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Identification of proteins that may directly interact with human RPA

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RPA, which consisted of three subunits (RPA1, 2 and 3), plays essential roles in DNA transactions. At the DNA replication forks, RPA binds to single-stranded DNA region to stabilize the structure and to assemble other replication proteins. Interactions between RPA and several replication proteins have been reported but the analysis is not comprehensive. We systematically performed the qualitative analysis to identify RPA interaction partners to understand the protein–protein interaction at the replication forks. We expressed in insect cells the three subunits of human RPA, together with one replication protein, which is present at the forks under normal conditions and/or under the replication stress conditions, to examine the interaction. Among 30 proteins examined in total, it was found that at least 14 proteins interacted with RPA. RPA interacted with MCM3-7, MCM-BP and CDC45 proteins among the proteins that play roles in the initiation and the elongation of the DNA replication. RPA bound with TIPIN, CLASPIN and RAD17, which are involved in the DNA replication checkpoint functions. RPA also bound with cyclin-dependent kinases and an amino-terminal fragment of Rb protein that negatively regulates DNA replication. These results suggest that RPA interacts with the specific proteins among those that play roles in the regulation of the replication fork progression.

Keywords: checkpoint proteins/MCM helicase/replication fork/RPA/single-stranded DNA-binding protein.

Abbreviation: ATR, ATM and Rad 3-related; ATRIP, ATR-interacting protein; CDC, cell division cycle; CDK, cyclin-dependent kinase; MCM, minichromosome maintenance; PSF, partner of SLD five; RAD, radiation; Rb, retinoblastoma; RPA, replication protein A; SLD, synthetic lethality with Dpb11-1; TIM, timeless; TIPIN, timeless-interacting protein.

The RPA complex, consisted of three subunits of RPA1, RPA2 and RPA3 (70, 34 and 11 kDa, respectively), has been identified as a cellular factor that plays an essential role in SV40 DNA replication *in vitro*. It has been shown that RPA plays roles in the DNA transactions of DNA replication, transcription and recombination in eukaryotic cells by interacting with single-stranded DNA (*1*, *2*). Both RPA1 and RPA2 subunits contain OB-fold structures that bind to single-stranded DNA (*3*). Collaboration of RPA with T antigen DNA helicase and DNA polymerase α/primase complex is required for the initiation of DNA synthesis in SV40 DNA replication (*4*). At cellular DNA replication, it is known that MCM DNA helicase, which is generated from MCM2-7 complex with the assistance of CDC45 protein and GINS complex, functions as a replicative DNA helicase (*5*, *6*). At the initiation and the elongation of cellular DNA replication, RPA binds to the unwound single-stranded DNA region generated by the action of MCM helicase to stabilize the structure and to assemble other proteins. At the time when the DNA synthesis is perturbed at the elongation of DNA replication, the DNA replication checkpoint system is induced. This system functions not only for retarding the cell cycle progression but also for inhibiting the initiation of DNA replication and protecting the replication fork structures. The checkpoint system initiates from recognition by sensor proteins including RAD17 of a relatively long single-stranded DNA region that is generated by uncoupling of the fork movement and DNA synthesis (*7*). RPA binds the single-stranded DNA region to assemble the proteins involved in the signal transduction system (*8*, *9*). ATRIP binds to this region by interacting with RPA, and ATR/ATRIP complex phosphorylates CHK1 kinase for its activation (*10*). TIPIN bound with TIM, together with Claspin, facilitates the CHK1 phosphorylation with ATR/ATRIP. The activated CHK1 phosphorylates CDC25 to inhibit its function to activate CDK. The interactions of RPA with RAD17 (*8*) and ATRIP (*10*, *11*) have been reported and a relatively stable interaction with RPA has been reported for TIPIN (*12*, *13*).

TIPIN/TIM complex and Claspin seem to migrate with the replication forks under normal conditions without the replication stress. Several other proteins including MCM10 co-migrate with the forks (*6*). Rb protein, which plays a negative role in G1/S transition by interacting with E2F transcription factor, interacts with MCM proteins to inhibit the replication fork progression in a DNA replication system using *Xenopus* egg extracts (*14*). CDK that plays essential roles in the G1/S transition and in the initiation of DNA replication has central roles in preventing re-initiation

of DNA replication in a single cell cycle (*15*). It has been shown that CDK phosphorylates CDC6 and CDT1, both of which are required for assembling MCM2-7 protein complex at the replication origin, to inhibit their functions and it also phosphorylates MCM proteins. It is suggested that RPA function is regulated by the phosphorylation with CDK, ATR and DNA-PK (*16*, *17*). The function of the RPA phosphorylation with CDK is not fully understood, but it is probable that the RPA function is regulated by the phosphorylation.

We systematically performed the qualitative analysis to identify RPA interaction partners to understand the protein–protein interaction at the replication forks. We expressed in insect cells the three subunits of human RPA, together with one human replication protein that is present at the forks under normal conditions and/or under the replication stress conditions using the baculovirus protein expression system. Interaction between RPA and the human replication protein was examined by immuno-precipitation using the infected cell lysate. Among 30 proteins examined in total, it was found that at least 14 proteins interact with RPA. The results suggest that RPA interacts with several specific proteins among those that play roles in the regulation of replication fork progression.

Materials and Methods

Cloning of human RPA genes

cDNAs for human RPA1, RPA2 and RPA3 were synthesized from mRNA isolated from HeLa cells by RT–PCR method (Invitrogen, Carlsbad, CA, USA), and they were cloned into the baculovirus vectors, pVL1393, pAcUW31 and pVL1393, respectively. The nucleotide sequences of the cloned genes were determined by DNA sequencing in OpenGene system (Veritas, Tokyo, Japan). RPA1 was cloned to be expressed as a $(His)_6$-RPA1 fusion protein, and RPA2 was as a FLAG-RPA2 fusion protein. In comparison to the known sequences in NCBI, several nucleotide changes were identified in the cloned RPA1 and 2 genes (Table I).

Cloning of other genes

cDNAs for human CDC45, MCM10, PSF1, PSF2, PSF3, SLD5, CLASPIN, CDC6 and MCM-BP were synthesized and cloned into the baculovirus vectors. DNA sequencing of these genes was performed, except for CDC6. List of the results is shown in Table I. Six nucleotides in middle part of MCM-BP gene were deleted in comparison to the banked sequence. The cloning and sequencing of an amino-terminal fragment of human Rb, TIM, TIPIN, RAD17 and RECQL4 genes have been reported (*18*, *19*). Human CDT1 gene with nucleotides for 3× HA tags, which had been cloned into pcDNA3.1, was kindly provided by Nishitani, H. and this gene was re-cloned into pVL1392. Recombinant baculovirus for CHK1 expression was provided by Nakanishi, M., those for CDK1 and CDK2 were provided by Laskey, R., that for WRN was by Furuichi, Y., that for CDK4 was by Kitagawa M. and those for DNA polymerase α subunits were by Grosse, F.

