hCDC47, a Human Member of the MCM Family

DISSOCIATION OF THE NUCLEUS-BOUND FORM DURING S PHASE*

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hCDC47 is a putative human homologue of yeast CDC47 and a member of the MCM protein family, which has been implicated in the regulatory machinery causing DNA to replicate only once in the S phase. In the present study, we performed an initial characterization of hCDC47. We found that hCDC47 protein was present in the nucleus of cultured human cells in two different forms: one extractable by a non-ionic detergent and the other resistant to such extraction and tightly associated with the nucleus. The levels of the nucleus-bound form gradually diminished during S phase progression, although the total amount of nuclear hCDC47 protein remained relatively constant, suggesting that the nucleus-bound form becomes dissociated from the nuclear structure during DNA replication. This behavior of hCDC47 protein is very similar to that of other mammalian MCM proteins reported recently. We also found that expression of hCDC47 mRNA was repressed in quiescent cells but was induced at the late G1 to S phase by growth factor stimulation. Together, these findings indicate that hCDC47 protein together with other MCM proteins participates in the regulation of mammalian DNA replication.

Replication in eukaryotic cells is a precisely regulated event, which occurs only once in the S phase of the cell cycle to maintain the integrity of the genome. DNA replication proceeds in two stages: initiation of the replication fork and elongation of the new strands. Little is known so far about cellular factors that regulate the initiation of chromosomal DNA replication.

By using mutants that fail to maintain minichromosomes with autonomously replicating sequences (ARSs), several genes termed MCM have been isolated in budding yeast (1). Mutations in MCM affect the function of each ARS in an ARS-specific manner. This unique phenotype indicates that MCM gene products are involved in the initiation of DNA replication at ARSs rather than in the elongation step (2). Among them, MCM2, MCM3, and MCM5 (identical to CDC46), together with the fission yeast cdc21, share striking homology and compose a protein family implicated in the initiation of DNA replication (3-6) and conserved even in higher eukaryotes (7, 8).

CDC46/MCM5, MCM2, and MCM3 proteins show unique behavior during the cell cycle; they are localized in the nucleus during G1 phase but disappear from this site at the beginning of the S phase, to reappear at the end of the M phase (9, 10). This behavior is reminiscent of that of the putative replication licensing factor, which was first proposed from results with an in vitro replication system with Xenopus egg extracts (11). In this latter model, the licensing factor can enter the nucleus during nuclear membrane breakdown in mitosis. It is excluded, however, by an intact nuclear membrane and allows DNA to replicate only once in the S phase by evoking the initiation step and afterward being immediately inactivated.

Recently, a number of mammalian homologues of the MCM family have been identified, including MCM2, MCM3, CDC46/MCM5, and cdc21 (7, 12-19). Microinjection of antibodies against murine MCM3 (mMCM3) or human MCM2 (hMCM2) protein inhibits cellular DNA synthesis, suggesting that both proteins are related to replication in mammalian cells (13, 14). mMCM3 protein is present in the nucleus as a soluble form as well as in tight association with nuclear structures, becoming dissociated during S phase (14, 19). Similar observations were also obtained for hMCM2 (20). Like yeast MCM proteins, mammalian MCM proteins have been also implicated in the regulatory machinery allowing DNA to replicate only once in the S phase (7, 12-20).

We have isolated a cDNA, named hCDC47 (human CDC47), encoding a putative human homologue of budding yeast CDC47 replication protein. A part of hCDC47 cDNA was previously identified as P1.1Mcm3, a member of the mammalian MCM homologues (12). CDC47 belongs to the yeast MCM family, and its cell cycle-dependent subcellular localization pattern closely resembles that of other yeast MCM proteins (21, 22). In the present study, we performed an initial characterization of hCDC47.

