Molecular cloning of cDNA encoding mouse Cdc21 and CDC46 homologs and characterization of the products: physical interaction between P1(MCM3) and CDC46 proteins

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ABSTRACT

Two new mouse genes encoding proteins that belong to the yeast minichromosome maintenance (MCM) protein family, which is involved in the initiation of DNA replication, were isolated and their nucleotide sequence was determined. They were a putative CDC46/MCM5 homolog and a putative cdc21 homolog. About 30% amino acid identity was obtained between members in the family, and >40% between the putative mouse and yeast homologs. The expression of these genes was cell-cycle specific at the late G₁ to S phase. Immunochemical analyses showed the physical interaction between mouse P1MCM3 and CDC46 protein. These results suggest that MCM proteins function in co-ordination for DNA replication.

INTRODUCTION

Replication of all eukaryotic cell DNA occurs once during a single S phase. However, little is known about the regulatory mechanism of the co-ordinated initiation of DNA replication for the multiple replication origin. Budding yeast minichromosome maintenance (MCM) proteins have been postulated to be involved in the initiation of DNA replication by acting at the replication origin, since the mutation of these genes showed an autonomously replicating sequence (ARS)-specific minichromosome maintenance defect in addition to phenotypes with increased chromosome loss and recombination (1-5). Recently, inhibition of the initiation not only at an ARS on a minichromosome but at a specific chromosomal origin of DNA replication was shown in *mcm2-1* and *mcm3-1* mutants (6).

The Mcm gene family is conserved from yeast (3,4,7-11) to mammals (12-15) and consists of at least six members (Table 1). All genes examined in yeasts are essential for cell growth and their mutation causes cell cycle arrest in S phase (3,4,6-10,16). In mammalian cells, DNA replication is inhibited when antibody against human MCM2 homolog, BM28 (14) or mouse P1MCM3 (15) is microinjected into the nucleus. Thus MCM proteins

appear to play an essential role in DNA replication throughout eukaryotes, but their biochemical function still remains unknown. Koonin (17) suggested that MCM proteins may have DNA helicase or DNA unwinding activity because a putative DNA-dependent ATPase motif is found in the most conserved region, although no such activity has been experimentally proven so far.

Cytological studies using specific antibodies (6,16) suggested that these proteins are implicated in insuring the single round of DNA replication during a cell cycle. The subcellular localization of the yeast CDC46 (16), MCM2 and MCM3 proteins (6) changes during the cell cycle. They appear in the nucleus at the end of the M phase, remain there throughout the G₁ phase and disappear at the onset of S phase. This behavior is consistent with that of the 'licensing factor' (18), a hypothetical factor responsible for the once-per-cell-cycle DNA replication. Blow and Laskey (18) considered that the factor allows the chromatin to replicate by binding during the M phase, since it is not capable of passing the nuclear membrane; it is degraded or inactivated after the initiation of DNA replication during S phase (18). Mouse P1MCM3 protein exhibits changes in the subnuclear localization during the S phase (15). It exists in the nucleus throughout the interphase but the underphosphorylated form bound to a certain nuclear structure disappears from the region of replicated chromatin, first from euchromatin and next from heterochromatin, during the S phase. We reported data suggesting that P1MCM3 dissociated from the nuclear structure by hyperphosphorylation with the progression of DNA replication (15). Thus, these MCM proteins have been suggested to have an important role in the regulation of the once-per-cell-cycle DNA replication (6,7,15, 16). In this study, we isolated new mouse genes belonging to this family and noted the physical interaction between P1MCM3 and mCDC46 proteins.

MATERIALS AND METHODS

Isolation of mouse MCM genes

All recombinant DNA techniques were according to Sambrook *et al.* (19) or as described (15). The putative mouse CDC46 and

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Cdc21 homologs, mCDC46 and mcdc21, were isolated by polymerase chain reaction (PCR) and plaque hybridization (15). Briefly, PCR was performed with a λ gt10 FM3A cDNA library and a set of degenerated primers, F1 and R1, corresponding to a restriction enzyme site and a nucleotide sequence coding the amino acids RGDIN(V/I/L)L [amino acids 400–406 of yeast MCM3 (3)] and IDEFDKM (472–478). The nucleotide sequence of the primers was as follows:

F1: CCGAATTCCGTGGAGA(T/C)AT(A/T/C)AA(T/C)(G/A/C)T(G/A/T/C)CT,

R1: CCAAGCTTATCTT(G/A)TC(G/A)AA(T/C)TC(G/A)TC(G/A)AA(T/C)TC(G/A)TC(G/A/T)AT.