Protein expression and immuno-precipitation

High5 cells (7×10^6 cells) were co-infected with the three viruses expressing the RPA1, 2 and 3 proteins (0.2 ml of viral stock solution) and one replication protein (0.4 ml of viral stock solution) for 2 days. In the experiments to examine the interaction between a RPA subunit and a replication protein, 0.25 ml of each viral stock solution was added to the cells. The cells were suspended in a 500 µl 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 $Na_4P_2O_7$ and protease inhibitors (Pharmingen, BD, San Jose, CA, USA). The mixture was incubated for 40 min on ice, and insoluble components were separated by centrifugation at 40,000 r.p.m. (TLS55; Beckman, Fullerton, CA, USA) for 40 min at 4°C. Supernatant of Triton-soluble (S) was recovered, and the precipitate was suspended with 100 µl of lysis buffer to obtain Triton-insoluble (I) fraction. The recovered supernatant (200 µl) was mixed for 3 h at 4°C with protein G-Sepharose-antibody beads that had been prepared by incubation of 4 µg of anti-RPA1, anti-FLAG, anti-Rb antibodies or culture supernatant of hybridoma cells producing anti-MCM-BP antibodies with protein G-Sepharose (20–30 µl) (Amersham Biosciences, Piscataway, NJ, USA) for 1 h at 4°C. After spin, proteins unbound to the Sepharose beads were recovered (U). The beads was washed 8–15 times with 100 µl of PBS containing 0.1% Triton X-100, and supernatant after the final spin was recovered (W). The proteins bound to the beads were eluted three times with 20–30 µl of elution buffer (0.1 M glycine, pH 2.5 and 0.15 M NaCl) (E1, E2 and E3). These eluates were neutralized by adding 1/10 volume of 2 M Tris–HCl, pH 8.0. Proteins were separated by 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 by 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 first antibodies in the diluted blocking buffer. After washing the membrane with TBS containing Triton X-100, it was incubated for

Table I. Summary of nucleotide sequencing of genes.

Gene	NCBI number							
RPA1	BC018126	406, C to T	446, C to T	589, T to A				
		136, P to S	149, S to L	197, W to R				
RPA2	BC021157	284, A to G						
		95, D to G						
RPA3	BC005264	None						
CDC45	AF062495	522, G to A	637, T to C	1036, C to G	1114, C to T			
		174, R to R	213, L to L	346, Q to E	372, L to L			
MCM10	NM_182751	None						
PSF1	BC012542	289, A to G						
		97, I to V						
PSF2	NM_016095	None						
PSF3	BC014437	None						
SLD5	NM_032336	None						
MCM-BP	NM_002388	393, C to T	1000–1005 deleted					
		131, H to H	334(C), 335(K) deleted					
CLASPIN	NM_022111	1574, A to G	1644, T to C	1710, A to C	2141, C to T	2622, A to G	3741, T to C	3839, C to T
		525, N to S	548, G to G	570, A to A	714, S to F	874, A to A	1247, A to A	1280, S to L

Nucleotide sequences of the cloned genes were compared with banked sequences. In the top, differences in nucleotide sequences are shown, and in the bottom, deduced amino acids changes are indicated.

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-MCM2 antibodies were prepared by immunizing full-size human MCM2 to rabbit. Anti-MCM3, -MCM4 and -MCM5 antibodies were prepared as reported (*20*, *21*). Anti-RPA1 (Calbiochem, NA13), Anti-MCM6 (Santa Cruz, Bio, sc-9843), anti-MCM7 (Santa Cruz Bio, sc-9966), anti-RAD17 (MBL, K0120-3), anti-CHK1 (Santa Cruz Bio, sc-8408), anti-CDK1 (Santa Cruz Bio, sc-747), anti-CDK2 (Santa Cruz Bio, sc-163), anti-CDK4 (Santa Cruz Bio, sc-749), anti-p27 (Santa Cruz Bio, sc-1641), anti-Rb (Santa Cruz Bio, sc-102), anti-CDC6 (Santa Cruz Bio, sc-8341), anti-p48 (NeoMarkers, RB-061-PO), anti-p58 (NeoMarkers, RB-062-PO) and anti-FLAG (Sigma, F-3165) antibodies were purchased. Antibodies against Werner helicase were kindly provided by Furuichi, Y. and those against CDT1 were by Nishitani, M. For detection of 180 kDa subunit of DNA polymerase α, SJK237 antibody was used.

Antibody production

Synthetic peptides of 20 amino acids (Sigma-Genosys) from the amino-terminus and the carboxyl-terminus of MCM10, CDC45, MCM-BP and CLASPIN were coupled to keyhole limpet haemocyanin and used to immunize mice (*22*). After generating hybridomas, clones were screened by ELISA.

Results

Interaction of human RPA with MCM2-7 proteins

The progression of the DNA replication forks is driven by the MCM helicase that is generated from MCM2-7 complex with the assistance of CDC45 and GINS complex. RPA binds to the single-stranded DNA region unwound with the MCM helicase. Three subunits of human RPA (His-RPA1, Flag-RPA2 and RPA3) were co-expressed with one of human MCM2-7 proteins in insect cells. The expressed RPA1 protein was mainly detected in Triton-insoluble (I) fraction, and the RPA2 protein was detected in Triton-soluble (S) and -insoluble (I) fractions. RPA1 in the S fraction was immuno-precipitated with anti-RPA1 antibodies (Fig. 1A, top). RPA2 was co-precipitated with RPA1. It is probable that RPA3 is also co-precipitated. A RPA complex consisted of RPA1, RPA2 and RPA3 proteins can be purified from the insect cells expressing these three proteins, and the purified complex exhibits an oligonucleotide-displacing activity *in vitro* (*19*). The co-expressed MCM2-7 proteins were detected in S and I fractions, except for MCM4 that was only detectable in I fraction (Fig. 1A, bottom). Among the MCM2-7 proteins, MCM5 and MCM6 proteins were co-precipitated with RPA1 and RPA2 proteins, but MCM2, MCM3, MCM4 and MCM7 proteins were not detected in the eluted fractions. As control experiments, each of MCM5 and MCM6 proteins was expressed in the absence of RPA1, and immuno-precipitation using anti-RPA1 antibody was performed. Negligible amounts of MCM5 and MCM6 proteins were detected in the precipitate, indicating the precipitation of MCM5 and MCM6 proteins in the above experiment is dependent upon the presence of co-expressed RPA1 protein (data not presented). Next, in the co-expression of MCM5 with RPA1 subunit, MCM5 protein was co-precipitated with RPA1 subunit (Fig. 1B). In similar experiment, MCM6 protein was co-precipitated with RPA1 subunit. Since it appears that the levels of MCM2-7 proteins co-expressed with RPA proteins are varied in the experiments of Fig. 1A, we re-examined the interactions of RPA2 with all of MCM2-7 proteins by immuno-precipiation (Fig. 1C). Although MCM2 was not co-precipitated with RPA2, MCM3, 4, 5, 6 and 7 proteins were co-precipitated with RPA2. These interactions were confirmed by the finding that MCM3-7 in the eluted fractions were not detected in the absence of co-expressed RPA2. These results suggest that MCM5 and MCM6 proteins may directly bind with both of RPA1 and RPA2 proteins, and MCM3, 4 and 7 proteins may directly bind with RPA2 protein.

