Functional domains involved in the interaction between Orc1 and transcriptional repressor AlF-C that bind to an origin/promoter of the rat aldolase B gene.

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Functional domains involved in the interaction between Orc1 and transcriptional repressor AlF-C that bind to an origin/promoter of the rat aldolase B gene

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ABSTRACT

The promoter of the rat aldolase B (AldB) gene functions in vivo as an origin of DNA replication in the cells in which transcription of the gene is repressed. Previously, we identified two closely related DNA-binding proteins, AlF-C1 and AlF-C2, which repressed the AldB gene promoter. We also reported that the binding site of these proteins, site C, is one of the required DNA elements of the AldB gene origin/promoter for autonomously replicating activity in transfected cells. In the present study, we show that AlF-C1 and AlF-C2 bind directly to Orc1, a subunit of the origin recognition complex (ORC). Deletion analyses revealed a functional domain in AlF-C2 for binding to Orc1, which is located separately from the DNA-binding domain. In addition, we found a novel protein-interacting domain in Orc1 required for the binding of AlF-C2, which was conserved in human, mouse and Chinese hamster, but not in Drosophila, frog and yeast. Thus, it is assumed that in mammalian cells, sequence-specific DNA-binding proteins are involved in recruiting ORC to regulate replication initiation and/or transcription repression.

INTRODUCTION

It has been generally accepted that origins of DNA replication in higher eukaryotes do not share common specific sequences. Nevertheless, the positions of origins are not distributed randomly on the chromosomal DNA (1). This origin spacing might involve recognition of specific sites by the origin recognition complex (ORC), a six-subunit protein, the role of which is to mark and activate origins (2). ORC subunits are highly conserved from yeast to plant to mammalian cells, indicating the conserved functions throughout the species (3). In budding yeast, ORC directly binds to a specific sequence called autonomously replicating sequence (ARS) consensus sequence (ACS) (4). However, in higher eukaryotes there is no direct evidence, to our knowledge, that ORC binds directly to specific DNA sequences while it is necessarily required for replication initiation. Therefore, what directs ORC to specific sites spaced at appropriate intervals is currently important to understand mechanisms operating in initiation of DNA replication. The aim of the present study is to investigate this point.

The promoter of the rat aldolase B (AldB) gene acts bifunctionally in vivo, but these functions are mutually exclusive; it functions as an origin of DNA replication in the rat hepatoma cells in which the gene is repressed, while in differentiated hepatic cells it functions as a cell type-specific transcription promoter (5,6). Our previous works identified two closely related proteins, AlF-C1 and AlF-C2, which bind to site C in the origin/promoter and are involved in the repression of the AldB gene (7). In addition, we showed that the origin/promoter fragment directs autonomous replication when transfected in Cos1 cells in a plasmid form. Site C is one of the necessarily required DNA elements for such an autonomously replicating activity (8). Therefore, we are interested in the association of AlF-C1 and AlF-C2 with ORC that might lead to the initiation of replication. In this report, we show that AlF-C1 and AlF-C2 bind directly to Orc1, a subunit of ORC, and discuss that in mammalian cells, sequence-specific DNA-binding proteins might be involved in recruiting ORC to regulate replication initiation and/or transcription repression.

MATERIALS AND METHODS

Plasmids

The plasmids carrying human ORC subunits were constructed as follows. The BamHI–NotI fragment of pGEX-ORC1 (9) was subcloned into the BamHI–NotI site of pcDNA3.1/HisC (Invitrogen) (referred to as pcDNA3.1/Orc1), the SmaI–NotI fragment of pGEX-ORC2 (9) into the EcoRV–NotI site of pcDNA3.1/HisA (Invitrogen) (pcDNA3.1/Orc2), the BamHI–

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Expression and purification of glutathione S-transferase (GST) fusion proteins

Recombinant Alf-C1, Alf-C2 and various Alf-C2 deletion mutants from pGEX vector were each expressed as a fusion protein with GST by culturing Escherichia coli BL21(DE3, pLysS) cells carrying pGEX/Alf-C1, pGEX/Alf-C2 and pGEX/Alf-C2 deletion mutants with 0.1 mM isopropyl-β-D-thiogalactopyranoside for 0.5–3 h at 20–37°C. The cells were collected and suspended in phosphate-buffered saline (PBS) containing 0.5% NP-40 and frozen at −80°C. The cell suspension was thawed on ice, treated with 12 U/ml DNase I (Takara) for 10 min. The cell lysate was cleared by centrifugation at 14 000 g for 30 min and mixed with glutathione–Sepharose 4B (Amersham) for 1 h on a rotary shaker at 4°C. The beads were washed four times with PBS containing 0.5% NP-40 before elution with 50 mM Tris–HCl (pH 9.6) containing 20 mM reduced glutathione. The eluted fraction was dialyzed against 20 mM Tris–HCl (pH 7.5) containing 20% glycerol, 20 mM NaCl, 0.2 mM EDTA, 1 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride. Protein concentrations were determined by comparison with

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The GST-fused proteins (200 ng) and 3 ng of 32P-labeled site C probe (labeled with [α-32P]dCTP by Klenow fragment) were incubated in a final volume of 15 μl containing 10 mM Tris–HCl (pH 7.9), 100 mM KCl, 0.5 mM EDTA, 2 mM dithiothreitol, 0.5 mg/ml RNase A, 10% glycerol and 33 μg/ml poly(dI–dC) for 40 min on ice. The DNA–protein complexes were resolved on 5% polyacrylamide gels in TBE buffer. The probe and competitors used are: site A, 5'-CAAT-CAGATTATGAATAAACACCTTC-3'; site C, 5'-GTG-AGCCTGATTACAAAGATTGGCTGTTCAC-3'.