The library was incubated at 70°C for 5 min, then mixed with buffer consisting of 50 mM Tris-HCl (pH 8.0), 40 mM KCl, 2.5 mM MgCl₂, 0.2 mM each dNTP, 4 μ M primers and 1.25 U *Taq* DNA polymerase in 50 μ l reaction mixture. The PCR schedule was: 94°C for 5 min, 45°C for 1 min, 74°C (1 cycle), 94°C for 1 min, 45°C for 1 min, 74°C for 2 min (34 cycles). The PCR products were cloned into pBluescriptII SK(+) (Stratagene) and their nucleotide sequence was determined using Sequenase Kit version 2 (US Biochemical). The λ gt10 library was screened with these cloned or original PCR products labeled with ³²P by a multipriming method (19). The inserts of the positive λ phages were subcloned into pBluescriptII KS(+) and their nucleotide sequence was determined using the Kit (US Biochemical) in both directions from the exonuclease III deletion series of the plasmids. The DNA sequence was analyzed using the GENE-TYX software (Software Development Co., Ltd, Tokyo). The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide databases with the accession number D26089 for mcdc21, D26090 for mCDC46.

Northern hybridization

Swiss 3T3 cells were synchronized by serum deprivation (15). Briefly, cells were plated at 10⁶ cells per 150 mm dish and the medium was replaced by MEM containing 0.2% FCS. After incubation for 72 h, cells were stimulated to enter the cell cycle with MEM containing 10% FCS. The total RNAs were recovered by the guanidinium thiocyanate–CsCl procedure (19) at 4 h intervals.

HeLa cells were synchronized at M phase by treatment with nocodazole (40 ng/ml) for 10 h followed by mitotic shake off (14). Mitotic cells thus collected were replated on the dish after washing with DMEM three times. The total RNAs were recovered by AGPC method (20) at 4 h intervals after replating.

DNA synthesis was monitored by the incorporation of bromodeoxyuridine for 30 min into synchronized cells at 4 h intervals by the BrdU Labeling and Detection Kit III (Boehringer Mannheim) (15).

RNA samples were separated on a 1% agarose gel containing 0.6 M formaldehyde and transferred to a positively charged nylon membrane (Hybond N⁺; Amersham) according to the instruction manual. The membrane was hybridized with probes labeled with ³²P by a multipriming method (19). The unedited signals in HeLa RNA were presented using a Fujix BAS2000 image analyzing system.

Preparation of antisera

β -galactosidase–mouse MCM fusion proteins were produced in *E. coli* for immunization.

mcdc21 and mCDC46 genes were ligated to pUR292 vector to make the fusion genes. pBluescriptII–mcdc21 was digested with *Bam*HI and *Sal*I and ligated to pUR292 digested with *Bam*HI and *Sal*I. Since the *Bam*HI site was located at –77 bp upstream of the putative initiation codon, the resulting fusion protein was expected to consist of β -galactosidase and full length mCdc21 protein with some additional amino acids. To construct the expression plasmid of β -galactosidase–mCDC46 fusion protein, mCDC46 cDNA was digested with *Sma*I and inserted into the *Sal*I site of pUR292 with a *Xho*I linker, CCTCGAGG. This construction was to code the nearly full length fusion protein lacking only seven amino acids at the N-terminus of mCDC46.

Since the fusion proteins were produced in the insoluble form, they were collected as inclusion bodies (19) and purified by SDS–PAGE using Prep Cell (BioRad). Exponentially growing *E. coli* XL1-Blue carrying the plasmid in LB medium (2 l) was induced with 1 mM isopropyl β -D-thiogalactopyranoside (IPTG) and further incubated at 30°C overnight. The cells were collected and suspended with 50 ml of 20 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF) and lysed by sonication on ice for a total of 10 min with five intervals. After centrifugation (10 000 g) for 20 min at 4°C, the precipitate was washed twice with 50 ml of 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 100 mM NaCl, 0.5% Triton X-100, and then solubilized with 50 ml of urea buffer (8 M urea, 0.5 M Tris-HCl (pH 8.0), 0.5 M NaCl, 1 mM EDTA, 30 mM 2-mercaptoethanol, 1 mM PMSF). After centrifugation (13 000 g) for 10 min at room temperature, the supernatant was dialyzed twice against 1 l of dialysis buffer [40 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, 50% glycerol] and then cleared by centrifugation (10 000 g) for 20 min at 4°C. The supernatant was applied to a Model 491 Prep Cell (BioRad). The fraction of ~200 kDa was collected and dialyzed against PBS. The rabbits received an injection of 300 μ g of the purified protein with complete Freund's adjuvant and two to four booster injections.

