The Human Homolog of *Saccharomyces cerevisiae* CDC45*

(Received for publication, March 9, 1998, and in revised form, May 21, 1998)

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In budding yeast *Saccharomyces cerevisiae* CDC45 is an essential gene required for initiation of DNA replication. A structurally related protein Tsd2 is necessary for DNA replication in *Ustilago maydis*. We have identified and cloned the gene for a human protein homologous to the fungal proteins. The human gene CDC45L is 30 kilobases long and contains 15 introns. The 16 exons encode a protein of 566 amino acids. The human protein is 52 and 49.5% similar to CDC45p and Tsd2p, respectively. The level of CDC45L mRNA peaks at G1-S transition, but total protein amount remains constant throughout the cell cycle. Consistent with a role of CDC45L protein in the initiation of DNA replication it co-immunoprecipitates from cell extracts with a putative replication initiation protein, human ORC2L. In addition, subcellular fractionation indicates that the association of the protein with the nuclear fraction becomes labile as S phase progresses. The CDC45L gene is located to chromosome 22q11.2 region by cytogenetics and by fluorescence in situ hybridization. This region, known as DiGeorge syndrome critical region, is a minimal area of 2 megabases, which is consistently deleted in DiGeorge syndrome and related disorders. The syndrome is marked by parathyroid hypoplasia, thymic aplasia, or hypoplasia and congenital cardiac abnormalities. CDC45L is the first gene mapped to the DiGeorge syndrome critical region interval whose loss may negatively affect cell proliferation.

Eukaryotic DNA replication is regulated during the cell cycle so that it occurs in S phase only once per cycle. This regulation occurs at the level of origin firing. In yeast *Saccharomyces cerevisiae*, origin recognition complex (ORC) consisting of six subunits (ORC1–6) binds to specific cis-acting DNA sequences (1, 2). In human, homologs of four of the ORC subunits (Orc1, Orc2, Orc4, and Orc5) have been identified (3, 4). Homologs of ORC proteins have been identified also in other eukaryotes (5). Although ORC subunits are essential for viability of yeast, their constant binding to replication origins throughout the cell cycle suggests that ORC alone cannot be responsible for the restriction of replication to once per cycle. Another protein, CDC6 in *S. cerevisiae* (6, 7) and Cdc18 in *Schizosaccharomyces pombe* (8), is essential for DNA replication and interacts with ORC and cyclin-Cdk. In yeast, CDC6/Cdc18 protein is degraded as the cell cycle progresses through S phase (9, 10). Overexpression of Cdc18 induces re-replication of DNA at S phase in *S. pombe* (11, 12). Homologs of CDC6 have been found in human (13, 14) and other eukaryotes (15). Studies with epitope-tagged human CDC6lp suggest that the human protein is regulated through the cell cycle by changes in subcellular localization (14). The epitope-tagged protein is nuclear in G1 and cytoplasmic in S phase. Like Cdc6p, MCM (mini chromosome maintenance) family of proteins are also implicated in the regulation of initiation of DNA replication. There are six polypeptides in this family (MCM2–7) and homologs identified in human, *Drosophila*, *Xenopus*, and *S. pombe* (5). In yeast, MCM proteins are cytoplasmic except in G1 phase during which the prereplicative complex is formed (16, 17). After mitosis, ORC and Cdc6p recruit MCM proteins to form the prereplicative complex in G1 phase, and DNA replication is initiated upon the activation of the complex by cyclin-Cdk and CDC7 kinases in S phase.

CDC45 is yet another gene whose function is required for the initiation of DNA replication in *S. cerevisiae* (18–21). CDC45 genetically interacts with MCM family members and with ORC2 and physically assembles in a complex containing Mcm5p (18–20). CDC45 protein in yeast is present at a constant level throughout the cell cycle and localized in nucleolus (18). CDC45, MCM, and CDC6 proteins together form a complex necessary for the initiation of DNA replication in eukaryotic cells. CDC45 protein is homologous to Tsd2, a protein that is required for DNA replication in *Ustilago maydis* (22).

