Identification of a Novel MCM3-associated Protein that Facilitates MCM3 Nuclear Localization*

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MCM3 is essential for the initiation of DNA replication, and also participates in controls that ensure DNA replication is initiated once per cell cycle. In a two-hybrid screen for proteins that interact with human MCM3, we identified and cloned a novel protein of which the calculated molecular weight is 80,291. A specific antibody against the protein identified a 80-kDa protein in HeLa cell extract, indicating the protein actually expressed in cells. The interaction of these proteins was confirmed by immunoprecipitation assay. Moreover, we clarified a nuclear localization signal of human MCM3, and we find that mutagenesis on the nuclear localization signal of MCM3 affected the binding of newly isolated MCM3-associated protein, Map80. Map80 was expressed in Escherichia coli as a fusion with His6 tag and purified with sequential column chromatographies. The addition of recombinant Map80 stimulated the amount of nuclear localized MCM3. These results suggest that Map80 is involved in the nuclear localization pathway of MCM3.

The chromatin structure of DNA replication origins changes during the cell cycle, alternating between the prereplicative complex during the G1 phase and postreplicative complex during the S, G2, and M phases (Refs. 1–3). Initiation of DNA assembly and maintenance of the complex requires the prior assembly of prereplicative complex and to understand their functions. Thus, a key issue in the initiation of DNA replication is to identify the proteins that are component of the prereplicative complex, and to understand their functions. It is currently thought that the origin recognition complex binds to the DNA origin directly and recruits Cdc6 protein, which in turn recruits MCM proteins on chromatin (5–7). The complex needs to be activated by protein kinases, Cdc7/Dbf4, cyclin/Cdc2, and so on, to trigger the onset of the S phase (8–10). MCM proteins associate with chromatin during the G1 phase and are released during the S phase, which could explain why G1 but not G2 nuclei were triggered to initiate DNA replication when cells are fused to S phase cells (11, 12).

In this paper, to identify new members of the prereplicative complex, we screened human liver cDNA library by a two-hybrid method using MCM3 as a bait, and we identified a novel MCM3-associated protein. A newly isolated MCM3-associated protein, Map80,† suggested the involvement of the nuclear localization of MCM3.

EXPERIMENTAL PROCEDURES

Two-hybrid Screening—All components were purchased from CLONTECH, and all assays were carried out as suggested by the manufacturer. To obtain the MCM3-associated protein, bait was prepared by inserting the DNA fragment of hMCM3 encoding amino acids 556–728 into the pGAT9 and introduced into Hfr7c. The pACT2 cDNA library prepared from human liver as a source of interacting proteins was introduced into the Hfr7c pGAT9-hMCM3 strain. Then, 1 × 10̇7 transformants were selected for tryptophan, leucine, and histidine prototrophy. Isolated colonies were tested for β-galactosidase activity (13, 14).

Antibody Production—Rabbit polyclonal anti-Map80 antibody was raised against the synthetic peptide corresponding to the sequence of amino acids 460–474 of Map80. Synthesis of peptide, immunization, and obtaining of the antisera were performed by Sawady Technology Inc. (Tokyo, Japan).

Western Blotting—The samples were separated by SDS-PAGE and transferred to a PVDF membrane at a constant voltage of 50 V for 1 h. The membrane was blocked with 5% non-fat milk in PBS and probed with antibody in 5% non-fat milk in PBS. The bound antibodies were detected with peroxidase-conjugated second antibody (Amersham Pharmacia Biotech) using 1/500 dilution in 5% non-fat milk in PBS, and the protein bands were visualized by the chemiluminescent method with 3,3′-diaminobenzidine as substrate (15).

Expression and Purification of Proteins—The DNA fragment of hMCM3 encoding amino acids 556–728 was inserted into the pEGFP C2 vector (CLONTECH). The fragment of GFP and hMCM3 cDNA was amplified by the PCR method with an appropriate primer set. The amplified fragment was inserted into the pTrcHis vector (Invitrogen). The induced protein was purified with a nickel-nitrilotriacetic acid superflow column (Qiagen). The fractions eluded with 250 mM imidazole were dialyzed against crude extract with a nickel-nitrilotriacetic acid superflow column. The amplified protein was purified with antibody in 5% non-fat milk in PBS. The bound antibodies were detected with peroxidase-conjugated second antibody (Amersham Pharmacia Biotech) using 1/500 dilution in 5% non-fat milk in PBS, and the protein bands were visualized by the chemiluminescent method with 3,3′-diaminobenzidine as substrate (15).