Western blotting and immunoprecipitation

Mouse FM3A cells were routinely grown in DMEM supplemented with 5% fetal calf serum, and 10⁸ cells were subjected to fractionation as described (15). A fraction of extracts was separated by 8% SDS–PAGE followed by blotting onto a nitrocellulose filter (Gelman Sci.). The filter was washed with TBST [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20], incubated with TBST containing 5% non-fat dried milk overnight at 4°C. The filter was incubated with a primary rabbit antiserum diluted to 1/2000 in TBST containing 5% skimmed milk for 1 h at room temperature. After four washings with TBST, the filter was incubated with an alkaline phosphatase-conjugated goat anti-rabbit antibody (BioRad) diluted to 1/4000 in TBST containing 5% skimmed milk for 1 h at room temperature, washed four times with TBST, then incubated with BCIP/NBT.

A hypotonic extract of 10⁷ cells was subjected to anti-P1MCM3 immunoprecipitation as described (15). Briefly, the extract (200 μ l) was pre-adsorbed with 10 μ l of pre-immune antiserum and 10 μ l of packed *Staphylococcus aureus* cells (Pansorbin Cells; Calbiochem) and half of the lysate was immunoprecipitated with 1 μ l of anti-P1MCM3 antiserum and 5 μ l of packed Pansorbin Cells. The lysate was recovered by centrifugation and the precipitate was washed three times with

NET-gel buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% NP-40, 1 mM EDTA, 0.25% gelatin, 0.02% sodium azide] and then once with Tris-NP-40 buffer [10 mM Tris-HCl (pH 7.5), 0.1% NP-40]. The pre-cleared lysate, adsorbed lysate with anti-P1MCM3 antibody, and the precipitated materials suspended to the original volume were separated on 6% SDS-polyacrylamide gels followed by immunoblotting. In the experiment shown in Figure 4A (lanes 3 and 4), the NET-gel buffer was replaced by RIPA buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS].

The protein size standard used in this study was BioRad pre-stained marker, which was calibrated by the manufacturer and caused a different mobility between batch-to-batch (for example, Fig. 4A and B), except one (Promega) in Figure 2.

Synchronization of HeLa cells at G₁-S boundary

One day after plating 10⁶ HeLa cells onto 90 mm dishes, thymidine was added to the medium at a final concentration of 2.5 mM. Cells were washed three times with DMEM after 26 h and incubated with fresh medium. Hydroxyurea (1 mM) was added 9 h later and cells were further incubated for 14 h. Cells were released from the block by washing with DMEM three times. Nocodazole (40 ng/ml) was added 3 h after the release to prevent rapid cycling cells to enter next G₁ phase. At intervals of 0, 3 and 8 h after the release, synchronized cells were harvested and divided into two tubes.

Proteins were extracted from cells in one of the tubes (15). Cells were lysed with the buffer containing Triton X-100 [10 mM HEPES-KOH (pH 7.5), 7 mM MgCl₂, 1.5 mM KCl, 1 mM DTT, 1% Triton X-100] for 30 min on ice. After microcentrifugation for 30 min, the supernatant was recovered. The precipitate was washed once with the same buffer and suspended with salt buffer [10 mM HEPES-KOH (pH 7.5), 7 mM MgCl₂, 1.5 mM KCl, 1 mM DTT, 0.5 M NaCl] and left to stand on ice for 30 min. After microcentrifugation for 30 min, the supernatant was recovered. The protein concentration of both extracts was measured by BioRad Protein Assay using bovine serum albumin (BSA) as the standard. Aliquots of the extracts (10 µg) were loaded onto a 6% SDS-polyacrylamide gel and subjected to Western blotting.

Cells in the other tube were subjected to cell cycle analysis by FACScan (Beckton-Dickinson). Cells were fixed with 1 ml of ice-cold 70% ethanol for 30 min, washed with PBS, incubated with 2 ml of PBS containing 10 µg/ml RNase A and 50 µg/ml propidium iodide for 30 min at 37°C and proceeded to FACScan analysis.