We have identified and cloned a human homolog of yeast CDC45 and Tsd2 genes. The CDC45L mRNA level increases during G1-S transition, but the amount of protein is unchanged throughout the cell cycle. Consistent with a role of the protein in the initiation of DNA replication, it is physically associated with human ORC2L protein, and its affinity for a nuclear structure diminishes as DNA replication proceeds during S phase. The gene is located in chromosome 22q11.2 in the minimal region that is deleted in DiGeorge syndrome. DiGeorge syndrome is associated with congenital cardiac abnormalities, hypocalcemia arising from parathyroid hypoplasia, and primary immunodeficiency arising from thymic aplasia. The phenotype may arise from defects in the development of the pharyngeal arches and pouches during embryogenesis (23–25). Several genes have been identified in the minimal region (2 megabases) commonly deleted. These include a putative transcription factor TUPLE1 (TUP-like enhancer of split gene 1), a potential adhesion receptor protein, a serine threonine kinase DGS-G, and a few genes of unknown function. CDC45L is the first gene consistently deleted in DiGeorge syndrome that may be directly involved in cell proliferation.

*This work was supported by Grant CA60499 from the National Institutes of Health and by a grant from the Charlotte Geyer Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF053074.

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MATERIALS AND METHODS

Cloning of CDC45L—The EST data base was searched with *S. cerevisiae* CDC45 nucleotide sequence to look for homologous sequence in human. Two human EST clones had significant matches (T34235 and T31599). The EST clones were obtained from Research Genetics, Inc. Sequencing of the two clones were performed, and the sequences of both clones were the same. The sequence has been deposited in GenBank™ (accession number AF053074) and is the same as another sequence submitted while this manuscript was under review (GenBank™ accession number AJ223728).

Raising Antibody—CDC45L cDNA was cloned into pRSET-C plasmid (Invitrogen) between *Bam*HI and *Xho*I sites, and the protein was expressed as His6 tag in *E. coli*. The overexpressed protein was purified over nickel-agarose affinity column and used for raising polyclonal antiserum in rabbit (Cocalico Biologicals Inc.). For expressing GST-tagged CDC45L in mammalian cells, pEBG-CDC45L was created by cloning the cDNA into *Bam*HI and *Kpn*I sites of pEBG plasmid (26).

Other Techniques—Fluorescence in situ hybridization (FISH) was carried out as described (27) on metaphase chromosome preparations from peripheral blood lymphocytes obtained from normal males and from patients with DiGeorge syndrome known to carry a deletion on one chromosome 21 at the DGCR. HeLa cells were synchronized at mitosis with 50 ng/ml nocodazole (Aldrich) for 24 h. For synchronization in G1-S HeLa cells were blocked with 2 mM thymidine for 12–14 h, released into thymidine-free medium for 12 h, and blocked again with 1 mM hydroxyurea for 12–14 h. The subcellular fractionation was done as described before (28). A *Pst*I and *Xho*I fragment of CDC45L was used to probe the Northern blots.

![Alignment of protein sequences of human CDC45L, budding yeast CDC45, and *U. maydis* TSD2](attachment://alignment.png)

**FIG. 1.** Alignment of protein sequences of human CDC45L, budding yeast CDC45, and *U. maydis* TSD2. The alignment was done using PILEUP program in GCG package. The shading was carried out using GeneDoc program. Identical residues are indicated by dark shading, and similar residues are indicated by light shading. The acidic amino acid-rich region is marked by a solid line, and the putative bipartite nuclear localization signal is shown by a broken line.
RESULTS

Cloning and Sequence Analysis of Human CDC45L—The sequences of the two clones are identical. The 1.86-kilobase cDNA has one long open reading frame, which encodes a protein of 566 amino acids having a theoretical molecular mass of 64 kDa. The polypeptide is highly homologous to CDC45p of S. cerevisiae (27.6% identical and 52% similar) and Ts2p of U. maydis (26.8% identical and 49.5% similar). As shown in Fig. 1, there is significant homology over the entire length of the human protein with CDC45 and Ts2. Like the fungal proteins, the human contains a stretch of acidic amino acids (136–166) and a putative bipartite nuclear localization signal (156–172) (Fig. 1). The newly identified protein has no significant sequence homology with any other protein in the data base. Considering its high homology with the yeast CDC45 and Ts2, we identify the protein as human homolog of the yeast CDC45 (CDC45L).

