressed MCM2-7 proteins were detected in the S, I and U fractions. Each of MCM2-7 proteins was significantly detected in the precipitate. As control experiments, each of MCM2-7 proteins was expressed in the absence of TIM, and immuno-precipitation using anti-Flag antibody was performed (Supplementary Fig. 2). Bands of MCM2, 5, 6 and 7 proteins were not detected in the precipitate. Faint bands of MCM3 and MCM4 were detected but their band intensities were 4–5% of those precipitated in the presence of Flag-TIM. These results indicate the precipitation of MCM2-7 proteins in the above experiment is dependent upon the presence of co-expressed TIM protein. The amounts of MCM2-7 proteins in the precipitate in the complete experiments were quantified using purified MCM proteins as a standard (Fig. 3G). The highest amount was detected for MCM4 and the amounts decreased by the order of MCM5, MCM6, MCM3, MCM7 and MCM2. The band intensity of precipitated Flag-TIM was compared to the intensity in the S fraction in the co-expression of Flag-TIM and MCM3. Such the relative intensity of the precipitated Flag-TIM band in this case was lower than those in other cases, suggesting that the amounts of MCM3 co-precipitated with Flag-TIM are underestimated.

Next, TIPIN was co-expressed with each of MCM2, MCM3, His-MCM4, His-MCM5, MCM6 and His-MCM7 proteins, and immuno-precipitation using anti-TIPIN antibody was performed (Fig. 4A–G). Each of MCM2-7 proteins was co-precipitated with TIPIN. As control experiments, each of MCM2-7 proteins was expressed in the absence of TIPIN, and immuno-precipitation using anti-TIPIN antibody was performed (Supplementary Fig. 3). Bands of MCM2, 5, 6 and 7 proteins were not detected in the precipitate. Faint bands of MCM3 and MCM4 were detected but their band intensities were 6% and 23% of those precipitated in the presence of TIPIN, respectively. These results indicate the precipitation of MCM2-7 proteins in the above experiment is basically dependent upon the presence of co-expressed TIPIN protein. Quantification of the precipitated MCM2-7 proteins in the complete experiments shows that the highest amount was detected for MCM7, and the levels decreased by the order of MCM3, MCM5, MCM4, MCM6 and MCM2. These results suggest that TIM