Construction of epitope-tagged expression plasmids and transfection

The C-terminal haemagglutinin (YPYDVPDYA)-tagged mouse P1MCM3 (P1-HA) was constructed by PCR using a set of primers (PIHAF and PIHAR) and P1MCM3 cDNA. The PCR schedule was: 94°C, 1 min; 55°C, 1 min; 74°C, 2 min for 15 cycles supplemented with *Taq* polymerase (Promega) and *Pfu* polymerase (Stratagene). The PCR product was digested with *Eco*RI, subcloned into pBluescriptII, confirmed by partial nucleotide sequencing and then ligated downstream of the SRα promoter (21). The C-terminal FLAG (DYKDDDDK)-tagged mCDC46 (CDC46-FLAG) was constructed as follows: PCR was performed to add the *Xho*I site at the C-terminus of mCDC46 cDNA using primers (46FLAGF and 46FLAGR) by the same schedule. The FLAG linker, which is the annealed product of

FLAG1 and FLAG2, was inserted into the *Xho*I site of the PCR product cloned into the pUC-derived vector by *Eco*RI sites and confirmed by sequencing. The mCDC46-FLAG was then ligated with pcDNA1 (Invitrogen) to be driven by CMV IE promoter.

The nucleotide sequence of synthetic oligonucleotides were as follows:

PIHAF: GGGAATTCGCGGCCGCCACCATGGCGGGCACA-GTAGTGCTGGAT,

PIHAR: GGGAATTCGCGGCCGCTTAGGCGTAATCAGGA-ACGTCATAAGGGTAGATAAGGAAGACGATGCCCTCA,

46FLAGF: GGGAATTCAGGTACAGTCATGTCGGG,

46FLAGR: GGGAATTCAGTTCGAGGCGATAGAGCACCTT,

FLAG1: TCGACTACAAGGACGACGATGACAAGC,

FLAG2: TCGAGCTTGTTCATCGTCGTCCTTCTAG.

Human 293 (2 × 10⁵) or monkey COS7 (10⁵) cells plated on a 60 mm dish were transfected with 8 µg DNA by the standard CaPO₄ method (19). Three days later, the cells were lysed in hypotonic buffer with 1% Triton X-100 and the extracts were mixed with 20 µl anti-FLAG M2 beads (2 mg/ml) (IBI Kodak) or control mouse IgG coupled with Sepharose 4B (2 mg/ml) at 4°C for 1 h. The beads were washed three times with NET-gel buffer and once with Tris-NP-40 buffer and then boiled with SDS gel-loading buffer for 5 min.

RESULTS

Isolation of mouse Mcm genes

We identified three mouse Mcm genes by PCR and screened a mouse cDNA library by the PCR products (15). Several positive clones were isolated and longer inserts were subcloned for their nucleotide sequencing. The primer extension analyses and the Northern hybridization, in which the hybridized signal size appeared to correspond to the length of the cloned cDNAs, showed that these cDNA clones were nearly full length (data not shown) and contained the initiation codon which gave a large open reading frame. One encoded P1MCM3 protein, having 42% amino acid homology with the budding yeast MCM3 (3) and named DNA polymerase α associated protein P1 or P1 protein in previous studies (12,15). The others were new genes including mCDC46 and mcDC21 (15) (Table 1). Although there was ~30% amino acid identity in the entire length between the members in the MCM family, there was >40% amino acid identity between the putative mouse and yeast homologs. mCDC46, a putative mouse homolog of yeast CDC46, consisted of 733 amino acids having 47% amino acid identity with the budding yeast CDC46 protein (7) and having 97% identity with the partial amino acid sequence of the human P1_{CDC46} protein (13). In addition, a short sequence of amino acids in mouse CDC46 described by Hennessy *et al.* (7) was completely identical to amino acids 337-381 of the mCDC46 protein. mcDC21, a putative mouse cdc21 homolog consisting of 863 amino acids, had 43% identity with the fission yeast cdc21 protein (8). The mCdc21 protein also had strong identity (98%) over a 200-amino-acid stretch of human P1_{cdc21} (13). The N-terminus of the mCdc21 protein had 11 (S/T)Pxx motifs including three (S/T)Px(R/K), which are good target sites for cyclin-dependent kinases, similar to yeast Cdc21 protein (8). The central region of these proteins was also strongly conserved as was the DNA-dependent ATPase motif (17), including DEAD-box (13), although the NTP binding or the ATPase activity has not yet been shown.