MATERIALS AND METHODS

Cloning of hCDC47 cDNA—During attempts to identify the gene encoding a protein that interacts with human papilloma virus type-18 E6 protein using a yeast two-hybrid system, we isolated a 630-base pair cDNA fragment as a fusion gene with a GAL4 activation domain, later found to correspond to the 3'-end of the hCDC47 cDNA encoding amino acids 562-719. A 2.4-kilobase cDNA was then isolated, which encodes a protein of 719 amino acids with a calculated molecular mass of 81.3 kDa. hCDC47 protein has 49% amino acid identity with the budding yeast CDC47 protein. Details of these studies will be published elsewhere.2

Cell Culture and Synchronization—Human normal diploid fibroblast cell line WI38 (obtained from the) Japanese Cancer Research Resources

1 The abbreviations used are: ARS, autonomously replicating sequence; FC5, fetal calf serum; PI, propidium iodide; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen.

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Bank) was cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum (FCS). Cervical cancer-derived lines, HeLa, SiHa, and C3A, were grown in Dulbecco's modified Eagle's medium with 5% FCS.

For synchronization, exponentially growing WI 38 cells were starved of serum for 48 h prior to addition of fresh medium with 10% FCS. HeLa cells were synchronized at the Go/G1 boundary by means of a double thymidine block. HeLa cells released during the G1/S phase were incubated with 2.5 mM thymidine for 22 h, released for 10 h, and then treated with 1.5 mM hydroxyurea for a further 16 h. The cells were washed and incubated in fresh medium with 5% FCS to reenter the cell cycle.

Cell Cycle Analysis with Flow Cytometry—Cells were harvested by trypsinization, and the nuclei were stained with propidium iodide (PI) using a cytofix kit (Becton-Dickinson) according to the manufacturer's instructions. Cells (1 x 10^6) were analyzed with a FACSkan (Becton-Dickinson), and the percentages in each phase were calculated with the SOBR model in the CELFIT program.

RNA Extraction and Northern Blot Analysis—Total RNA isolation, Northern blot hybridization, and probe labeling were performed as described previously (23). The 630-base pair fragment corresponding to the 3'-end of the hCDC47 cDNA, whose sequence is relatively distinct among hMCMs, was used as a probe. This gave a clear single band corresponding to a length of approximately 2.8 kilobases with various human cell lines. Probed membranes were analyzed with a Bio-Imaging analyzer BAS2000 (Fuji, Japan).

Secondary Antibodies—A His-tagged fusion protein containing amino acids 562-719 of hCDC47 was produced by inserting the 60-base pair fragment into pQE31 (Qiagen) and introducing it into Escherichia coli strain XL1-Blue. After induction with isopropyl-β-D-thiogalactopyranoside (0.1 mM), the overexpressed 20-kDa fusion protein was isolated as inclusion bodies, subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), excised from the gel, and eluted into phosphate-buffered saline (PBS) containing 0.1% SDS. A rabbit was injected with approximately 200 μg of the purified protein emulsified in complete Freund's adjuvant and subsequently with the protein in incomplete adjuvant.

For affinity purification of anti-hCDC47 antibodies, the glutathione S-transferase-fused hCDC47 protein produced in E. coli was coupled to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instruction. The antisera was applied to the glutathione S-transferase-hCDC47 Sepharose column, washed, and eluted.

Rabbit polyclonal antibodies against the oligopeptide VCVIDEFDK-His (amino acids 107-119) from the hCDC47 protein but not His-tagged or glutathione S-transferase-fused unrelated proteins by immunoblot assay (data not shown).

Immunoprecipitation—Immunoprecipitation was performed by standard procedures (24). Cells growing in 60-mm plates were harvested by 0.2% Triton X-100 for 2 min. The samples were washed twice with PBS, and the samples were reacted with 20 μg/ml fluorescin isothiocyanate-conjugated goat anti-rabbit IgG antibodies (affinity purified, Zymed) for 1 h, followed by treatment with 500 μg/ml RNase and 10 μg/ml PI for 30 min. The samples were mounted and analyzed by conventional microscopy or confocal laser microscopy (LSM-G2000, Olympus, Japan).