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The abbreviations used are: Map80, MCM3-associated protein 80; EGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; HA, influenza hemagglutinin monoclonal antibody epitope; MCM3, human MCM3; hMCM3, human MCM3; GFP, fusion HA, influenza hemagglutinin monoclonal antibody epitope; EGFP, nucleotide-binding GTPase family, member D; MCM3, Xenopus MCM3; MCM3, Xenopus MCM3; MCM3, Xenopus MCM3.
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RESULTS AND DISCUSSION

Cloning of Map80 and Sequence Analysis—While screening for proteins that interact with the hMCM3 in a two-hybrid assay, we discovered a novel interacting protein. The newly identified protein, Map80 (MCM3-associated protein 80), is 721 amino acids long (Fig. 1A), with a calculated molecular weight of 80,291 and an estimated pI of 5.14. Comparison of the DNA sequence with sequences in the GenBank™ data base reveals strong homology to the human STS sequences (GenBank™/EBI/DDBJ accession numbers G15578 and G34586). We prepared the antisera for immunoblotting of a HeLa cell extract, in which the HA-tagged Map80 was expressed (Fig. 2B). The anti-HA tag antibody when the HA-tagged Map80 was expressed (Fig. 2B). The anti-HA tag antibody failed to precipitate the hMCM3 in the control cell extract, in which the HA-tagged Map80 was absent (data not shown). These results indicated that Map80 can bind to the intact hMCM3 expressed in HeLa cells.

Furthermore, we examined whether Map80 can bind to the other members of human MCM protein family. Yeast two-hybrid assay showed that the hMCM3 could induce the β-galactosidase activity in the presence of Map80, whereas each of the other MCM proteins, MCM2, MCM5, and MCM7, failed to induce (Fig. 3). These results indicate that Map80 can bind only to the hMCM3 in a two-hybrid system.

Map80 May Be Involved in the Localization Pathway of MCM3—Xenopus MCM3 (XMCM3) was shown not to be imported into the nucleus, in contrast to hMCM3 (18). The amino acid sequences corresponding to the putative NLS region were compared. A cluster of basic amino acid residues, which is upstream from the putative initiation codon (see GenBank™/EBI/DDBJ accession number AB005543), these results indicate that the cDNA obtained encodes the full length of Map80 cDNA.

Map80 Interacts with Intact MCM3—To confirm the binding of Map80 to the hMCM3, Map80 was expressed in HeLa cells as a fusion protein with an amino-terminal HA-epitope tag (Fig. 2A). The hMCM3 was precipitated with the anti-HA tag antibody when the HA-tagged Map80 was expressed (Fig. 2B). The anti-HA tag antibody failed to precipitate the hMCM3 in the control cell extract, in which the HA-tagged Map80 was absent (data not shown). These results indicated that Map80 can bind to the intact hMCM3 expressed in HeLa cells.

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Map80 May Be Involved in the Localization Pathway of MCM3—Xenopus MCM3 (XMCM3) was shown not to be imported into the nucleus, in contrast to hMCM3 (18). The amino acid sequences corresponding to the putative NLS region were compared. A cluster of basic amino acid residues, which is important for the function of the NLS (19), was identified in hMCM3 but not in XMCM3 (Fig. 4A). We prepared “mut. MCM3,” which is the same as hMCM3 except that 4 amino acids have been replaced with the corresponding amino acids of XMCM3 (Fig. 4A), and compared the cellular localization of these three MCM3 proteins, i.e., hMCM3, XMCM3, and mut. MCM3. As shown in Fig. 4B, hMCM3 was localized in the nucleus, whereas XMCM3 was in the cytoplasm, and mut. MCM3 was in both the nucleus and the cytoplasm. The mutagenesis on putative NLS of hMCM3 affected the nuclear localization of hMCM3, indicating that the sequence was responsible for the localization of MCM3. Furthermore, as shown in Fig. 4C, the mutagenesis affected the interaction between Map80 and hMCM3 in a two-hybrid system. Map80 interacted with hMCM3 and weakly with mut. MCM3, but not with...
Fig. 3. Two-hybrid assay of MCM protein and Map80. Map80 and full length of human MCM protein indicated were expressed in yeast SFY526 strain as a fusion protein with an amino-terminal GAL4 activation domain and GAL4 DNA binding domain, respectively. After incubation, the β-galactosidase activity of three colonies was analyzed employing a colony-lift filter assay using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside as substrate.