Interaction of RPA with proteins involved in the fork progression

In addition to MCM10 and GINS, CDC45 and DNA polymerase α/primase are required for the DNA replication fork progression. The three subunits of RPA1, 2 and 3 were co-expressed with MCM10 protein, and FLAG-RPA2 in the cell lysate was immunoprecipitated with anti-FLAG antibody. RPA1 was co-precipitated with RPA2 (Fig. 2A, top). MCM10 was not co-precipitated with RPA2. GINS complex, which is required for assembly of DNA polymerase ε onto the replication origins and also for activation of the MCM2-7 complex, consists of PSF1, PSF2, PSF3 and SLD5 proteins. One of these human GINS subunits was co-expressed with the three RPA subunits (Fig. 2A). When RPA1 in the cell lysate prepared was immno-precipitated, RPA2 was co-precipitated (data not presented). The results in Fig. 2A suggest that PSF1 but not PSF2, PSF3 or SLD5 was co-precipitated with RPA1. However, it should be noted that the detected bands of ~26 kDa may come from the light chain of immunoglobulin but not of PSF1, since bands with similar molecular weights were detected in the absence of co-expression of PSF1 (data not presented). In addition, immunoprecipitation with anti-HA antibody did not precipitate RPA2 in the same lysate containing RPA and PSF1. Thus it is concluded that RPA does not interact with PSF1. DNA polymerase α/primase complex is consisted of four subunits of 48, 58, 70 and 180 kDa. RPA was co-expressed with one of three subunits (48, 58 and 180 kDa) of human DNA polymerase α/primase complex, and immuno-precipitation was performed using either anti-RPA1 or anti-FLAG antibodies (Fig. 2A). None of these three subunits was co-precipitated with RPA1 and RPA2 proteins. The three subunits of RPA1, 2 and 3 were co-expressed with CDC45 protein, and FLAG-RPA2 in the cell lysate was immuno-precipitated with anti-FLAG antibody (Fig. 2B, top). CDC45 was co-precipitated with RPA2 but it was not precipitated in the absence of co-expressed RPA2. Next, in the co-expression of CDC45 either with RPA1 or RPA2 subunit, CDC45 protein was not co-precipitated with RPA1 subunit (Fig. 2B, middle) but it was co-precipitated with RPA2 subunit (Fig. 2B, bottom). These results suggest that only CDC45 among the proteins

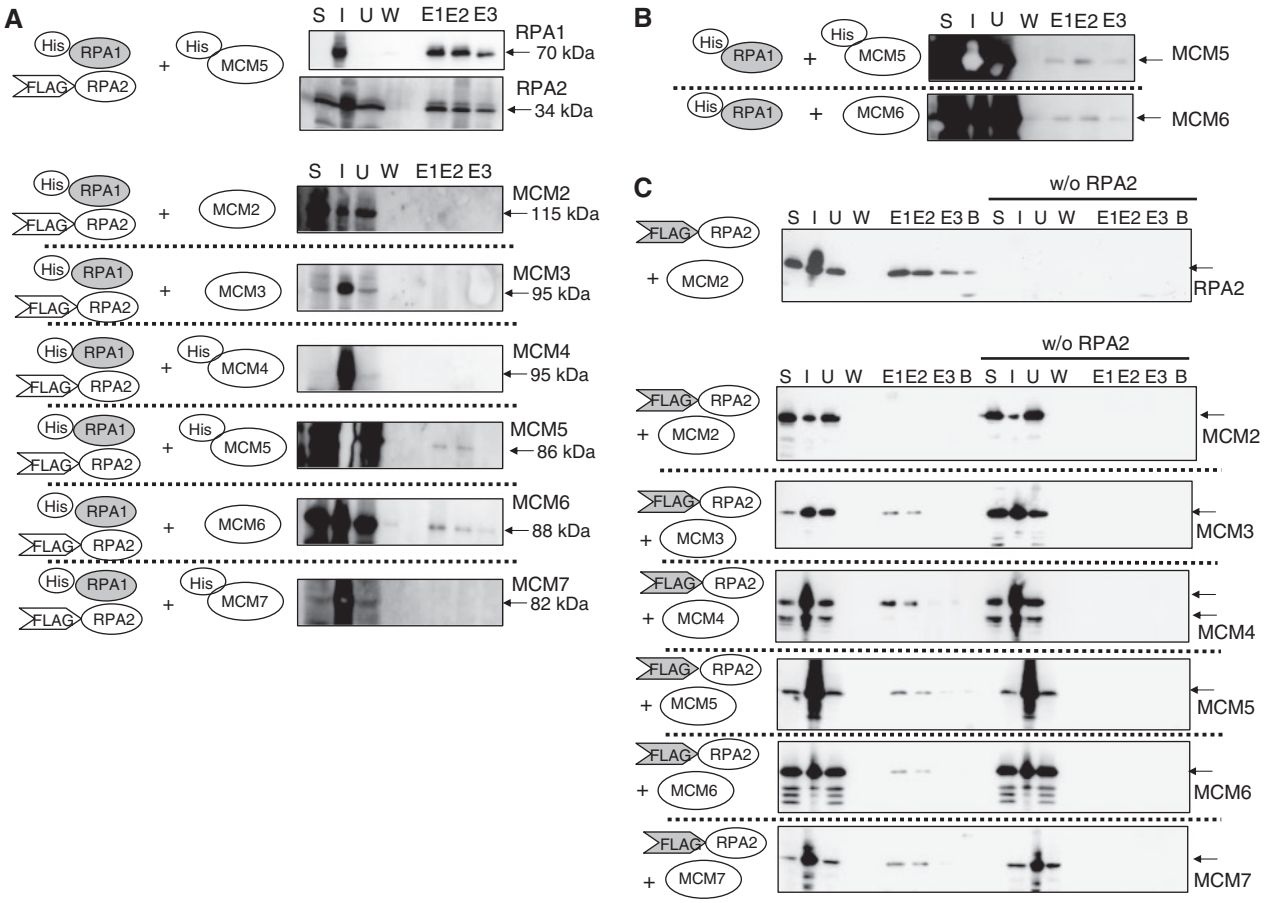

Fig. 1 Interaction of RPA with MCM2-7 proteins. (A) Three subunits of His-RPA1, FLAG-RPA2 and RPA3 were co-expressed with one of MCM2-7 proteins in High5 cells and the cells were fractionated into Triton-soluble (S) and -insoluble (I) fractions. Proteins in the Triton-soluble fraction was incubated with anti-RPA1 antibodies bound with protein G-Sepharose. After spin, supernatant was recovered (U). The Sepharose beads were extensively washed and supernatant after spin at the final wash was recovered (W). The proteins bound to the beads were eluted by incubating with elution buffer (E1, E2 and E3). The proteins in these fractions were electrophoresed in 10% acrylamide gel containing SDS and they were transferred to a membrane filtre. Loaded sample volumes are 5 μl for S and U fractions, 3 μl for I fraction, 10 μl for W, E1, E2 and E3 fractions. (top) The filter was proved with anti-RPA1 and anti-RPA2 antibodies in the co-expression experiment of RPA1, RPA2, RPA3 and MCM5 proteins, as indicated at the right. (bottom) Combinations of expressed proteins are depicted by the circles with or without tags, except for RPA3. RPA1 protein that was the target of immuno-precipitation was indicated by shadowed circle. Proteins on the filter were proved with anti-MCM2-7 antibodies, as indicated at the right. Due to over-loading, a MCM5 band in I fraction was not detected. (B) Each of MCM5 and MCM6 protein was expressed with His-RPA1 and immuno-precipitation was performed with anti-RPA1 or anti-FLAG antibodies. Proteins in the fractions (S, I, U, W, E1–3) were analysed by western-blotting using anti-MCM antibodies. Due to over-loading, MCM5 bands in I fraction were not detected (top). (C) Each of MCM2, 3, 4, 5, 6 and 7 proteins was expressed with or without FLAG-RPA2 as indicated. Immuno-precipitation was performed with anti-FLAG antibodies, as indicated by shadowed FLAG tags. Proteins in the fractions (S, I, U, W, E1–3) and those (B) eluted from the gels by treatment with SDS-sample buffer after the elution, were analysed by western-blotting using anti-MCM antibodies. At the top, distribution of RPA2 proteins in the co-expression experiment with MCM2 was shown. Loaded sample volumes are 2.5 μl for S, U and I fraction and 10 μl for W, E1, E2 and E3 fractions.

involved in the DNA replication fork progression binds RPA2.