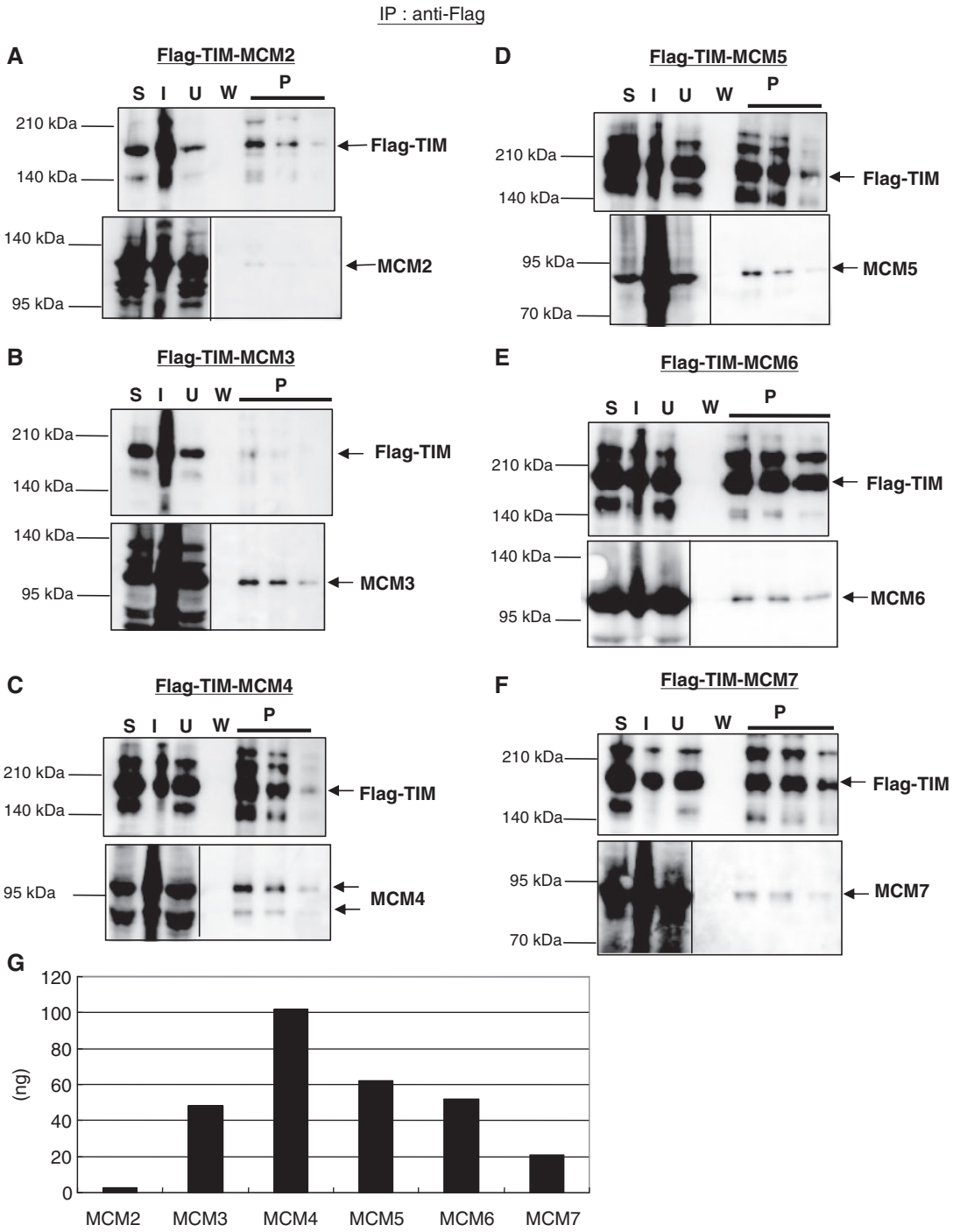

Fig. 3 Interaction of TIM with MCM2-7 proteins. Flag-TIM protein was co-expressed with MCM2 (A), MCM3 (B), His-MCM4 (C), His-MCM5 (D), MCM6 (E) or His-MCM7 (F) protein in insect cells. The insect cells were lysed to obtain Triton-soluble (S) and -insoluble (I) proteins. Flag-TIM in the S fraction was immuno-precipitated with anti-Flag antibody, and unbound (U) proteins were recovered. After wash (W), precipitated proteins were eluted three times from protein G-Sepharose beads (P). Proteins in S, I, W and P fractions were electrophoresed and probed with anti-Flag antibody and anti-MCM-specific antibodies. Precipitated Flag-TIM was shown in (A–F). Co-precipitated MCM proteins in (A–F) were quantified and presented in (G).

and TIPIN directly interact with MCM2-7 proteins by different affinities.

Interaction of an amino-terminal fragment of Rb with MCM2-7 proteins

Interaction between an amino-terminal fragment of human Rb protein (Rb-N) and human MCM7 has been observed by two-hybrid system in which the fragment was used as bait (*21*). The carboxyl-terminal 137 amino acids region of MCM7 binds to the Rb-N fragment. The addition of the Rb-N fragment to *Xenopus* egg extracts inhibits the DNA replication (*21*), suggesting that the Rb-N fragment interacts with MCM7 to inhibit MCM helicase activity. In order to examine the