Figure 1. Northern blot analysis by synchronized cells. Total RNA was periodically recovered, electrophoresed and hybridized with the indicated probes. Positions of 28S and 18S ribosomal RNA are shown. Expression of histone H3 coupled with DNA replication shows that cells were in the S phase. Hybridization with β -actin probe is shown as a loading control. (A) Swiss 3T3 cells were synchronized by serum deprivation. Total RNA (5 μ g) recovered at the indicated time after the serum stimulation was loaded on each lane. (B) HeLa cells in the mitotic phase were collected by shake off after the nocodazole treatment and then replated. Total RNA was recovered at the indicated time and 20 μ g samples were loaded.

Table 1. The MCM-P1 family

	MCM2	MCM3	CDC46	yBR1441	Cdc21	Mis5
<i>S.cerevisiae</i>	MCM2(4)	MCM3(3)	CDC46(7)/MCM5(5)	yBR1441(11)	Hcd21?(8)	
<i>S.pombe</i>	Nda1(9)	Mcm3?(8)	Nda4(9)		Cdc21(8)	Mis5(10)
<i>X.laevis</i>		MCM3/P1(29)			?(8)	
mouse		P1MCM3(12,15)	mCDC46 ^a	mBR1441 ^b	mCdc21 ^a	
human	BM28(14)	P1MCM3(12,29)	P1CDC46(13)	P1.1MCM3(13)	P1cdc21(13)	

?, Possible homolog identified by short sequencing.

^aThis study.

^bH. Kimura, unpublished data.

Mouse Mcm genes are expressed at the late G₁ to S phase

Swiss 3T3 cells synchronized at the G₀ phase by serum deprivation were stimulated to enter the cell cycle. The total RNA was isolated at 4 h intervals and subjected to Northern hybridization. The expression of mCDC46 and mcdc21 genes were first found 8 h after serum stimulation, before that of the histone H3 gene, which showed S phase specific expression at 12 h (Fig. 1A). The DNA synthesis obtained by monitoring with BrdU incorporation was first observed 12 h after stimulation and reached a peak at 20 h, which is in agreement with the expression pattern of histone H3 (data not shown). This suggested that these mouse Mcm genes were not expressed in quiescent cells and expressed at the late G₁ to S phase as described for the P1MCM3 gene (12). However, the result could not exclude the possibility that the expression was induced at the point of G₀ to G₁ transition not G₁ to S phase. Hence, further study was performed using mitotically arrested HeLa cells in order to confirm the cell cycle specific expression. The accumulation of mcdc21, mCDC46 and P1MCM3 mRNA was first observed 12 h after replating and the level of expression decreased after the peaks at 16 and 20 h

(Fig. 1B). The expression of histone H3 coupled with DNA replication was found 16 h after replating and peaked at 20 and 24 h (Fig. 1B). This result supported the view that the expression of these Mcm genes is cell cycle specific at the late G₁ to S phase. The expression may be regulated in a similar manner among the family and cooperatively play some roles in the initiation of DNA replication. In fact, two E2F binding sites were observed at the 5' region of the P1 gene (15). E2F is considered to be the nearest analog of yeast DSC1 that controls the expression of the DNA synthesis genes (22). It may be interesting to know the 5' flanking sequences and the regulation of gene expression of these Mcm genes. However, the amount of these MCM proteins did not vary considerably throughout the cell cycle (data not shown). Therefore, it remains unknown whether *de novo* synthesis of proteins is needed for the progression of the S phase within the cell cycle.

Antibody against mCDC46 and mCdc21 recognized 90 and 100 kDa proteins

We prepared antisera against mouse MCM proteins to assess their function and interaction. Western blottings showed that mCDC46

Figure 2. Western blottings of mouse FM3A extracts with anti-mCDC46 or anti-mCdc21 antiserum. Mouse FM3A cells were homogenized with hypotonic buffer and the cytoplasmic extract and nuclei were separated by low speed centrifugation. The cytoplasmic extract was cleared by high speed centrifugation. The nuclear sediment was extracted with the same volume of the hypotonic buffer containing NaCl at the indicated concentrations. Extract from 10^5 cells was loaded onto each lane of a 8% SDS-polyacrylamide gel and subjected to immunoblotting with anti-mCDC46 or anti-mCdc21 antiserum.