RESULTS

AIF-C1 and AIF-C2 proteins interact with Orc1
To examine whether AIF-C proteins interact with ORC, we carried out a GST pull-down assay. AIF-C1 and AIF-C2 proteins were synthesized in E.coli as fusion proteins of GST and affinity-purified by glutathione–Sepharose. We employed these fusion proteins as a bait for probing T7-tagged human ORC subunits translated in vitro. Binding assays were carried out in 80 mM KCl, and the proteins bound to glutathione–Sepharose via GST–AIF-C1 or GST–AIF-C2 were eluted and detected by western blotting using an anti-T7 tag antibody (Fig. 1A and B). Under this condition, non-specific binding of T7-tagged lacZ to GST–AIF-C1 was not observed (Fig. 1C, lane 2). Of the four ORC subunits tested, Orc1 specifically bound to both AIF-C1 and AIF-C2 proteins but not to GST alone (Fig. 1A and B, lanes 1 and 2). Orc2 and Orc4 bound to neither AIF-C1 nor AIF-C2 (Fig. 1A and B, lanes 3 and 5). A weak interaction of Orc5 with AIF-C proteins was observed (Fig. 1A and B, lane 7). However, the amount of Orc5 in the supernatant was much greater than that of Orc1. Significantly greater quantities of Orc1 in the bound fraction (Fig. 1A and 1B, lanes 1 and 7) suggested that the affinity of Orc1 to AIF-C is much higher than that of Orc5. We then examined the interaction of GST–AIF-C1 and T7-Orc1 under increasing concentrations of KCl (Fig. 1C) to examine the stability of interaction. At 80–200 mM KCl, stable binding of Orc1 to AIF-C1 was detected (lanes 1, 3 and 5). However, the only detectable amount of binding was observed at 250 mM KCl (lane 7). Under these conditions, non-specific binding of T7-tagged lacZ to GST–AIF-C1 was not observed (Fig. 1C, lanes 2, 4, 6 and 8). These results clearly indicated that Orc1

Figure 1. Orc1 interacted with AIF-C1 and AIF-C2. AIF-C1 (A) and AIF-C2 (B) were expressed as GST-fusion proteins in E.coli and purified. The GST-fusion proteins (lanes 1, 3, 5 and 7), or GST alone (lanes 2, 4, 6 and 8), were bound to the glutathione beads. The fusion-protein-bound glutathione beads were incubated in in vitro translated T7-tagged Orc1 (lanes 1 and 2), Orc2 (lanes 3 and 4), Orc4 (lanes 5 and 6) and Orc5 (lanes 7 and 8). After extensive washing, the protein bound to the beads were separated on an SDS–PAGE and blotted with an anti-T7 antibody (upper panel). The 1/10 volume of unbound fractions (supernatant) was also analyzed (lower panel). Arrows indicate the positions of Orc1, Orc2, Orc4 and Orc5. (C) The GST-fused AIF-C1 protein and T7-tagged Orc1 (lanes 1, 3, 5 and 7), or lacZ (lanes 2, 4, 6 and 8) were incubated with 80 mM (lanes 1 and 2), 150 mM (lanes 3 and 4), 200 mM (lanes 5 and 6) and 250 mM (lanes 7 and 8) KCl, and their binding activities were tested as in (A). Arrows indicate the positions of Orc1 and lacZ.
directly bound to AlF-C1 and AlF-C2 proteins with a relatively strong binding affinity.

**Orc1 domain responsible to AlF-C2 binding**

To define the domain of Orc1 required for the interaction with AlF-C2, a series of T7-tagged Orc1 deletion mutants were translated in vitro, and the products were applied to the GST–AlF-C2-coupled glutathione beads. The Orc1 mutant proteins bound to the beads via GST–AlF-C2 were eluted and detected as described in Figure 1 (Fig. 2). As shown in Figure 2A (lane 1), full length Orc1 protein [Orc1(1-862)] bound to AlF-C2. Orc1(1-208) containing BAH (bromo-adjacent homology) domain (11), did not bind to AlF-C2 (Fig. 2A, lane 2), whereas Orc1(1-239), Orc1(1-278) and Orc1(210-511) bound (Fig. 2A, lanes 3–5). Orc1(512-862), which contains Cdc6 homology region and NTP-binding domain (Fig. 2B), did not bind to AlF-C2 (Fig. 2A, lane 6). These results indicated that amino acids 210–239 in Orc1 were required for the interaction with AlF-C2 (Fig. 2B). Comparison of amino acid sequences of Orc1 from different species revealed that the amino acids 210–239 were conserved among human, mouse and Chinese hamster, but not in yeast, *Xenopus laevis* and *Drosophila* (Fig. 3).