The mRNA Level of CDC45L Increases at G1-S Transition, but Protein Level Is Unchanged throughout the Cell Cycle—Northern blot analysis of the mRNA from HeLa cells synchronously released from a mitotic block shows that the level of CDC45L mRNA increases at G1-S phase transition (indicated by the increased cyclin E expression and diminished cyclin B expression (29, 30)) and decreases in mitosis (indicated by the increased expression of cyclin B message) (Fig. 2A). GAPDH mRNA serves as the loading control. The polyclonal antiserum produced against bacterially expressed His6-tagged CDC45Lp specifically recognizes the bacterially expressed antigen and recombinant CDC45Lp expressed in Hi5 insect cells by baculovirus infection (Fig. 2B). In mammalian cell extract the antiserum recognizes a 60-kDa protein band close to the theoretical size of CDC45L. Western blot analysis of the protein extracts of HeLa cells at various stages of cell cycle shows that although the cells cycle normally (as indicated by the cyclins A and B), the total level of CDC45Lp is unchanged throughout the cell cycle (Fig. 2C). RPA1 protein is used as a loading control.

Subcellular fractionation of asynchronously growing human osteosarcoma U2OS cells indicated that CDC45L protein is present in both the cytosolic and nuclear fractions. Blocking of cells at G1-S phase by aphidicolin (Fig. 2D, lanes 2 and 5) or hydroxyurea (Fig. 2E, 0 h lane) showed that a significant fraction of the protein was associated with the nuclear fraction. However, blocking of cells in mitosis with nocodazole reveals that the CDC45L protein is now mostly absent from the nuclear fraction (Fig. 2D, lanes 3 and 6). MCM7 protein follows a similar pattern. Thus, like MCM proteins, the affinity of CDC45L protein to a nuclear tether is significantly diminished as DNA replication proceeds.

To follow the decrease in nuclear affinity of CDC45Lp during S phase in greater detail, HeLa cells were released from a hydroxyurea block and fractionated at various time points (Fig. 2F). Immunoprecipitation of CDC45L from nuclear fractions with anti-CDC45L polyclonal antibodies reveals that the CDC45L protein is present in both the nuclear and cytosolic fractions at G1-S phase (Fig. 2F, lanes 1 and 2). However, a significant amount of the CDC45L protein is released from the nuclear fraction at S phase (Fig. 2F, lanes 3 and 4). The latter was further extracted with 0.4 M NaCl and 0.02% Nonidet P-40 containing 20 mM HEPES, pH 7.8, 5 mM potassium acetate, 0.5 mM MgCl2, and 1 mM dithiothreitol. Immunoblot analysis was carried out with the antibodies against the indicated proteins. CDC45Lp was absent in the nuclear insoluble fraction (not shown). E, CDC45L protein is released from the nuclear fraction as S phase proceeds. HeLa cells were released from a hydroxyurea block and fractionated at indicated time points. Anti-CDC45Lp, anti-MCM7p, and anti-proliferating cell nuclear antigen antibodies were used to immunoblot the nuclear soluble fraction.-human protein with CDC45 and Tsd2. Like the fungal proteins,
FIG. 3. FISH with CDC45L cDNA of metaphase chromosome spread from a patient with DiGeorge syndrome. Arrows point to the site of hybridization of the digoxigenin labeled human CDC45L (short tail) and LSI 22q13.3 (long tail) probes. Hybridization was observed with a Zeiss Axiophot microscope and photographs prepared using the Cyto Vision Imaging System (Applied Imaging).

2E). Pulse labeling with $^3$H_thymidine indicates that S phase ends at 8 h after release from hydroxyurea block (not shown). Both MCM7p and CDC45Lp are progressively lost from the nuclear fraction as S phase proceeds. Proliferating cell nuclear antigen present in the nuclear fraction was relatively constant at the various time points and serves as a loading and fractionation control. The MCM7p appears to be lost from the nuclear fraction earlier than CDC45Lp, suggesting that the two proteins are released from the nuclear fraction by different mechanisms. The earlier release of human MCMp relative to CDC45Lp agrees well with the recently reported time of release of CDC7 kinase activate the prereplicative complex to initiate DNA replication and also induce the disassembly of initiation complex so that origins cannot be fired for a second time in the same S phase. The homologs of MCM proteins and four of the ORC proteins have been identified in human and other higher eukaryotes. We and others have identified human CDC45L protein in the initiation of DNA replication.