and mCdc21 were 90 and 100 kDa proteins, respectively, present both in the cytoplasmic and nuclear fractions (Fig. 2). Most of these proteins were extracted with 0.5 M NaCl as P1MCM3 (15). This was not inconsistent with their expected function involved in DNA replication. DNA replication proteins, such as DNA polymerase α , proliferating cell nuclear antigen and RP-A, were also extracted in the cytoplasmic fraction although they exist and act in the nucleus (23–26). It is considered that the excess proteins exist as a pool not in association with replication machinery attached to a nuclear skeleton (24,27). The proteins in the nuclear fraction, which are not extracted with the buffer containing Triton X-100, may play a role in DNA replication (see below). Although their molecular weight obtained by SDS-PAGE was larger than their calculated values (82 and 97 kDa for mCDC46 and mCdc21, respectively), such a discrepancy is observed throughout MCM proteins (3,7,15). The blot with the anti-mCDC21 also showed a signal at 75 kDa in the cytoplasmic and the wash fractions. We considered that it was a degraded product because it was not observed in a quickly prepared extract (data not shown). The antibodies against mCDC46 and mCdc21 also recognized 90 and 100 kDa proteins in the human cell extract, respectively (Fig. 3B), although the specificity of anti-mCDC46 was not so high. This is in agreement with the strong conservation of the nucleotide sequence of these Mcm genes among mammals (13).

Alteration of the extraction profile of mouse MCM proteins during the cell cycle

We have already shown that P1MCM3 is a nuclear phosphoprotein and changes the nuclear distribution during the S phase, in addition to the fact that the microinjected P1MCM3-specific antibody inhibits DNA replication in mouse 3T3 cells (15). We showed that at least two forms of P1MCM3 existed in the nucleus,

an underphosphorylated form associated with a particular nuclear structure which is resistant to extraction with Triton X-100 and DNase treatment, and a hyperphosphorylated form extractable with Triton X-100 (15). The underphosphorylated P1MCM3 seemed to allow DNA to replicate once per cell cycle like a 'license' (28) of DNA replication, since the underphosphorylated form was dissociated sequentially first from the euchromatin, and next from the heterochromatin regions (15). It was expected that the Triton X-100-insoluble underphosphorylated form would decrease during the progression of S phase. We examined this possibility by Western blotting of extracts from synchronized HeLa cells by double block with thymidine and hydroxyurea. When cells were at the G₁-S boundary, P1MCM3 was more abundant in the Triton X-100-insoluble pellet (Triton pellet) than in the soluble fraction (Triton supernatant) (Fig. 3). The amount of P1MCM3 in the Triton pellet slightly decreased when most cells entered S phase 3 h after the release from the hydroxyurea block. Eight hours after the release, when the majority of cells were in the late S to G₂ phase, much more P1MCM3 was found in the Triton supernatant than in the pellet (Fig. 3). The accumulation of P1MCM3 in the Triton supernatant may not reflect the protein synthesis directly (Fig. 1B) but a change in the property of the protein. This result supported the view that the underphosphorylated P1MCM3 dissociated from the nuclear structure by phosphorylation during the S phase. Similar extraction profiles dependent on the cell cycle were found in both mCDC46 and mCdc21 (Fig. 3B middle and lower panel), suggesting that these MCM proteins were regulated in a similar manner and/or functioned as a complex by binding each other. Although we were interested in cellular localization of mCdc21 and mCDC46 proteins by immunofluorescence, our antisera against these proteins could not be used for immunofluorescence or immunoprecipitation.

mCDC46 was physically associated with P1MCM3 protein

We obtained evidence for the physical interaction between mouse MCM proteins. A 90 kDa protein, p90, was co-precipitated with P1MCM3 when FM3A hypotonic extract was precipitated with the P1MCM3-specific antibody (Fig. 4A; 14). Co-precipitation was detected even after washing with a higher stringent buffer such as RIPA (Fig. 4A, lane 4), suggesting that P1MCM3 is tightly associated with the p90. The genetic interaction between Mcm genes (4,10), similar extraction profile (Fig. 2 and 3) and identical molecular weight between the p90 and mCDC46 prompted us to test whether the p90 is a member of the MCM protein family using antisera we raised. The p90 was recognized with anti-mCDC46 antibody and the amount of mCDC46 protein was much less in the supernatant fraction after the anti-P1MCM3 immunoprecipitation (Fig. 4B). However, mCdc21 protein was not co-precipitated under the same conditions (Fig. 4B). This suggested tight association between P1MCM3 and mCDC46, but the possibility of a cross-reaction between the anti-P1MCM3 antibody and mCDC46 could not be ruled out. The antibody might react with the conserved central region of mCDC46 under the native condition, although the anti-P1MCM3 immunoblot showed only a 105 kDa P1MCM3 protein (15). We confirmed the P1MCM3-mCDC46 interaction by epitope tagged-proteins produced transiently by COS7 or 293 cells. Endogenous P1MCM3 protein, even though small in amount, was co-precipitated when overproduced mCDC46-FLAG protein was precipitated by anti-