Interaction of RPA with the checkpoint proteins

A number of proteins are involved in the DNA replication checkpoint functions. RAD17, which functions as one of the sensor proteins in this system, assembles onto the RPA-coated single-stranded DNA region at the forks that is generated by the uncoupling of the two reactions of DNA unwinding with MCM helicase and DNA synthesis with DNA polymerases (*7*). It has been shown that ATR/ATRIP complex assembled to this region by the interaction with RPA phosphorylates CHK1, an effecter kinase, for its activation. The three proteins of TIPIN, TIM and CLASPIN play roles in facilitating the phosphorylation of CHK1 by ATR/ATRIP. The proteins of TIPIN, TIM1 and CLASPIN also play roles in protecting the arrested fork structure. It has been suggested that these proteins co-migrate with the replication forks under the conditions without the replication stress (*23*).

RPA subunits were co-expressed either with TIPIN, TIM, CLASPIN, RAD17 or CHK1, and then RPA1 or FLAG-RPA2 was immuno-precipitated either with anti-RPA1 or anti-FLAG antibodies (Fig. 3A). The three proteins of TIPIN, CLASPIN and RAD17 were co-precipitated with RPA1 and RPA2, but TIM were not co-precipitated. CHK1 protein band was not detected in the elution fractions. As a control experiment, RAD17 was expressed in the absence of FLAG-RPA2, and immuno-precipitation with anti-FLAG antibody was performed (Supplementary Fig. S1).

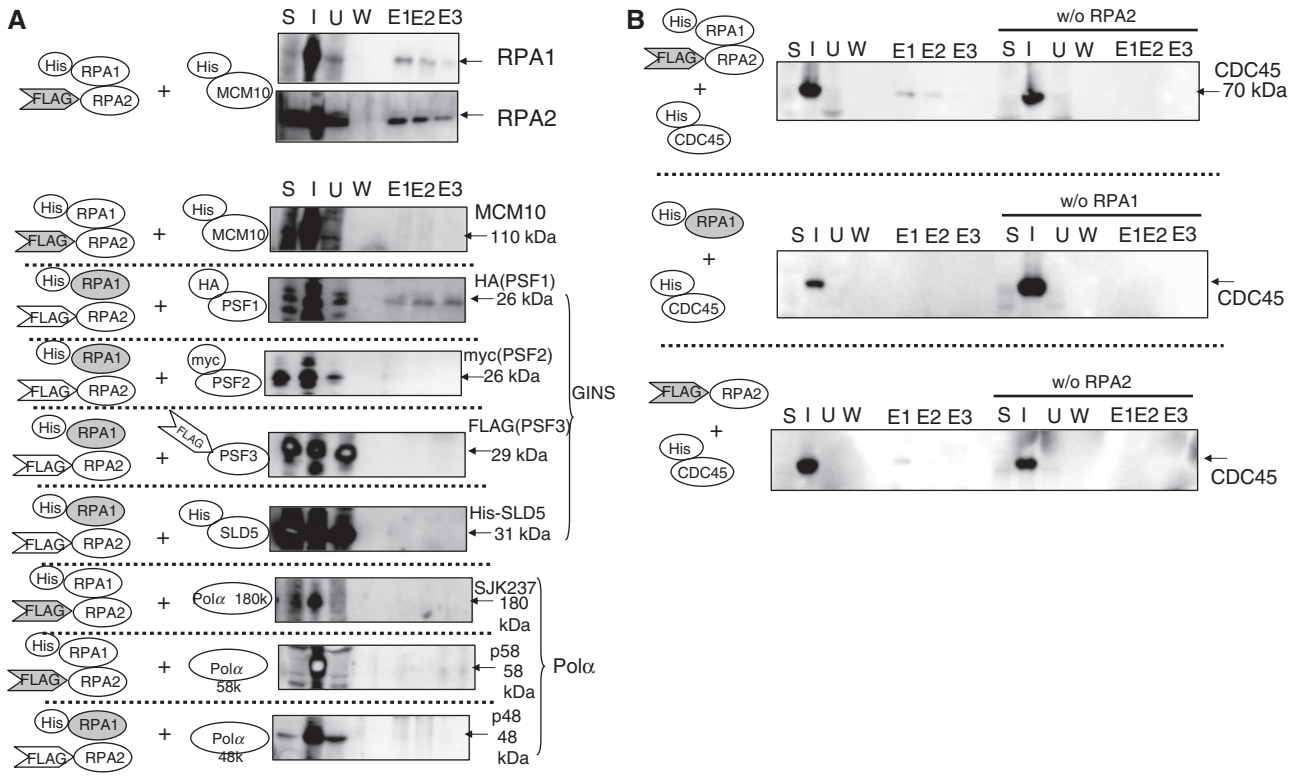

Fig. 2 Interaction of RPA with proteins involved in the initiation of DNA replication. (A) Three subunits of His-RPA1, FLAG-RPA2 and RPA3 were co-expressed either with His-MCM10, Myc-PSF1, FLAG-PSF2, His-PSF3, HA-SLD5 or three subunits of DNA polymerase α/primase in High5 cells. The Triton-soluble (S) fraction was incubated either with anti-RPA1 antibodies or anti-FLAG antibodies, as indicated by shadowed circles and FLAG tags. After the Sepharose beads were washed, the proteins bound to the beads were eluted (E1, E2 and E3). The proteins in these fractions were electrophoresed in polyacrylamide gel containing SDS. Loaded sample volumes are 5 μl for S and U fractions, 3 μl for I fraction, 10 μl for E1, E2 and E3 fractions. (top) The filter was proved with anti-RPA1 and anti-RPA2 antibodies in the co-expression experiment of RPA1, RPA2, RPA3 and His-MCM10 proteins. (bottom) The filters were proved with the antibodies to detect each of the proteins, as indicated at the right. For detection of GINS subunits, proteins were electrophoresed in the absence of dithiothreitol in order to prevent dissociation of immunoglobulin chains in samples. (B) (top) CDC45 protein was co-expressed with RPA subunits in the presence or absence of RPA2 subunit as indicated, and immuno-precipitation with anti-FLAG antibodies was performed. Loaded sample volumes are 2 μl for S, U and I fraction and 10 μl for W, E1, E2 and E3 fractions. (middle) CDC45 was expressed in the presence or absence of RPA1, and immuno-precipitation with anti-RPA1 antibodies was performed. (bottom) CDC45 was expressed in the presence or absence of RPA2, and immuno-precipitation using anti-FLAG antibodies was performed. Distribution of CDC45 proteins in the obtained fractions was examined by using anti-CDC45 antibodies.