Immunofluorescence Analysis—All staining procedures were carried out at room temperature. Cells grown in culture chambers (Lab-Tek Chamber Slide, Nunc) were fixed for 10 min with 3.7% formaldehyde and then permeabilized with 0.2% Triton X-100 for 2 min. The samples were incubated with either anti-hCDC47 antisera or the preimmune serum at a 1:1000 dilution in PBS with 10% normal goat serum (Zymed) for 1 h. In some experiments, the purified antibodies were used at 8 μg/ml. After washing three times with PBS, the samples were reacted with 20 μg/ml fluorescin isothiocyanate-conjugated goat anti-rabbit IgG antibodies (affinity purified, Zymed) for 1 h, followed by treatment with 500 μg/ml RNase and 10 μg/ml PI for 30 min. The samples were mounted and analyzed by conventional microscopy or confocal laser microscopy (LSM-G2000, Olympus, Japan).

Differential Extraction of hCDC47 Protein—Cells cultured in 60-mm plates were washed twice with ice-cold PBS and lysed for 20 min on ice with ice-cold hypotonic lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 10 mM MgCl2, 0.5% Nonidet P-40) containing 1 mM phenylmethylsulfonyl fluoride, aprotinin, leupeptin, and sodium orthovanadate. The cell lysates were collected and centrifuged at 5000 rpm for 5 min at 4 °C. The supernatant was clarified by centrifugation at 15,000 rpm for 5 min to obtain the Nonidet P-40-extractable fraction. The nuclear pellet fraction was washed once with hypotonic lysis buffer and directly added to SDS-sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% ␤-mercaptoethanol, 10% glycerol, 0.01% bromphenol blue). Alternatively, the pellet was incubated in hypotonic lysis buffer adjusted to the appropriate NaCl concentration on ice for 20 min.

RESULTS

hCDC47 mRNA Is Expressed at the Late G1 to S Phase—Normal diploid human fibroblast line WI 38 cells were arrested by serum depletion for 48 h, induced to start the cell cycle by adding serum, and harvested sequentially at the indicated times.

Flow cytometric analysis revealed that 80% of the cells were arrested in G0 by serum deprivation, and 20 h after the stimulation, about half of the cells began to enter the S phase (Fig. 1A). In parallel, RNA was isolated and subjected to Northern analysis, demonstrating that the hCDC47 mRNA level started to increase at 12 h and reached to peak at 16 h, just before the onset of the S phase, when it was five times higher than that in the quiescent state (Fig. 1B).

The Level of hCDC47 Protein Does Not Vary Greatly during the Cell Cycle—Rabbit antisera was raised against bacterially produced His-tagged hCDC47, which reacted against the immunized protein and glutathione S-transferase-fused hCDC47 protein but not His-tagged or glutathione S-transferase-fused unrelated proteins by immunoblot assay (data not shown). This antisera, but not a preimmune serum, recognized a protein migrating at approximately 83 kDa on SDS-PAGE by immunoprecipitation from a 35S-labeled extract of WI 38 (Fig. 2A). Some weak additional bands were also observed after immunoprecipitation with the anti-hCDC47 antisera. With immunoblot analysis, the antisera also detected an 83-kDa protein in lysates prepared from two tumor cell lines as well as WI 38 (Fig. 2B). Affinity-purified polyclonal antibodies gave essentially the same results as that with the antisera (data not shown). The 83-kDa protein immunoprecipitated from the [35S]Met-labeled HeLa cell extract with the anti-hCDC47 antibodies was subjected to SDS-PAGE, and its digestion pattern was compared with the one of in vitro translated [35S]Met-labeled hCDC47 (Fig. 2C). The molecular size of the in vitro translated hCDC47 estimated by SDS-PAGE had good concordance with that of the cellular protein detected with the anti-hCDC47 antibodies, and the two maps after partial proteolysis were virtually identical. Together, these findings led us to conclude that the 83-kDa pro-

3 M. Fujita and M. Ishibashi, unpublished observations.
tein detected by our polyclonal antiserum is the hCDC47 protein. Both the cellular and in vitro translated hCDC47 protein often resolved into a doublet. However, the underlying mechanism is obscure at present; we could not discriminate these two bands with regard to cell cycle dependence or detergent extractability (see below).

Using the antiserum, we examined whether the total protein levels of hCDC47 changed during the cell cycle along with the mRNA levels in synchronized WI38 cells. hCDC47 protein was clearly identified even in the quiescent WI38 cells starved of serum for 48 h, and, when the cells entered S phase, only a slight increase in the amount of the protein was observed (Fig. 3).