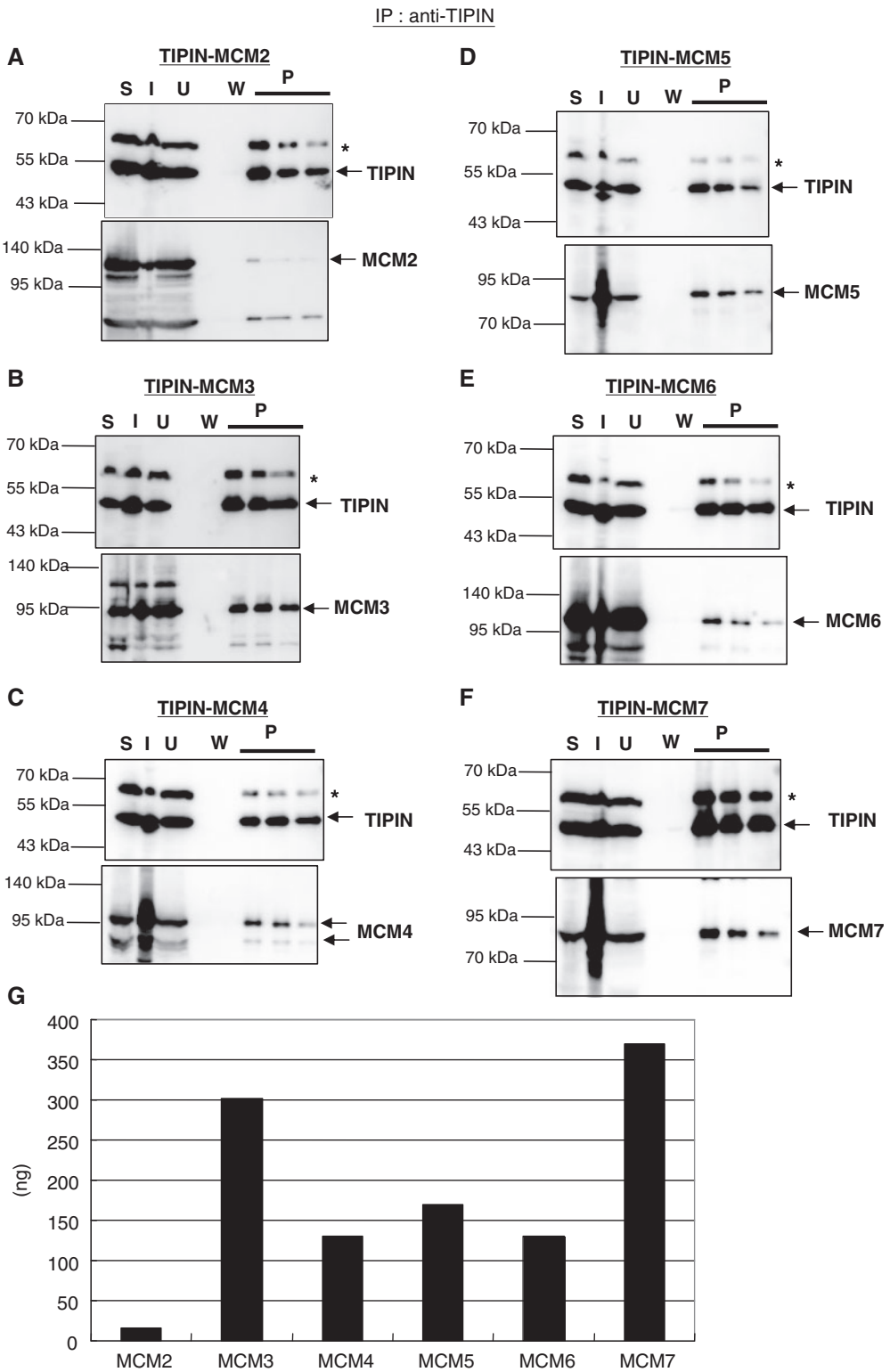

Fig. 4 Interaction of TIPIN with MCM2-7 proteins. His-TIPIN protein was co-expressed with MCM2 (A), MCM3 (B), His-MCM4 (C), His-MCM5 (D), MCM6 (E) or His-MCM7 (**F**) protein in insect cells. The insect cells were lysed to obtain Triton-soluble (S) and -insoluble (I) proteins. His-TIPIN in the S fraction was immuno-precipitated with anti-TIPIN antibody, and unbound (U) proteins were recovered. After wash (W), precipitated proteins were eluted three times from protein G-Sepharose beads (P). Proteins in S, I, W and P fractions were electrophoresed and they were probed with anti-TIPIN antibody and anti-MCM-specific antibodies. Precipitated TIPIN was shown in (A)–(F). Bands marked by asterisk indicate an unidentified one. Co-precipitated MCM proteins in (A–F) were quantified and presented in (G).