Negligible amounts of RAD17 protein were detected in the precipitate. When TIPIN was expressed in the absence of RPA1 and immuno-precipitation using anti-RPA1 antibody was performed, however, significant bands were detected in the precipitate fraction (data not presented). Thus, it is possible that the bands near 50 kDa in the elution fractions of RPA and TIPIN immuno-precipitation experiment come from light chains of immunoglobulin. In order to overcome this difficulty, immuno-precipitation with TIPIN antibody was performed in the experiments to examine the interaction of TIPIN with RPA at subunit levels (Fig. 3B and C). In the co-expression experiment of TIPIN and RPA1, RPA1 was co-precipitated with TIPIN (Fig. 3B). In the co-expression of TIPIN and RPA2, RPA2 was co-precipitated with TIPIN (Fig. 3C). In the absence of co-expressed TIPIN, RPA2 was not detected in the elution fractions. These results suggest that TIPIN interacts with RPA1 and RPA2. Neither CLASPIN nor RAD17 was co-precipitated with RPA1 (Fig. 3B) but these proteins were co-precipitated with RPA2 (Fig. 3C). As a control experiment, CLASPIN was expressed in the absence of FLAG-RPA2, and immuno-precipitation with anti-FLAG antibody was performed (Supplementary Fig. S1). Negligible amounts of CLASPIN were detected in the elution fractions. RPA was co-expressed either with Werner helicase or RECQL4 helicase, both of which belong to RECQ family that play roles in the regulation of genomic recombination (Fig. 3A). It has been reported that RPA and Werner physically and functionally interact (*24*). Human RECQL4 has a region homologous to Sld2 in *Saccharomyces cerevisiae* that plays an essential role in assembling DNA polymerase ε onto the replication origin. Neither Werner helicase nor RECQL4 helicase was co-precipitated with RPA under the present conditions. These results suggest that RPA interacts with the checkpoint proteins of TIPIN, CLASPIN and RAD17 among the analysed proteins that are involved in the DNA replication checkpoint system and in the regulation of the fork progression.

Interaction of RPA with cell cycle proteins and MCM-related proteins

G1/S transition of the cell cycle is driven by CDK2 and CDK4, both of which phosphorylate Rb protein to activate E2F transcription factor. CDK2 also plays

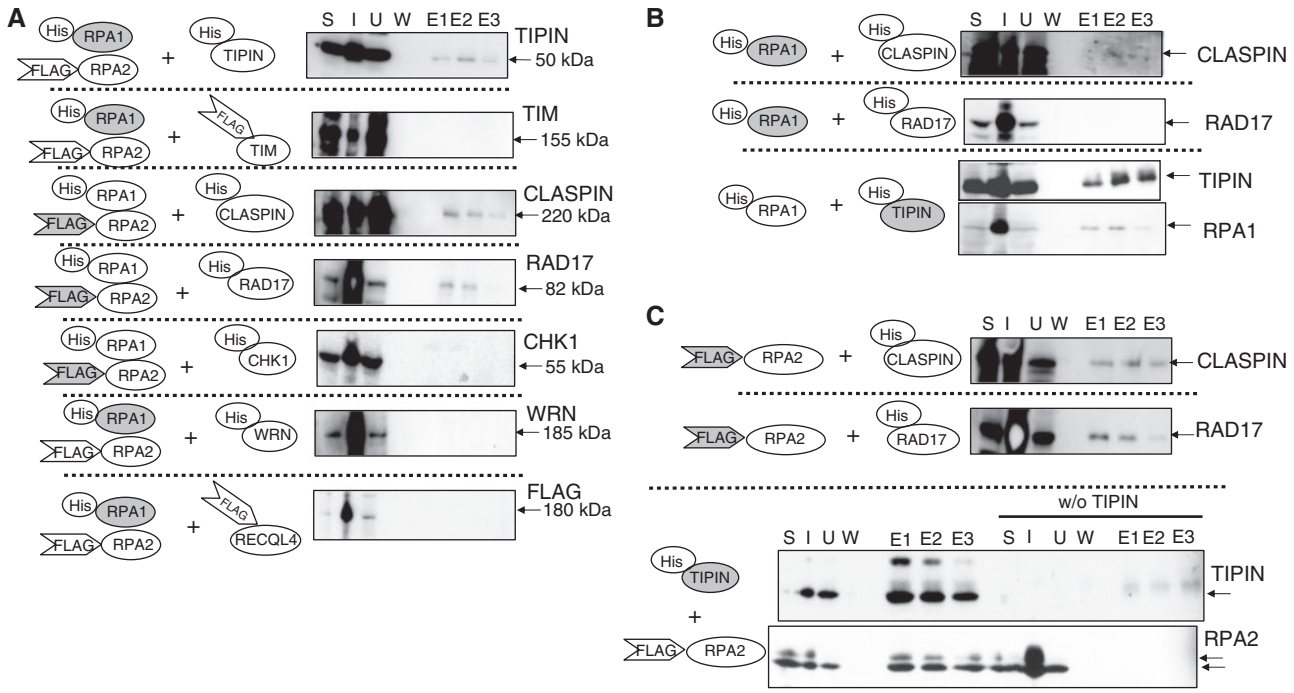

Fig. 3 Interaction of RPA with checkpoint proteins. (A) Three subunits of His-RPA1, FLAG-RPA2 and RPA3 were co-expressed either with TIPIN, TIM1, CLASPIN, RAD17, CHK1, Werner and RECQL4 proteins in High5 cells. The proteins in the Triton-soluble fraction were incubated either with anti-RPA1 antibodies or anti-FLAG antibodies, as indicated by shadowed circles and FLAG tags. Sample volumes loaded to SDS-gel are 5 μl for S and U fractions, 3 μl for I fraction, 10 μl for E1, E2 and E3 fractions. In the co-expression of RPA and CHK1, loaded sample volumes are 7 μl for S, U and I fraction, 10 μl for W and 12 μl for E1, E2 and E3 fractions. Proteins obtained in the immuno-precipitation of CHK1 and RPAs were electrophoresed in the absence of dithiothreitol. After electrophoresis, the filter was proved with the antibodies, as indicated at the right. (B) Each of TIPIN, CLASPIN and RAD17 protein was expressed with His-RPA1. Immuno-precipitation was perfomed using anti-RPA1 or anti-TIPIN antibodies, as indicated and distribution of proteins in obtained fractions was examined. (C) Each of CLASPIN and RAD17 protein was expressed with FLAG-RPA2. TIPIN was expressed in the presence or absence of FLAG-RPA2. Immuno-precipitation was perfomed using anti-FLAG or anti-TIPIN antibodies as indicated, and distribution of proteins was examined. Loaded sample volumes are 2.5 μl for S, U and I fraction and 10 μl for W, E1, E2 and E3 fractions for CLASPIN and RAD17 experiments, and they are 7 μl for S and U fractions, 5 μl for I fraction, 10 μl for W fraction and 12 μl for E1, E2 and E3 fractions for TIPIN experiment.

central roles in preventing re-initiation of DNA replication in a single cell cycle. G2/M transition of the cell cycle is driven by CDK1 that phosphorylates several proteins including condensin subunits and lamin B. It has been reported that RPA2 is phosphorylated with CDK at the specific sites during S phase (*17*). The function of CDK2 is regulated by p27, one of CKI. Both p27 (*25*) and Rb (*14*) negatively regulate DNA replication by interacting with MCM7. From telophase in M phase to G1 phase, MCM2-7 complex binds to the DNA replication origin bound with ORC. The proteins of CDC6 and CDT1 are required for loading MCM2-7 complex onto the origin. Recently, three novel MCM members of MCM8, MCM9 and MCM-BP (*26*) have been identified. MCM9 plays a role in assembling the MCM2-7 complex onto the origin (*27*). The function of MCM-BP remains to be determined but recently it has been reported that MCM-BP plays a role in the cohesion of replicated chromosomes (*28*).