Subcellular Localization of hCDC47 Protein—To investigate the localization of hCDC47 protein, indirect immunofluorescence staining with the antiserum was applied to growing WI38 cells. After the immunostaining, the cells were further treated with RNase and counterstained with PI to identify the nuclei. As shown in Fig. 4, the antiserum gave essentially nuclear staining in WI38 cells, although there was some variation in the signal intensity among the cells. In contrast, no specific staining was observed with the preimmune serum. Similar nuclear staining was also observed in G1/S- or late S/G2-enriched HeLa cells with anti-hCDC47 antibodies (Fig. 6B, see below).
Nuclear mMCM3 protein consists of two forms that can be differentiated by non-ionic detergent extraction (14). We were, therefore, interested in whether this was also the case for hCDC47. Asynchronously growing WI38 cells were first extracted with hypotonic buffer containing 0.5% Nonidet P-40, and then the pellets were successively extracted with the same buffer containing NaCl at the indicated concentrations. The amount of hCDC47 protein in each sample was determined by immunoblotting (Fig. 5). The supernatant fraction of the Nonidet P-40 extraction contained three-fourths of the total hCDC47 protein amount, and most of the remaining protein in the pellet fraction was extractable with 0.5M NaCl, demonstrating that hCDC47 protein also has two forms like mMCM3, a form extractable by Nonidet P-40 and the nucleus-bound form.

Dissociation of Nucleus-bound hCDC47 Protein during S Phase—To gain insight into the behavior of hCDC47 protein during DNA replication, we chose to use HeLa cells because synchronized populations can thereby be easily obtained. HeLa cells were synchronized at the G1/S boundary by a thymidine-hydroxyurea double block and then released. Analysis of the DNA content of these cells by flow cytometry showed that near 100% of the cells were arrested in G1/S, while 4 h after release 80–90% were in mid S phase, and at 8 h they had reached late S/G2 phase (Fig. 6A). In the S/G2-enriched population, mitotic cells were less than 10% as determined by microscopy for formaldehyde-fixed and PI-stained cells (data not shown). When the localization of hCDC47 protein was examined by immunofluorescence microscopy of the G1/S or late S/G2-enriched HeLa cells, nuclei were predominantly stained in both populations (Fig. 6B).

Nonidet P-40-extracted supernatant and remaining pellet fractions were prepared from these three populations and subjected to SDS-PAGE followed by Coomassie Blue staining. The extraction efficiency was confirmed to be virtually equal (data not shown). Examination of the levels of hCDC47 protein in these samples by immunoblotting with the anti-hCDC47 antiserum demonstrated no great variation through the S phase in the supernatant fraction, which contained three-fourths of the total hCDC47 protein in the G1/S population. In contrast, the levels of hCDC47 protein in the pellet fraction decreased remarkably along with progression through the S phase (Fig. 7). In the late S/G2-enriched population, the amount of the nucleus-bound hCDC47 was one-fifth that in the G1/S population. With parallel immunoblotting, the levels of hMCM3 protein in the pellet fraction were found to be coincidentally decreased with that of hCDC47 during the S phase (Fig. 7).
PCNA, the auxiliary protein of DNA polymerase δ required for DNA replication (26, 27), also has two forms that can be differentiated by non-ionic detergent extraction. The detergent extraction-resistant form is probably associated with DNA replication sites and is thought to play a fundamental role in DNA synthesis (28). To establish whether the change in nuclear association of hCDC47 and hMCM3 during S phase is specific for these MCM proteins, we examined the levels of nucleus-bound PCNA with parallel immunoblotting (Fig. 7). PCNA also showed cell cycle-regulated association with the nucleus, but the kinetics of change proved to be different from those of hMCM proteins. The amount of the nucleus-bound PCNA was most abundant in the mid S-enriched population, and in the late S/G2-enriched population it decreased to only two-thirds that in the G1/S population. Therefore, the changing nuclear association pattern of hMCM proteins seems to be specific for them.

