Identification of HsORC4, a Member of the Human Origin of Replication Recognition Complex*

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A new member of human origin recognition complex (ORC) has been cloned and identified as the human homologue of *Saccharomyces cerevisiae* ORC4. HsORC4 is a 45-kDa protein encoded by a 2.2-kilobase mRNA whose amino acid sequence is 29% identical to ScORC4. HsORC4 has a putative nucleotide triphosphate binding motif that is not seen in ScORC4. HsORC4P also reveals an unsuspected homology to the ORC1-Cdc18 family of proteins. HsORC4 mRNA expression and protein levels remain constant through the cell cycle. HsORC4P is co-immunoprecipitated from cell extracts with another subunit of human ORC, HsORC2P, consistent with it being a part of the putative human origin recognition complex.

Initiation of eukaryotic DNA replication involves the controlled and simultaneous firing of numerous sites of initiation. In the budding yeast *Saccharomyces cerevisiae*, these sites are defined by specific sequences recognized by a multisubunit complex, the origin recognition complex (ORC)1–5. All six members of ORC identified in yeast are essential for cell viability (3, 6–11). ORC, in its pre-replicative or in its post-replicative form, is bound to DNA throughout the cell-cycle (1, 2) and could act as a platform for the recruitment of other proteins involved in the replication machinery.

One of the proteins believed to be recruited by ORC before the initiation of DNA replication is the CDC6/Cdc18 (TM) proteins involved in the replication machinery. The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™ Data base (National Center for Biotechnology Information), the partial sequence of a mouse cDNA (AA168456) was deposited with significant homology to a portion of ScORC4 from *S. cerevisiae*. A BLAST search with the AA168456 sequence revealed a homologous sequence human EST W23942, which in turn identified a mouse EST AA110785. A BLAST search with the latter identified a human EST T80329 with an internal portion with significant homology to amino acids 85–121 of ScORC4. T80329 represented the 5’ end of a cDNA clone 25172 (IMAGE, Integrated Molecular Analysis of Genomes and their Expression) obtained from human fetal brain mRNA. This clone was obtained and found to contain a 2.2-kilobase cDNA that corresponds in size to the mRNA detected by Northern blotting. The sequence will be deposited in GenBank™.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession numbers AF029210.

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1 The abbreviations used are: ORC, origin recognition complex; EST, Expressed Sequence Tag; GST, glutathione S-transferase.

Although DNA sequences defining an origin of replication have not yet been identified in higher eukaryotes, two members of a putative ORC complex homologous to yeast ORC1 and ORC2 have been identified so far in mammals, both in humans and in mice (13, 14), suggesting a universal mechanism of initiation of DNA replication in eukaryotes. We report here the identification of a novel member of human ORC homologous to *S. cerevisiae* ScORC4. Cloning the gene for a third member of the human origin recognition complex is an important step toward the ultimate goal of reconstituting the entire human ORC in vitro.

EXPERIMENTAL PROCEDURES

Cloning and Sequencing—In the Expressed Sequence Tag (EST) Data base (National Center for Biotechnology Information), the partial sequence of a mouse cDNA (AA168456) was deposited with significant homology to a portion of ScORC4 from *S. cerevisiae*. A BLAST search with the AA168456 sequence revealed a homologous sequence human EST W23942, which in turn identified a mouse EST AA110785. A BLAST search with the latter identified a human EST T80329 with an internal portion with significant homology to amino acids 85–121 of ScORC4. T80329 represented the 5’ end of a cDNA clone 25172 (IMAGE, Integrated Molecular Analysis of Genomes and their Expression) obtained from human fetal brain mRNA. This clone was obtained and found to contain a 2.2-kilobase cDNA that corresponds in size to the mRNA detected by Northern blotting. The sequence will be deposited in GenBank™.

Antibody, Immunoprecipitation, Immunoblotting—A 1-kilobase NcoI fragment from the cDNA encoding amino acids 57–388 of HsORC4P was cloned into the NcoI site of pRSETA. A 35-kDa fragment of HsORC4P was produced in bacteria fused to a 6-histidine epitope tag, purified on a nickel resin column, and used to raise antibodies in rabbits (Cocalico Biologicals).

Antibody against human ORC2 was raised against a recombinant His-tagged fragment of HsORC2P from amino acids 27–577, created by cloning the XbaI-Sacl fragment of HsORC2 cDNA into the pPOII site of pRSETC.

Where indicated, antibodies were cross-linked to protein A-Sepharose beads with dimethyl pimelimidate for use in immunoprecipitation experiments; immunoprecipitated proteins were released from the cross-linked antibodies with 100 mM triethylamine, pH 11.5, separated by centrifugation, and brought to Laemmli buffer conditions.