RPA1, 2 and 3 proteins were co-expressed either with CDT1, CDC6, MCM-BP, and then RPA1 or FLAG-RPA2 was immuno-precipitated either with RPA1 antibodies or anti-FLAG antibodies (Fig. 4A). Only MCM-BP protein was co-precipitated. When MCM-BP was expressed in the absence of RPA1 and immuno-precipitation using anti-RPA1 antibody was performed, MCM-BP was hardly detected in the elution fractions (Supplementary Fig. S1). However, MCM-BP was not co-precipitated with RPA1 subunit in the co-expression of MCM-BP and RPA1 (Fig. 4B). Only faint bands were detected in the elution fractions in this experiment but these are also detected in the absence of co-expressed RPA1 (data not presented). RPA2 was not co-precipitated with MCM-BP in the co-expression experiment of MCM-BP and RPA2 (data not presented). When RPA were co-expressed either with CDK1, CDK2 or CDK4, and then FLAG-RPA2 in the lysate was immuno-precipitated with anti-FLAG antibodies, all these CDK were co-precipitated with RPA (Fig. 4A). When each of CDK1, CDK2 and CDK4 was expressed in the absence of FLAG-RPA2 and immuno-precipitation using anti-FLAG antibody was performed, these proteins were hardly detected in the precipitate (Supplementary Fig. S2). Next, these CDKs were co-expressed either with RPA1 or FLAG-RPA2, and immuno-precipitation was performed using anti-RPA1 or anti-FLAG antibodies, respectively (Fig. 4B and C). All the CDK1, CDK2 and CDK4 were co-precipitated with FLAG-RPA2 (Fig. 4C). Only small amounts of CDK1, CDK2 and CDK4 proteins were co-precipitated with RPA1 (Fig. 4B). When RPA was co-expressed either with p27 or an amino-terminal

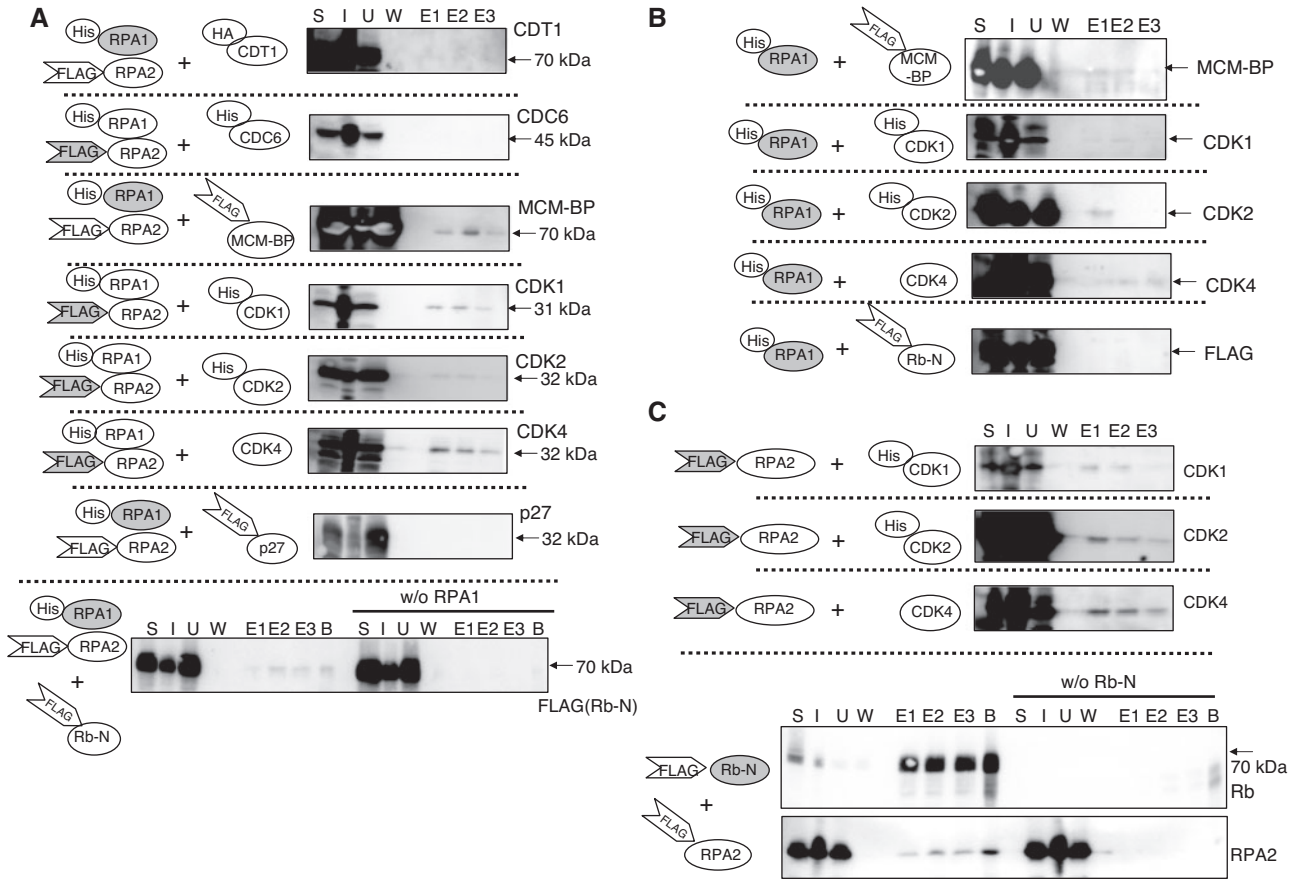

Fig. 4 Interaction of RPA with cell cycle proteins and MCM-related proteins. (A) Three subunits of His-RPA1, FLAG-RPA2 and RPA3 were co-expressed either with CDT1, CDC6, MCM-BP, CDK1, CDK2, CDK4, p27 or an amino-terminal fragment of Rb protein (Rb-N). The proteins in the S fraction were incubated either with anti-RPA1 antibodies or anti-FLAG antibodies, as indicated by shadowed circles and FLAG tags. Five microliters (5 μl) for S and U fractions, 3 μl for I fraction, 10 μl for E1, E2 and E3 fractions were loaded for electrophoresis. In the experiment using Rb-N, 2.5 μl for S, U and I fraction and 10 μl for W, E1, E2 and E3 fractions were electrophoresed, and proteins eluted from the gels by treatment with SDS-sample buffer after elution were also electrophoresed (B). After electrophoresis, proteins on the filter were proved with the antibodies, as indicated at the right. In the experiment to examine the binding of RPA and Rb-N, Rb-N was expressed with RPA2 and RPA3 in the presence or absence of RPA1 as indicated. (B) Each of MCM-BP, CDK1, CDK2, CDK4 and Rb-N fragment was expressed with His-RPA1. His-RPA1 in the cell lysate was immuno-precipitated with anti-RPA1 antibodies. (C) Each of CDK1, CDK2 and CDK4 was expressed with FLAG-RPA2, and FLAG-RPA2 in the cell lysate was immuno-precipitated with anti-FLAG antibodies. Rb-N was expressed with or without FLAG-RPA2 as indicated. Rb-N in the cell lysate was immuno-precipitated with anti-Rb antibodies. Distribution of proteins in obtained fractions was analyzed by western-blotting. Loaded sample volumes are 2.5 μl for S, U and I fraction and 10 μl for W, E1, E2 and E3 fractions.

fragment of Rb (Rb-N), and then RPA1 in the cell lysate was immuno-precipitated with anti-RPA1 antibodies, p27 was not co-precipitated with RPA but the Rb-N fragment was co-precipitated (Fig. 4A). The Rb-N fragment was not co-precipitated with RPA1 subunit (Fig. 4B) but RPA2 subunit was co-precipitated with the Rb-N fragment (Fig. 4C). These results indicate that CDK may bind RPA1 and RPA2, and Rb-N fragment may bind RPA2 protein, but the proteins involved in MCM2-7 loading and p27 do not directly bind RPA. The interaction of MCM-BP and RPA was only detected when RPA1, 2 and 3 subunits were all expressed. A possibility that MCM-BP binds with RPA3 remains to be examined.