direct interaction between the Rb-N fragment and MCM2-7 proteins, we performed co-immuno-precipitation experiment using insect cell lysates. The Flag-tagged Rb-N protein and one of MCM2, MCM3, His-MCM4, His-MCM5, MCM6 and His-MCM7 proteins were co-expressed in insect cells, and the Rb-N in the lysate was precipitated with anti-Flag antibody (Fig. 5A–F). The Rb-N fragment, which

migrates as 65 kDa protein in SDS-gel, was detected in the precipitate fractions. A band of MCM3 was detected in the precipitated fractions. Very faint bands of MCM6 and MCM7 were detected after prolonged exposure of filters. As control experiments, each of MCM2-7 proteins was expressed in the absence of the Rb-N fragment, and immuno-precipitation using anti-Flag antibody was performed (Supplementary Fig. 4). Bands of MCM2-7 proteins were not detected in the precipitate. The amounts of these MCM proteins in the complete experiments were quantified (Fig. 5G). The highest amount was detected for MCM3 and the amounts decreased by the order of MCM7 and MCM6. The amounts of MCM2, MCM4 and MCM5 proteins were below the detectable level. When the immuno-precipitation was performed with anti-Flag agarose beads instead of using anti-Flag antibody, co-precipitation of MCM3, MCM5, MCM6 and MCM7 with the Flag-Rb-N was observed (Supplementary Fig. 5). Since both MCM3 and MCM5 were co-expressed with the Rb-N fragment in this experiment, it is possible that MCM5 was co-precipitated not with Flag-Rb-N but with MCM3. The results suggest that Rb-N fragment directly binds with MCM3, MCM6 and MCM7.

Interaction of p27 with MCM2-7 proteins

p27 that is an inhibitor of CDK/cyclin interacts with MCM7 in two-hybrid and pull-down experiments (*23*). Furthermore, it has been reported that the interaction of p27 with MCM7 inhibits the initiation of cellular DNA replication. Direct interaction between Flag-p27 and one of MCM2, His-MCM4, MCM6 and His-MCM7 proteins was examined by co-immunoprecipation experiment (Fig. 6A–E). MCM3 and MCM5 proteins were co-expressed with p27 at the same time. It was confirmed that p27 in the S fraction was precipitated with anti-Flag antibody (Fig. 6A–E). However, none of the co-expressed MCM2, MCM3, MCM4, MCM5, MCM6, and MCM7 was detected in the precipitate. These findings were confirmed by the experiments where p27 was co-expressed with MCM4/6 proteins, MCM6/7 proteins, MCM2/7 proteins or MCM4/6/7 proteins (Supplementary Fig. 6). p27 was not detected in the precipitate in these experiments. In contrast, when CDK2 or CDK4 protein was co-expressed with p27, they were co-precipitated with p27 (Fig. 6F and G). Thus, these results indicate that p27 does not stably bind with MCM2-7 proteins under the present conditions.

Discussion

It has been suggested that a number of proteins interact with MCM2-7 proteins. Among them, we chose TIM, TIPIN, Rb and p27 proteins, and examined whether they can directly interact with MCM2-7 proteins. We performed the immuno-precipitation experiments using cell lysate of co-expressed insect cells. The data suggest that both TIM and TIPIN interact with MCM2-7 proteins with different affinities (Table I). The amino-terminal fragment of Rb interacted with MCM3, MCM6 and MCM7 proteins. In contract, the interaction between p27 and MCM2-7 proteins was not detected. These results suggest that TIM, TIPIN, Rb and p27 proteins interact with MCM2-7 with different affinities and with different specificities.