To ensure that co-immunoprecipitating proteins were not a result of cross-reacting antibody, interactions were disrupted by boiling the immunoprecipitates in 1% SDS. Samples were then diluted to RIPA buffer conditions and immunoprecipitated again with the same antibodies.

We expressed recombinant HsORC4P fused with glutathione S-transferase (GST) in mammalian cells using the pEBG expression plasmid. Polymerase chain reaction with plaque-forming unit polymerase was used to introduce a BamHI site into the HsORC4 cDNA three nucleotides upstream from the initiator methionine of HsORC4P (GGATCCGAAATG). This BamHI site was used to clone HsORC4 cDNA into the pEBG vector such that the GST coding region was fused in-frame to the HsORC4P coding sequence. 293T cells transiently transfected for 48 h with pEBG or pEBG-ORC4 were lysed, and the expressed GST or GST-HsORC4P was recovered by affinity purification on glutathione-agarose beads. Coprecipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting according to standard protocols.
Identification of Human ORC4

RESULTS AND DISCUSSION

A 45-kDa Human Protein Homologous to Yeast ORC4—Complete sequencing of the cDNA insert revealed an open reading frame encoding a predicted protein of 436 amino acids (Fig. 1), with an approximate molecular mass of 45 kDa. The initiator methionine is preceded by an untranslated leader sequence of 126 nucleotides that contains stop codons in all three reading frames. The alignment of the protein sequence reveals 29% sequence identity to ScORC4 (Fig. 1). We have tentatively identified this clone as human replication origin recognition complex ORC4 (HsORC4). Amino acids 67–73 of HsORC4 contain a putative NTP binding motif (GXXGGKT) (17).

Surprisingly, the sequence of HsORC4 was also significantly related to HsCDC18, SpCdc18, and HsORC1 over areas beyond the nucleotide binding motif (Fig. 2). Nearly 28% of HsORC4 residues were identical to at least one of the three Cdc18/ORC1 sequences shown and have been represented above the alignments as a “consensus sequence.” The identities of HsORC4P with ORC1 and Cdc18 extended over the whole length of the protein, including the six boxes of high homology noted between the Cdc18/ORC1 proteins. Comparing Figs. 1 and 2 suggests that ScORC4 contains several of the amino acids that in HsORC4 are related to the Cdc18/ORC1 family. One critical difference, however, is that ScORC4 does not contain the canonical nucleotide binding motif, although ScORC5 has been reported to contain such a sequence motif. Human ORC5 has not yet been identified. It is also clear that the Cdc18/ORC1 molecules are more closely related to each other (especially over the six boxes of homology) than to ORC4 and that the identity of HsORC4P with the HsORC1p or the HsCdc18p is lower (around 17%) than the identity with the budding yeast ORC4P (29%).

Antibodies to HsORC4P and to HsORC2P—To study the ORC4 and ORC2 proteins, we raised polyclonal rabbit antibodies to recombinant fragments of the two cloned genes. Fig. 3A shows that immunoprecipitation of cell lysates with the anti-ORC2 antibody (lane 2) isolates a 72-kDa polypeptide that can be detected by immunoblotting with the same antibody. This size is consistent with that reported for HsORC2P. To confirm the specificity of the anti-ORC2 antibody, we expressed Myc epitope-tagged ORC2P by transient transfection of 293T cells with pA3M-ORC2 and immunoprecipitated cell lysates with pre-immune, anti-ORC2, and anti-Myc epitope antibodies (lanes 3–5). The precipitates were immunoblotted with anti-ORC2 antibody. Addition of the Myc epitope to ORC2 produced a longer protein of about 76 kDa, which was precipitated by anti-ORC2 and by anti-Myc antibodies. Thus the anti-ORC2 antibody recognizes ORC2 protein in both immunoprecipitation and immunoblotting reactions.

Fig. 3B shows the specificity of the anti-ORC4 antibody. In this case, endogenous 45-kDa HsORC4 protein was detected by direct immunoblotting of cell lysates (lane 2). In most experiments, a doublet of 45 kDa is seen that could be due to post-translational modifications or to partial proteolysis of the protein. When GST-HsORC4P was expressed in 293T cells (Fig. 3B), a new 66-kDa protein was detected by the anti-ORC4 antibody. The size of the new protein and its affinity for glutathione-agarose beads (see below, Fig. 4B) suggests that it is GST-ORC4. This result also proves that the anti-ORC4 antibody recognizes the protein encoded by the cloned HsORC4 cDNA.