Figure 3. Alteration of a biochemical property of mouse MCM proteins during the cell cycle. HeLa cells were arrested at the G₁-S boundary by double block with thymidine and hydroxyurea (see Materials and Methods). Cells were subjected to: FACS analysis (A) and preparation of extract followed by Western blotting (B) at 0, 3 and 8 h after the release. (A) 10^4 cells were sorted after fixation and incubation with PBS containing RNase and propidium iodide (PI). The positions of 1C (G₁) and 2C (G₂/M) DNA contents are indicated. (B) Triton X-100 soluble (S; lanes 1, 3 and 6) and insoluble (P; lanes 2, 4 and 8) fractions were prepared from synchronized HeLa cells at 0 h (lanes 1 and 2), 3 h (lanes 3 and 4) and 8 h (lanes 5 and 6) after the release from the block. An equal amount (10 μ g) of extracts was separated by 6% SDS-PAGE followed by Western blotting with the indicated antisera. Arrowheads show the positions of human MCM proteins recognized by the antisera.

FLAG M2 antibody (Fig. 5, lane 3 and 8). It may be worth while mentioning that much more P1MCM3 was recovered after co-transfection with P1MCM3-HA and mCDC46-FLAG (Fig. 5, lane 4 and 9) than after transfection with mCDC46-FLAG alone. There were no signals in the control experiments (Fig. 5, lanes 1, 2, 5-7 and 10). This result strongly supports physical interaction of mCDC46 and P1MCM3 proteins. Although there remained the possibility of indirect association of P1MCM3 and mCDC46 proteins through other factors, it is unlikely because no other proteins as prominent as P1MCM3 and mCDC46 were observed in the anti-P1MCM3 immunoprecipitate (Fig. 4A). However, the *in vitro* binding should be examined to obtain evidence of a direct interaction in a future study. We have not been able to find any association of Cdc21 with P1MCM3-HA or with mCDC46-FLAG under the conditions used (data not shown).

DISCUSSION

MCM proteins are postulated to be involved in once-per-cell-cycle regulation of DNA replication in yeast and mice (6,7,15,16). However, their biochemical function and regulatory mechanism are not well understood. All yeast MCM genes are essential for viability and do not complement other *mcm* mutants (3,4,7-10). All the MCM genes must be identified to determine their function and regulation. Hu *et al.* reported seven polypeptides (130, 120, 105, 102, 90, 85 and 63 kDa) that reacted with an antiserum against the synthetic polypeptide CIDEFDKM corresponding to the most conserved region among MCM proteins in the nucleus of human cells (13). They cloned putative

human homologs of MCM3, CDC46, cdc21 and yBR1441, which they called P1_{MCM3}, P1_{CDC46}, P1_{cdc21} and P1.1_{MCM3}, respectively, by screening with the antiserum (13). Todorov *et al.* cloned a putative human homolog of MCM2, BM28, and characterized the product to be a 125 kDa protein, which would be p120 or p130 reported by Hu *et al.* (13), required for DNA replication and cell division (14). We showed that mCDC46 and mCdc21 are 90 and 100 kDa proteins from the immunoblottings, which is in agreement with the predicted amino acids from cDNA sequences, suggesting that they are p90 and p102 observed in the anti-CIDEFDKM immunoblotting (13), although Hu *et al.* considered that p63 was P1_{CDC46}. We have further cloned a mouse gene homologous to yBR1441 (11) and P1.1_{MCM3} (13) and the molecular mass calculated from the nucleotide sequence was 81 kDa, which may correspond to p85 (unpublished). One candidate for the gene for p130 or p120 would be a homolog of Mis5, a new member of the MCM gene family in fission yeast (10), although it has not been isolated from higher eukaryotes.