It has been reported that TIPIN is co-precipitated with MCM6 and MCM7 proteins (*18*,*19*). The present study suggests that both TIM and TIPIN can mainly interact with MCM3-7 proteins, although the regions in MCM3-7 that interact with TIM and TIPIN remain to be determined. This result is in contrast to the finding that CDT1 specifically interacts with MCM6 (*32*). The finding that TIM and TIPIN can directly interact with MCM3-7 proteins suggests a possibility that a TIM-TIPIN dimer induces a structural change of the MCM2-7 hexamer to inhibit its DNA helicase activity when the replication fork movement is perturbed. Among MCM3-7 proteins, TIM has the highest affinity for MCM4 that is a constituent of core subunit of MCM4/6/7 trimer in the MCM2-7 hexamer. The MCM4/6/7 hexamer, a dimer of MCM4/6/7 trimer, exhibits distinct DNA helicase activity, and adenosine triphosphate (ATP) binding motif of MCM4 plays a role in the activity (*33*). TIPIN has the highest affinity for MCM7 that plays a central role in ATP hydrolysis of the MCM4/6/7 helicase (*33*) and also of MCM2-7 complex (*34*). Binding of TIM and TIPIN to MCM4 and MCM7, respectively, may give serious effect on the MCM helicase activity. The amino-terminal fragment of Rb, which acts as a negative regulator of cell cycle at G1 phase, has been reported to interact with a carboxyl-terminal fragment of MCM7 using yeast two-hybrid system (*21*). The Rb fragment inhibits the unwinding of DNA duplex in *Xenopus* egg extracts and the inhibition is neutralized by addition of the carboxyl-terminal 137 amino acids fragment of MCM7 that interacts with the Rb fragment (*22*). Consistent with this result, our results indicate that the Rb fragment can bind to MCM7. It is possible that the interaction of the Rb fragment with MCM7 inhibits the ATPase activity of MCM7 to inhibit DNA replication in *Xenopus* egg extracts. In addition, we detected strong interaction of the Rb fragment with MCM3 and weak interaction with MCM6. It remains to be determined whether the changes in 12 amino acids of the Rb-N fragment used in this study may affect the interaction with MCM proteins.

p27, which is a negative regulator of cell cycle at G1 phase, has been reported to interact with MCM7 by two-hybrid system, pull-down experiment and immuno-precipitation experiment using mammalian cell lysates (*23*). The addition of the peptide, which blocks the interaction between p27 and MCM7, is shown to inhibit the DNA replication in human cells. In our experiment, however, the interaction between p27 and MCM2-7 proteins was not detected. It is probable that the interaction of p27 and MCM7 is not so strong to be detected under our experimental conditions; or, it is possible that p27 interacts with MCM complexes *in vivo*.