Discussion

In this article, it has been shown that 14 out of 30 proteins examined may directly interact with RPA (Table II). At the subunit level, 13 proteins may interact with RPA2, and three proteins may interact with RPA1. No protein that only interacts with RPA1 was detected. These results suggest that RPA2 plays a main role in interacting with other proteins, although it has been reported that several checkpoint proteins directly interact with RPA1 (*9*). CDK phosphorlates RPA2 at specific sites during the cell cycle (*17*, *29*). A relatively strong interaction between RPA2 and CDKs was detected in the present study and this may be explained by the relationship between substrate and enzyme. Other proteins that interacted with RPA are MCM3-7, CDC45, TIPIN, CLASPIN, RAD17, MCM-BP and Rb-N fragment. It is probable that all of these proteins play roles in regulation of the replication fork progression. Under normal conditions without replication stress, the interaction of RPA with MCM3-7 proteins may play roles in the progression of the replication forks. It should be noted, however, this finding is slightly different from the results that mouse RPA binds with all the MCM2-7 proteins

Table II. Summary of the proteins that can interact with RPA.

	RPA1,2 and 3	RPA1	RPA2	Reference
MCM2	x	nd	x	30
MCM3	x*	nd	o	30
MCM4	x*	nd	o	30
MCM5	o	o	o	30
MCM6	o	o	o	30
MCM7	x*	nd	o	30
Polα (p180)	x	nd	nd	
Polα (p58)	x	nd	nd	
Polα (p48)	x	nd	nd	
PSF1	x	nd	nd	
PSF2	x	nd	nd	
PSF3	x	nd	nd	
SLD5	x	nd	nd	
MCM10	x	nd	nd	
CDC45	o	x	o	
TIPIN	o	o	o	12,13
TIM	x	nd	nd	
CLASPIN	o	x	o	
RAD17	o	x	o	8
CHK1	x	nd	nd	
WRN	x	nd	nd	24
RECQL4	x	nd	nd	
CDT1	x	nd	nd	
CDC6	x	nd	nd	
MCM-BP	o	x	x	
CDK1	o	o/x	o	
CDK2	o	o/x	o	
CDK4	o	o/x	o	
p27	x	nd	nd	
Rb-N	o	x	o	

The proteins, which showed the interaction with RPAs, are shown by o and those that did not interact with RPAs are shown by x. Those that showed faint bands in the precipitated fractions are indicated by o/x. nd indicates not determined. As for MCM3, MCM4 and MCM7, the interactions with RPAs were not detected in the experiments where all the three RPA subunits were co-expressed with MCM. Since the interactions of RPA2 with these MCM members were detected, however, the above results should be carefully interpreted. Therefore, these interactions were shown by x*. In a right-end column, previous works to show the indicated interactions are cited.

in two-hybrid system (*30*). It is possible that MCM2 can interact with RPA but the interaction is less stable than the interactions with MCM3-7 with RPA. It remains to be determined whether these interactions can be detected using purified proteins in the presence of single-stranded DNA and what are their functional outcomes. In relation to the latter point, it has been reported that RPA stimulates ATPase activity of MCM4/6/7 complex (*31*).

Under the replication stress conditions, it is probable that the interaction of RPA with TIPIN, CLASPIN and RAD17 play roles in the assembly of these proteins at the fork and in the checkpoint functions. It has been reported that RPA interacts with several proteins involved in the DNA replication checkpoint system. They include RAD9, RAD17 and ATRIP proteins. It is probable that the interactions of RAD9 and RAD17 with RPA are involved in the assembly of the RAD9-HUS1-RAD1 complex at the fork under the replication stress conditions. TIPIN is identified as the protein interacting with RPA2 by two-hybrid system (*13*). It has been shown that TIPIN/TIM complex can bind RPA complex *in vitro*. The presented data on RAD17 and TIPIN are consistent with these published results, and support that direct interaction of RPA with these proteins. The interaction of RPA with CLASPIN may also play roles in the checkpoint function. It is suggested that RPA interact with CDC45 at the initiation of DNA replication in *S. cerevisiae* (*32*). Consistently, co-immuno-precipitation of RPA and CDC45 was detected in this study. RPA physically and functionally interacts with Werner helicase in a DNA helicase reaction (*24*). However, any interaction of RPA with the protein was not detected in this study. It is possible that only stable interactions of RPA with other proteins can be detected under our experimental conditions. Several specific sites in RPA2 are phosphorylated with the kinases of CDK, DNA-PK and ATM (*16*, *17*). The binding of RPA with proteins can be modulated by the RPA2 phosphorylation. It should be noted that RPA2 is mainly present as non-phosphorylated form in the present study.

MCM-BP has been identified as a novel MCM2-7 family protein (*26*). Recently, it has been reported that dysfunction of MCM-BP leads to defect in the cohesion of replicated chromosomes (*28*), suggesting that MCM-BP functions near the replication forks to facilitate the cohesion reaction. It appears that the present finding indicating that MCM-BP can interact with RPA is consistent with this notion. MCM-BP may be involved in the cohesion by interacting with MCM2-7 and RPA at the forks. It has been reported that Rb negatively regulates DNA replication by interacting with MCM7 (*14*). Recently, our group reported that the amino-terminal fragment of Rb (Rb-N) can interact with MCM3 and MCM6 in addition to MCM7 in immuno-precipitation experiments using co-expressed insect cell lysate (*18*). The present finding that Rb-N can bind with RPA supports the notion that Rb regulates DNA replication at the forks.

Thus the present study will give a framework to understand protein–protein interactions at the replication forks. In next steps, the interaction of RPA with the identified proteins including MCM3-7 in cells should be examined.

Supplementary Data

Supplementary Data are available at *JB* Online.

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Conflict of interest

None declared.