**DISCUSSION**

hCDC47 is a putative human homologue of yeast CDC47 (21, 22) and a member of the MCM protein family (7, 12–20). In this study, we investigated the biological properties of hCDC47. Immunochemical analysis of cultured human cells showed that hCDC47 protein is present in the nucleus in two different forms: one extractable by Nonidet P-40 and the other resistant to Nonidet P-40 extraction and thus tightly associated with the nucleus. In synchronized HeLa cells, the levels of the nucleus-bound form were gradually diminished during S phase progression, although the total amount of nuclear hCDC47 did not vary greatly, suggesting that the nucleus-bound hCDC47 is dissociated from nuclear structures during DNA replication. Although the question of whether the findings for hCDC47 observed in HeLa cells, which are transformed, can be generalized for normal cell cycle regulation remains, similar change in nuclear association of mammalian MCM3 to that found for hCDC47 has been observed not only in HeLa cells (18, 19) but also in untransformed murine Swiss 3T3 cells (14). Overall, these findings for hCDC47 closely resemble those reported for other mammalian MCMs (14, 18–20). Very recently, a Xenopus MCM (XMCM) complex including XMCM2, -3, and -5 was reported as a component of the licensing factor for replication in the Xenopus egg extract (19, 29, 30). In this system, only chromatin-bound XMCMs are active for the initiation of replication, becoming detached as replication proceeds. By analogy with the Xenopus system, the nucleus-bound form of mammalian MCMs including hCDC47 may be required for DNA replication. Recently, it has also been demonstrated that MCM proteins form complexes with each other in mammalian cells (16–18), which may be similarly regulated and function in the regulation of DNA replication. The precise role of the MCM proteins, however, still remains mostly unknown. In the case of yeast, different MCM mutants are arrested at different points of S phase, which indicates specific and indispensable functions of each MCM for DNA replication (3, 4, 6, 9, 21). It is very informative that the phenotype of mammalian cells in which each MCM is repressed be elucidated.

In higher eukaryotes, MCM proteins may play a regulatory role through their association-dissociation with the nuclear structure. Kimura et al. (14) has proposed that association of mMCM3 with the nuclear structure is regulated by its phosphorylation (14). On the other hand, it has been reported that mCDC46 coprecipitating with mMCM3 is not phosphorylated (14, 16, 18). Furthermore, while hcdc21 coprecipitating with hCDC47 is phosphorylated (17), hCDC47 was not evidently phosphorylated (14). On the other hand, it has been reported that hCDC47 is phosphorylated (17), hCDC47 was not evidently phosphorylated (14, 16, 18). On the other hand, it has been reported that hCDC47 is phosphorylated (17), hCDC47 was not evidently phosphorylated when the cells were labeled in vivo during S phase. 3 The mechanisms regulating the nuclear association of MCM proteins thus remain to be clarified.

In the present study, hCDC47 mRNA was not expressed in quiescent cells but was expressed at the late G1 to S phase, as reported for other mammalian MCM genes (7, 15, 18) and also for other DNA synthesis-related genes. The 5′-region of the mMCM3 gene has two binding sites for E2F (14), which is considered to control the expression of some genes involved in DNA synthesis (31). We also detected two E2F sites in the 5′-region of the hCDC47 gene2, and thus the expression of mammalian MCMs might be simultaneously regulated by factor(s) such as E2F. In contrast to the mRNA expression, the fluctuation in hCDC47 protein levels appeared small during S phase, which indicates specific and indispensable functions of each MCM for DNA replication (3, 4, 6, 9, 21). It is very informative that the phenotype of mammalian cells in which each MCM is repressed be elucidated.

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**FIG. 7. Dissociation of the nucleus-bound hCDC47 protein during the S phase in HeLa cells.** G1/S, mid S, and late S/G2-enriched HeLa cell populations, as prepared in Fig. 6A, were extracted with hypotonic Nonidet P-40 buffer to obtain the Nonidet P-40-extractable fraction (NP-40/sup) and the remaining pellet fraction (NP-40/pellet). Each fraction was immunoblotted with anti-hCDC47 antibodies, anti-hMCM3 antibodies, or anti-PCNA monoclonal antibody. The loaded amount of the Nonidet P-40-extractable fraction (equivalent to approximately 10^5 cells/lane) was set at one-third that of the Nonidet P-40/pellet fraction (approximately 30^5 cells/lane). 46–40 kDa.
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