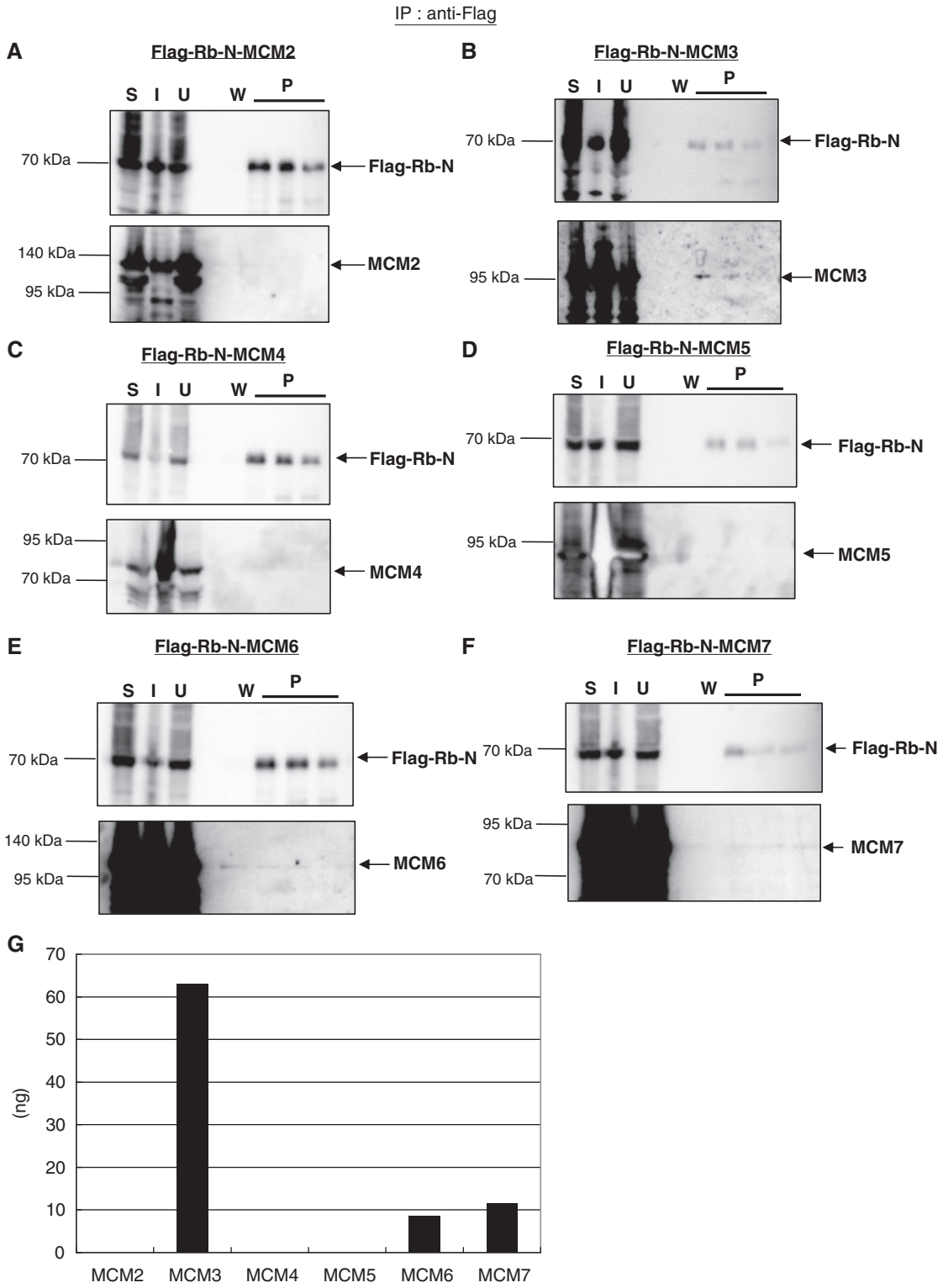

Fig. 5 Interaction of Rb-N fragment with MCM2-7 proteins. Flag-Rb-N fragment was co-expressed with MCM2 (A), MCM3 (B), His-MCM4 (C), His-MCM5 (D), MCM6 (E) or His-MCM7 (**F**) protein in insect cells. The insect cells were lysed to obtain Triton-soluble proteins (S). Flag-Rb-N in the S fraction was immuno-precipitated with anti-Flag antibody beads, and unbound (U) proteins were recovered. After the final wash (W), precipitated proteins were eluted from protein G-Sepharose beads (P). Proteins in S, I, W and P fractions were electrophoresed and they were probed with anti-Flag antibody and anti-MCM-specific antibodies (A–F). Precipitated Rb-N fragment was shown in (A–F). In (G), co-precipitated MCM proteins in (A–F) were quantified. The amounts of MCM2, MCM4 and MCM5 proteins were below the detectable level.