Fig. 4. The mutagenesis on putative NLS of MCM3 affects the binding of Map80. A, the amino acid sequence of the putative NLS of hMCM3 is compared with the corresponding sequence of XMCM3. An alignment was prepared by using the GENETYX-MAC version 8.0 program. Basic amino acids are indicated in bold type. Identities are indicated by asterisks. The mut. MCM3 indicates the mutation sites contained in the mutant hMCM3 used in the following assay. B, the fragments of hMCM3 corresponding to nucleotides 1715–2233, which encode the putative NLS and of XMCM3 corresponding to nucleotides 1966–2212, were inserted into the pEGFP C2 to be expressed in HeLa cells as a fusion protein with GFP. The mut. MCM3 is the same as the hMCM3 except for containing mutations of the 4 amino acids indicated above. After electroporation of these plasmids into HeLa cells, the cells were cultured for 24–30 h. The cells were observed by confocal microscopy. C, the cDNA of Map80 was inserted into the yeast expression plasmid pGAD424 and expressed in SFY526. The fragments of XMCM3 used above were inserted into the yeast expression plasmid pGBK7 and co-expressed with Map80 in the SFY526. β-Galactosidase activity was estimated by the liquid culture method with o-nitrophenyl-β-D-galactopyranoside as the substrate.

XMCM3. These results suggest that Map80 is necessary for the nuclear localization of hMCM3.

Map80 was expressed in E. coli and purified by several sequential column chromatographies (Fig. 5A). We prepared a fusion protein of hMCM3 and GFP (hMCM3-GFP) as a substrate for the nuclear import assay. The import of hMCM3-GFP was clearly observed in the presence of human red blood cell cytosol, ATP, and GTP (Fig. 5B). The recombinant Map80 alone failed to import the hMCM3-GFP into the nucleus in the absence of cytosol (Fig. 5C). In the presence of smaller amounts of the cytosol, the nuclear import of the hMCM3-GFP became much weaker (Fig. 5D) than the standard (Fig. 5B); however, the recombinant Map80 noticeably facilitated the import even in the presence of smaller amounts of the cytosol (Fig. 5E). These results indicate that Map80 plays a role related to the localization of hMCM3.

We demonstrated that the newly isolated Map80 could bind to the hMCM3 and also indicated that Map80 is involved in the nuclear localization of MCM3. Although Map80 only interacted with MCM3 but not with other MCM proteins (Fig. 3), the data cannot deny the possibility that Map80 participates in the localization mechanism of other MCM proteins, because the MCM proteins are now considered to exist as heterocomplexes (20–23).

Import of karyopheric protein from cytosol to nucleus is mediated by soluble proteins of the importin/karyopherin α and β families (24–29). The importin/karyopherin α binds to both the NLS within the karyopheric protein and importin/karyopherin β, simultaneously, and the importin/karyopherin β interacts with nucleoporins of the cytoplasmic face of a nuclear pore. We could not find any homologous sequence between Map80 and importin/karyopherin (Fig. 1A), whereas the mutagenesis on the NLS of MCM3 affected the binding of Map80 (Fig. 4). Map80 may not participate in the import of MCM3 but stabilize the nuclear localization of MCM3 by interacting with other karyopheric materials, such as chromatin or other karyopheric protein. It is tempting to speculate that Map80 has some function in the nucleus, because a fusion protein of Map80 and DNA binding domain of GAL4 activated the reporter gene transcription in yeast (data not shown).

The MCM protein is identified as a member of the prereplicative complex, licensing machinery, which prevents reinitiation of replication within a single cell cycle (22, 23). Further investigation will clarify whether Map80 also participates in the prereplication complex.

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