Lysates from different numbers of HeLa cells were compared with different amounts of purified recombinant HsORC4 by immunoblotting with affinity-purified anti-HsORC4 antibody. Comparison of the relative intensities of signal indicates that approximately 5 × 106 HsORC4 molecules may be present per HeLa cell (data not shown).

ORC4 and ORC2 Are Each Associated with Multiple Cellular Proteins—293T cells were metabolically labeled with [35S]methionine and harvested 4 h after reinoculation. The cell cycle stages of synchronous cells were obtained by nocodazole shake-off as described above and harvested 4 h after re-inoculation. The cell cycle-specific cyclin B.

Cell Culture—Human 293T embryonic kidney cells or HeLa cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% donor calf serum. Transfections were done by the standard calcium phosphate method. For metabolic labeling of proteins, 293T cells were incubated in methionine-free medium for 4 h. Then 200 µCi of [35S]methionine (NEN Life Science Products) was added, and cells were incubated for 6 h before harvesting.

Cell-staged human cell cultures were prepared from exponentially growing HeLa cells. Cells were arrested in M phase with 40 ng/ml nocodazole for 18 h. Motic cells were then selected by shake-off, washed twice in warm PHEM buffer (60 mM PIPES, pH 6.8, 25 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM dithiothreitol, 0.1 mM pepstatin, 50 mM NaF, 1 mM Na3VO4) at 4 °C. Where indicated, 200 µg/ml leupeptin, 50 mM Na3VO4, 1 mM Na3VO4, 10 mM hydroxyurea (S) or 40 ng/ml nocodazole (M) for 18 h. G1 release. Alternatively, cells were blocked at different cell cycle phases with fresh medium, and harvested at indicated time points after re-inoculation. The cell cycle stages of synchronous cells were obtained by nocodazole shake-off as described above and harvested 4 h after re-inoculation.

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ORC4 and ORC2 Are Each Associated with Multiple Cellular Proteins—293T cells were metabolically labeled with [35S]me-
thionine, and cellular proteins were immunoprecipitated with anti-ORC4 and anti-ORC2 antibodies (Fig. 4A). The anti-ORC4 antibody immunoprecipitated a doublet of 45 kDa and also precipitated polypeptides of 100, 72, 35, and 31 kDa (lanes 2 and 5). With the exception of the 72-kDa band, the intensities of the other three bands are significantly lower than the 45-kDa band. This can be for different reasons. They can be interacting in substoichiometric amounts, they may not be recovered quantitatively, or they can have a slow rate of synthesis. The 45-kDa protein co-migrated with a 45-kDa polypeptide produced by in vitro transcription translation of the Hs-ORC4 cDNA (not shown). When the immunoprecipitate was denatured and re-precipitated with anti-ORC4 antibody, only the 45-kDa protein was re-precipitated (lane 3). Taken together with the immunoblotting results in Fig. 3B, the ORC4 antibody appears to directly recognize only the 45-kDa HsORC4 protein and co-precipitate the other cellular polypeptides because they are associated with HsORC4.

The anti-ORC2 antibody precipitates a protein of an apparent molecular mass of 72 kDa (Fig. 4A, lane 7), which was the only protein re-immunoprecipitated by the same antibody after denaturation of the proteins (lane 8). Additional polypeptides of 45, 55, 80, and 100 kDa were reproducibly present in the anti-ORC2 immunoprecipitates and are likely to represent proteins associated with HsORC2P. The higher molecular mass polypeptides are better resolved in lane 10.

HsORC4P and HsORC2P Are Associated with Each Other in Vivo—To demonstrate that the 72-kDa protein present in the ORC4 immunoprecipitate was indeed ORC2 and the 45-kDa protein coimmunoprecipitating with ORC2 was ORC4, we repeated the immunoprecipitations with antibodies covalently cross-linked to protein A-Sepharose beads.
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Fig. 3. Antibodies against HsORC2P and HsORC4P. A, lysates from 293T cells, untransformed (lanes 1 and 2) or transformed with pA3-ScORC2 (lanes 3–5), were immunoprecipitated with rabbit preimmune (Pre) or anti-HsORC2 antisera (lanes 1 and 3 and lanes 2 and 4, respectively) or with anti-Myc epitope mouse monoclonal antibodies (lane 5). All immunoprecipitates were immunoblotted with the rabbit anti-HsORC2 antiserum. B, cell lysates from pEBG-transfected (lanes 1 and 2) or pEBG-HsORC4-transfected (lanes 3 and 4) 293T cells immunoblotted with rabbit anti-HsORC4 antiserum. Two bands of apparent molecular mass around 45–47 kDa are specifically seen in the lysates probed with the immune antiserum. An additional band of apparent molecular mass around 66 kDa, compatible with GST-HsORC4P, was detected in the lysates from cells transfected with pEBG-HsORC4.