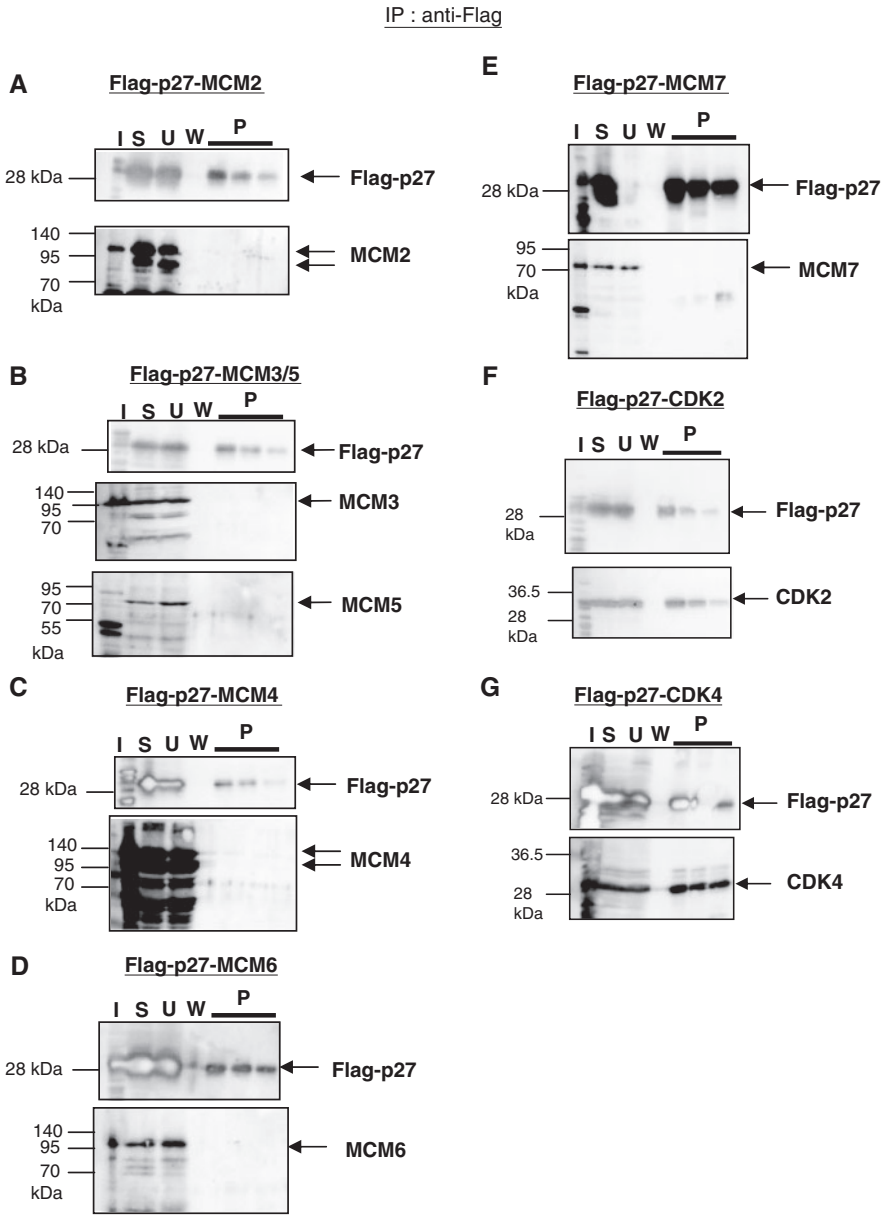

Fig. 6 Interaction of p27 with MCM2-7 proteins. Flag-p27 protein was co-expressed with MCM2 (A), MCM3 and His-MCM5 (B), His-MCM4 (C), MCM6 (D) or His-MCM7 (E) protein in insect cells. The insect cells were lysed to obtain Triton-soluble proteins (S). Flag-p27 in the S fraction was precipitated with anti-Flag antibody beads and unbound (U) proteins were recovered. After wash (W), precipitated proteins were eluted from protein G-Sepharose beads (P). Proteins in S, I, W and P fractions were electrophoresed and probed with anti-Flag antibody and anti-MCM-specific antibodies (A–F). Precipitated p27 was shown in (A–E). In (B), full-sized MCM5 protein was not detected in (I) fraction. It is probable that MCM5 protein was degraded by unknown reasons. Flag-p27 protein was co-expressed with CDK2 (F) or CDK4 (G) protein in insect cells. Proteins in S, I, W and P fractions were electrophoresed and they were probed with anti-Flag antibody and anti-CDK-specific antibodies.

Table I. Summary of the interactions.

	MCM7	TIM	TIPIN	Rb-N	p27	RAD17
MCM2	–	+/–	+/–	–	–	nd
MCM3	+++	++	+++	++	–	nd
MCM4	++	+++	++	–	–	nd
MCM5	+	++	++	–	–	nd
MCM6	+	++	++	+	–	nd
MCM7	nd	+	+++	+	–	+

The level of the interaction was expressed as, –, +/–, +, ++, +++. nd: not determined.

It has been reported that several mouse proteins present in the replication forks can interact with several members of MCM2-7 in two-hybrid system (*15*). The present study reveals novel interactions of human TIM, TIPIN and Rb proteins with MCM3-7 proteins in insect cells. Elucidation of detail of these interactions and their outcomes will give a hint to understand the mechanism of regulation of MCM2-7 helicase complex by these MCM-interacting proteins.

Supplementary Data

Supplementary data are available at *JB* online.

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Conflict of interest

None declared.

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