References

1. Wold, M.S. (1997) Replication protein A: a heterotrimeric, single-stranded DNA-binding protein required for eukaryotic DNA metabolism. *Annu. Rev. Biochem.* **66**, 61–92
2. Iftode, C., Daniely, Y., and Borowiec, J. (1999) Replication protein A (RPA): the eukaryotic SSB. *Crit. Rev. Biochem. Mol. Biol.* **34**, 141–180
3. Bochkarev, A. and Bochkareva, E. (2004) From RPA to BRCA2: lessons from single-stranded DNA binding by the OB-fold. *Curr. Opin. Struc. Biol.* **14**, 36–42
4. Dornreiter, I., Erdile, L.F., Gilbert, I.U., von Winkler, D., Kelly, T.J., and Fanning, E. (1992) Interaction of DNA polymerase α-primase with cellular replication protein A and SV40 T antigen. *EMBO J.* **11**, 769–778
5. Moyer, S.E., Lewis, P.W., and Botchan, M.R. (2006) Isolation of the Cdc45-MCM2-7-GINS (CMG) complex, a candidate for the eukaryotic DNA replication fork helicase. *Proc. Natl Acad. Sci. USA* **103**, 10236–10241
6. Labib, K. and Gambus, A. (2007) A keyrole for the GINS complex at DNA replication forks. *Trends Cell Biol.* **17**, 271–278
7. Osborn, A.J., Elledge, S.J., and Zou, L. (2002) Checking on the fork: the DNA-replication stress-response pathway. *Trends Cell Biol.* **12**, 509–516
8. Zou, L., Liu, D., and Elledge, S.J. (2003) Replication protein A-mediated recruitment and activation of Rad17 complexes. *Proc. Natl Acad. Sci. USA* **100**, 13827–13832
9. Xu, X., Vaithiyalingam, S., Grick, G.G., Mordes, D.A., Chazin, W.J., and Cortez, D. (2008) The basic cleft of RPA70N binds multiple checkpoint proteins, including RAD9, to regulate ATR signaling. *Mol. Cell Biol.* **28**, 7345–7353
10. Zou, L. and Elledge, S.J. (2003) Sensing DNA damage through ATRIP recognition of RPA-ssDNA complex. *Science* **300**, 1542–1548
11. Ball, H.L., Ehrhardt, M.R., Mordes, D.A., Glick, G.G., Chazin, W.J., and Cortez, D. (2007) Function of a conserved checkpoint recruitment domain in ATPIP proteins. *Mol. Cell. Biol.* **27**, 3367–3377
12. Ünsal-Kaçmaz, K., Chastain, P.D., Qu, P.-P., Minoo, P., Cordeiro-Stone, M., Sancar, A., and Kaufmann, W.K. (2007) The human Tim/Tipin complex coordinates an intra-S checkpoint response to UV that slows replication fork displacement. *Mol. Cell Biol.* **27**, 3131–3142
13. Gotter, A.L., Suppa, C., and Emanuel, B.S. (2007) Mammalian TIMELESS and Tipin are evolutionarily conserved replication fork-associated factors. *J. Mol. Biol.* **366**, 38–52
14. Sterner, J.M., Dew-Knight, S., Musahl, C., Kornbluth, S., and Horowitz, J.M. (1998) Negative regulation of DNA replication by the retinoblastoma protein is medicated by its association with MCM7. *Mol. Cell. Biol.* **18**, 2748–2757
15. Diffley, J.F. (2004) Regulation of early events in chromosome replication. *Curr. Biol.* **14**, R778–R786
16. Zou, Y., Liu, Y., Wu, X., and Shell, S.M. (2006) Functions of human replication protein A (RPA): from DNA replication to DNA damage and stress responses. *J. Cell. Phys.* **208**, 267–273
17. Binz, S.K., Sheehan, A.M., and Wold, M.S. (2004) Replication protein A phosphorylation and the cellular response to DNA damage. *DNA Repair* **3**, 1015–1024
18. Numata, Y., Ishihara, S., Hasegawa, N., Nozaki, N., and Ishimi, Y. (2010) Interaction of human MCM2-7 proteins with TIM, TIPIN and Rb. *J. Biochem.* **147**, 917–927
19. Suzuki, T., Kohno, T., and Ishimi, Y. (2009) DNA helicase activity in purified human RECQL4 protein. *J. Biochem.* **146**, 327–335
20. Kimura, H., Nozaki, N., and Sugimoto, K. (1994) DNA polymerase α associated protein P1, a murine homolog of yeast MCM3, changes its intranuclear distribution during the DNA synthetic period. *EMBO J.* **13**, 4311–4320
21. Kimura, H., Takizawa, N., Nozaki, N., and Sugimoto, K. (1995) Molecular cloning of cDNA encoding mouse Cdc21 and CDC46 homologs and characterization of the products: physical interaction between P1 (MCM3) and CDC46 proteins. *Nucleic Acids. Res.* **23**, 2097–2104
22. Kimura, K., Nozaki, N., Saijo, M., Kikuchi, A., Ui, M., and Enomoto, T. (1994) Identification of the nature of modification that causes the shift of DNA topoisomerase II beta to apparent higher molecular weight forms in the M phase. *J. Biol. Chem.* **269**, 24523–24526
23. Katou, Y., Kanoh, Y., Bando, M., Noguchi, H., Tanaka, H., Ashikari, T., Sugimoto, K., and Shirahige, K. (2003) S-phase checkpoint proteins Tof1 and Mrc1 form a stable replication-pausing complex. *Nature* **424**, 1078–1083
24. Brosh, R.M. Jr., Orren, D.K., Nehlin, J.O., Ravn, P.H., Kenny, M.K., Machwe, A., and Bohr, V.A. (1999) Functional and physical interaction between WRN helicase and human replication protein A. *J. Biol. Chem.* **274**, 18341–18350
25. Nallamshetty, S., Crook, M., Boehm, M., Yoshimoto, T., Olive, M., and Nabel, E.G. (2005) The cell cycle regulator $p27^{Kip1}$ interacts with MCM7, a DNA replication licensing factor, to inhibit initiation of DNA replication. *FEBS Lett.* **579**, 6529–6536
26. Sakwe, A.M., Nguyen, T., Athanasopoulos, V., Shire, K., and Frappier, L. (2007) Identification and characterization of a novel component of the human minichromosome maintenance complex. *Mol. Cell Biol.* **27**, 3044–3055
27. Lutzmann, M. and Mechali, M. (2008) MCM9 binds Cdt1 and is required for the assembly of prereplication complexes. *Mol. Cell* **31**, 190–200
28. Takahashi, N., Quimbaya, M., Schubert, V., Lammens, T., Vandepoele, K., Schubert, I., Matsui, M., Inze, D., Berx, G., and Veylder, L.D. (2010) The MCM-binding protein ETG1 aids sister chromatid cohesion required for postreplicative homologous recombination repair. *ProS Genet.* **6**, e1000817
29. Stephan, H., Concannon, C., Kremmer, E., Carty, M.P., and Nasheuer, H.-P. (2009) Ionizing radiation-dependent and independent phosphorylation of the 32-kDa subunit of replication protein A during mitosis. *Nucleic Acids Res.* **37**, 6028–6041
30. Kneissl, M., Pütter, V., Szalay, A.A., and Grummt, F. (2003) Interaction and assembly of murine pre-replicative complex proteins in yeast and mouse cells. *J. Mol. Biol.* **327**, 111–128
31. Biswas-Fiss, E.E., Khopde, S.M., and Biswas, S.B. (2005) The Mcm467 complex of *Saccharomyces serevisiae* is preferentially activated by autonomously replicating DNA sequences. *Biochemistry* **44**, 2916–2925
32. Zou, L. and Stillman, B. (2000) Assembly of a complex containing Cdc45p, replication protein A, and Mcm2p at replication origins controlled by S-phase cyclin-dependent kinases and Cdc7p-Dbf4p kinase. *Mol. Cell Biol.* **20**, 3086–3096