Fig. 4. Cellular proteins coimmunoprecipitating with HsORC2P or with HsORC4P. A, [35S]methionine-labeled 293T cell lysates were immunoprecipitated with the following sera: anti-ORC4 (lanes 2, 3, and 5) or corresponding preimmune (pre) (lanes 1 and 4); anti-ORC2 (lanes 7, 8, and 10) or corresponding preimmune (lanes 6 and 9). Lanes 3 and 8 show the result of re-immunoprecipitation with the ORC antibodies under denaturing (denat.) conditions aimed to disrupt protein-protein interactions (see “Experimental Procedures”). Lanes 4 and 5 are an overexposure of the lower half of 1 and 2, to demonstrate the 35- and 31-kDa co-precipitating proteins. Lanes 9 and 10 are the same as 6 and 7, except that they were run longer to resolve the bands in the 70–100 kDa area. B, unlabeled 293T cell lysates were immunoprecipitated (IP) with anti-HsORC4 antibodies and immunoblotted with anti-HsORC2 antibodies (lane 2, top panel). Similarly, lane 2 in the bottom panel displays the immunoprecipitation results with anti-HsORC2 probed with anti-HsORC4 antibodies. The identical coimmunoprecipitation of HsORC2P and HsORC4P seen in the presence of 200 μg/ml ethidium bromide (EtBr) (lane 3) supports a protein-protein interaction not mediated by DNA. The results of glutathione-agarose bead purification of pEBG-HsORC4P-transfected (lane 4) or pEBG-transfected (lane 5) 293T cells is shown after anti-HsORC2 immunoblotting (top panel) or after Ponceau S staining (bottom panel).

In summary, we report here the identification of a third member of the human replication origin recognition complex homologous to ScORC4, after which we name this gene HsORC4. The homology observed between human ORC4 and human Cdc18 and S. pombe Cdc18 supports a protein-protein interaction not mediated by DNA. The results of glutathione-agarose bead purification of pEBG-HsORC4P-transfected (lane 4) or pEBG-transfected (lane 5) 293T cells is shown after anti-HsORC2 immunoblotting (top panel) or after Ponceau S staining (bottom panel).

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anti-ORC2 immunoprecipitates (data not shown). This negative result may be explained by HsORC1P being recovered in substoichiometric amounts or by the anti-ORC2 or anti-ORC4 antibodies specifically disrupting the association of ORC1 with the ORC2-ORC4 subcomplex.

Examination of the HsORC4P-associated proteins and HsORC2P-associated proteins reveals another conundrum. Although the two proteins are clearly associated with each other, most of the other proteins present in the respective immunoprecipitates are different. HsORC4P is coimmunoprecipitated with proteins of 100, 35, and 31 kDa in addition to HsORC2P. However, HsORC2P is coimmunoprecipitated with proteins of 100, 80, and 55 kDa besides HsORC4P. It is possible that all these proteins are part of human ORC and have been dissociated during cell lysis or immunoprecipitation into two subcomplexes. Alternatively, the proteins associated with HsORC4P and HsORC2P are not members of human ORC but are other accessory proteins involved in functions different from the initiation of DNA replication. These possibilities can be resolved only after the molecular identification of the coprecipitating proteins.

In humans, the number of origins has been estimated to be about 3–5 × 10⁴/cell on the basis of the observed spacing between active origins of about 100 kilobases (19). Although the estimate of HsORC4P (5 × 10³/cell) is very approximate, comparison of the two estimates suggests either of the following two cases. Human cells may contain two pools of HsORC4P, only one of which is in ORC capable of activating origins of DNA replication. Alternatively, all the HsORC4P is in ORC bound to DNA, but only a fraction of these sites are active as origins of DNA replication, consistent with the Jesuit model (many are called, but few are chosen) of origin selection.

HsORC4 is the third ORC member identified in humans, after HsORC1 and HsORC2 (13). Discovery of ORC in yeast was made possible by the identification of specific DNA sequence elements that constitute the replicator (reviewed in Ref. 6). Identification of analogous sequences in higher eukaryotes has so far remained elusive. We foresee the discovery of additional human ORC members, so that ultimately it should be possible to work in the reverse direction to determine whether human ORC binds specific DNA sequences and whether such sequences constitute true human replicators.

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