In this study, we showed that the co-precipitated 90 kDa protein with P1MCM3 (15) was mCDC46, presenting evidence, for the first time, of the physical interaction between MCM proteins. The interaction between P1MCM3 and mCDC46 was very tight and maintained throughout the cell cycle (15). Since the phosphorylation of mCDC46 was not observed (15), it is possible that the function, including the association with the nuclear structure, of the protein complex is regulated by the phosphorylation of P1MCM3 (15). We could not find any interaction between mCdc21 and other proteins. However, mCdc21 may also be involved in the regulation of once-per-cell-cycle DNA replication

Figure 4. Co-precipitation of mCDC46 protein with anti-P1MCM3 antibody. (A) Mouse FM3A hypotonic extract was precipitated with the pre-immune (lanes 1 and 3) or anti-P1MCM3 (lanes 2 and 4) serum followed by Pansorbin cells and washed with NET-gel buffer (lanes 1 and 2) or RIPA buffer (lanes 3 and 4). The immunoprecipitated materials solubilized with SDS-gel loading buffer was separated by 8% SDS-PAGE and silver-stained. P1MCM3 (P1), co-precipitated 90 kDa protein (p90) and rabbit immunoglobulin (Ig) are indicated. (B) Mouse FM3A hypotonic extract (T) was precipitated with anti-P1MCM3 antibody followed by Pansorbin cells. After brief centrifugation, the supernatant (S) was recovered. The precipitated material (P) was washed with NET-gel buffer followed by Tris-NP-40 buffer and then suspended to the original volume. Immunoblotting with the indicated antibody was performed after 6% SDS-PAGE. The positions of P1MCM3 (left), mCDC46 (middle) and mCdc21 (right) are indicated by arrowheads.

in indirect association with the P1MCM3-mCDC46 complex because this complex and mCdc21 become soluble to Triton X-100 almost simultaneously at the late S and G₂ phase (Fig. 3). The physical association of P1MCM3 and mCDC46 was consistent with the genetic interaction of yeast Mcm genes, which is observed between *Mcm2* and *Mcm3* (4) and between *mis5* and *cdc21* (10). Since the *cdc46* mutants were allele specific suppressors of *cdc45* or *cdc54* (7), *CDC45* and *CDC54* may also be involved in the Mcm gene family. These findings suggested

Figure 5. Co-precipitation of P1MCM3 with C-terminal FLAG-tagged mCDC46 with anti-FLAG antibody. Monkey COS7 and human 293 cells were transfected with the mixture (8 μ g) of two plasmids (each 4 μ g). (Lanes 1 and 6) pSR α -P1HA and pcDNA1, which are the vectors with no insert. (Lanes 2 and 7) pSR α -P1HA, which is an expression plasmid of HA-tagged P1MCM3, and pcDNA1. (Lanes 3 and 8) pSR α and pcDNA1-mCDC46FLAG, which is an expression plasmid of FLAG tagged mCDC46. (Lanes 4, 5, 9 and 10) pSR α -P1HA and pcDNA1-mCDC46FLAG. Three days after transfection, the cells were lysed with the hypotonic buffer containing Triton X-100. The extract was immunoprecipitated with anti-FLAG (lanes 1-4 and 6-9) or control mouse IgG (lanes 5 and 10) beads. The immunoprecipitated proteins were separated by 8% SDS-PAGE followed by immunoblotting with the anti-P1MCM3 antiserum (anti-P1; upper gel) or with the mixture of anti-P1MCM3 and anti-mCDC46 antisera (anti-P1 + anti-mCDC46; lower gel). The positions of P1MCM3 (P1) and mCDC46 are indicated. Probable degraded products are observed below 90 kDa. The 115 kDa signal observed in lanes 1 and 2 in the lower gel is a trace of β -galactosidase, which is recognized by the antisera raised against its fusion proteins, used as the pre-stained size marker (BioRad).

that MCM proteins make a large complex for the initiation of DNA replication.

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NOTE ADDED IN PROOF

After submission of this paper, two related papers were published. The physical interaction of human P1MCM3 and P1CDC46 was shown by Burkhart *et al.* [*Eur. J. Biochem.*, **228**, 431–438 (1995)]. Dalton and Whitbread [*Proc. Natl. Acad. Sci. USA*, **92**, 2514–2518 (1995)] showed that budding yeast CDC47 gene is identical to yBR1441.