TABLE I

Sequences of the exon-intron (HSA) CDC45L

| Exon | (bp) | Location |
|------|------|----------|
| I    | (15-59) | 5'UTR-5'-CAGGGCGGCTGAGA-GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA

I). The mRNA is transcribed from 19 exons spanned over a 30-kilobase region in the gene.

DISCUSSION

In budding yeast CDC45 is an essential gene. The genetic and physical interactions of CDC45 protein with several ORC and MCM proteins suggest its involvement in the initiation of DNA replication. In cdc45-1 cells chromosome origins are fired less frequently at nonpermissive temperature. The overall model for initiation of eukaryotic DNA replication is that ORC is bound to replicator origins constitutively. In G1 phase other initiation factors like CDC6, MCM proteins, and CDC45 associate with the ORC at origins to form the licensed prereplicative complex. The S phase-promoting factors cyclin-Cdk and CDC7 kinase activate the prereplicative complex to initiate DNA replication and also induce the disassembly of initiation complex so that origins cannot be fired for a second time in the same S phase. The homologs of MCM proteins and four of the ORC proteins have been identified in human and other higher eukaryotes. We and others have identified human CDC46L. In G1 phase other initiation factors like CDC6, MCM proteins, and CDC45 associate with the ORC at origins to form the licensed prereplicative complex. The S phase-promoting factors cyclin-Cdk and CDC7 kinase activate the prereplicative complex to initiate DNA replication and also induce the disassembly of initiation complex so that origins cannot be fired for a second time in the same S phase. The homologs of MCM proteins and four of the ORC proteins have been identified in human and other higher eukaryotes.

Another intriguing observation is that CDC45L is located at Chromosome 22q11.2, which is frequently deleted in DiGeorge syndrome (DGS). In fact, one copy of CDC45L is deleted in DGS patients (Fig. 3). DGS is a developmental anomaly of the derivatives of third and fourth pharyngeal pouches in the embryo. It is associated with aplasia or hypoplasia of thymus and parathyroid glands and with conotruncal cardiac abnormalities. The majority of patients with DGS have deletion in 22q11. Other syndromes with a similar cytogenetic lesion include Shprintzen syndrome, which is marked by the craniofacial and palatal abnormalities, and Takao syndrome, which has mostly cardiac anomalies. Several genes have been identified in the
DGCR, including a putative transcription factor, a receptor for adhesion molecule, a serine-threonine kinase, and several proteins with unknown functions (25). The presence of a number of genes in the common deleted region and variability in the phenotypes raised the possibility that the phenotype may be attributed to more than one gene encompassed by a deletion. CDC45L is the first gene identified in the DGCR that is directly required for cell division. The loss of one copy of CDC45L selectively impairs cell proliferation in specific tissues during specific development stages. Alternatively, mutations, polymorphisms, or changes in methylation status of the remaining allele of CDC45L may result in limiting quantities of the protein being produced in specific tissues resulting in hypoproliferation and the observed developmental anomalies. At this point we cannot rule out the alternative possibility that CDC45L is a bystander gene, which is deleted because of its close proximity to some other gene whose loss is primarily responsible for the DGS phenotype and that deletion of one copy of CDC45L has no effect on cell proliferation or on development. Future experiments will examine the status of the intact allele of CDC45L in DGS patients and will also be directed at determining the phenotype of mice with only one copy of CDC45L selectively deleted by homologous recombination.

In summary, we have identified a human homolog of budding yeast CDC45p and U. maydis Tsd2p, which are involved in DNA replication initiation. The RNA level of CDC45L increases at the G1-S transition point, but protein level remains constant throughout the cell cycle. However, association of the protein with ORC2L and diminished association with a nuclear tether as S phase proceeds support a role of the protein in the initiation of mammalian DNA replication. The gene is located in DGCR, and one copy of CDC45L is deleted in DGS, raising the possibility that this loss may contribute to the phenotype of DGS.

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