**AlF-C2 domain responsible to Orc1 binding**

AlF-C1 and AlF-C2 share an identical amino acid sequence except for a 47-residue-insertion in AlF-C1 (7). As described above, both proteins bound to Orc1 with strong affinity. In order to clarify AlF-C1 and AlF-C2 domains required for binding to Orc1, the AlF-C2 deletion mutants were similarly examined by in vitro pull-down assays (Fig. 4).

A series of deletion mutants of AlF-C2 were prepared as GST-tagged proteins (Fig. 4B), and each mutant protein was individually bound to glutathione beads. To these beads, T7-tagged Orc1 (full length) was added and the amount of Orc1 bound to the mutant AlF-C2 proteins was determined by western blotting using anti-T7 tag antibody. In these experiments, in vitro translated T7-tagged Orc2 was included along with Orc1 protein in the binding reactions, as Orc2 could not bind to AlF-C1 and AlF-C2. Interaction of Orc2 with any of the AlF-C2 deletion mutants was not observed (Fig. 4A, upper panel), indicating that non-specific binding of in vitro translated Orc2 protein to AlF-C2 did not occur under our experimental condition. The results further confirm specific interaction between Orc1 and AlF-C proteins.

Deletion of C-terminal amino acids 207–284 [AlF-C2(1-206)] abolished the Orc1 binding (Fig. 4A, lane 1). In contrast, AlF-C2(207-284) containing C-terminal amino acids 207–284 (Fig. 4B) showed binding to Orc1 (Fig. 4A, lane 2). Then, a series of binding assays for N-terminal deletion mutants of AlF-C2(207-284) were performed (Fig. 4B). AlF-C2(207-284) and AlF-C2(249-284) bound to Orc1 (Fig. 4A, lanes 3 and 4), but AlF-C2(264-284) did not (Fig. 4A, lane 5). The results indicated that amino acid sequence 249–263 in AlF-C2 was a prerequisite for the
interaction with Orc1 (Fig. 4B). We also constructed a series of C-terminal deletion mutants from AlF-C2(207-284) (Fig. 4B). AlF-C2(207-272) and AlF-C2(207-263) bound to Orc1 (Fig. 4A, lanes 6 and 7). However, AlF-C2(207-238) abolished the Orc1-binding activity (Fig. 4A, lane 8). Taking these results together, amino acids 249–263 in AlF-C2 were responsible for the interaction with Orc1.

**AlF-C2 domain responsible for sequence-specific DNA binding**

AlF-C1 and AlF-C2 have been identified as sequence-specific DNA-binding proteins (7). To define the region of AlF-C proteins responsible for bind site C DNA, a series of deletion mutants of AlF-C proteins were prepared as GST-fusion proteins (Fig. 5A) and these fusion proteins were subjected to EMSA with site C DNA as a probe (Fig. 5B).

As shown in Figure 5B, both AlF-C1 and AlF-C2 proteins bind to site C DNA (lanes 1 and 4). The GST tag alone yielded no shifted band (data not shown). In competitive binding assays, the addition of 100-fold molar excess amount of non-labeled site C DNA abolished the shifted band (Fig. 5B, lanes 2 and 5), while addition of the same amount of site A DNA of the AldB promoter did not (Fig. 5B, lanes 3 and 6). Therefore, both AlF-C1 and AlF-C2 bound sequence-specifically to site C DNA. It should be noted here that, in our previous paper, we described that the AlF-C2 protein expressed in *E. coli* without tag bound to site C only weakly (7). In this work, we expressed GST-tagged AlF-C1 and AlF-C2 as soluble proteins in *E. coli*. In the former study, AlF-C proteins without tags were expressed in the inclusion body of *E. coli*, solubilized with guanidine–HCl and renatured by dialysis. Thus, it is possible that denaturation and renaturation caused lowered binding activity of AlF-C2 to site C.

A series of deletion mutants of AlF-C2 were prepared as GST-fusion proteins and subjected to EMSA. AlF-C2(1-206), AlF-C2(1-179) and AlF-C2(1-159) bound to site C DNA (lanes 7, 10 and 13). The addition of non-labeled site C DNA efficiently diminished the shifted bands (lanes 8, 11 and 14), while site A DNA did so only partially (lanes 9, 12 and 15). In contrast, AlF-C2(1-146) did not bind to site C (lanes 16). Thus, the amino acids 147–159 were indispensable for sequence-specific binding to site C. Protein motif search with Blocks (12) identified HMG14/HMG17 non-histone chromatin protein family motif (Block name: IPB000079) in 129–159 and histone H5 signature (Block name: PR00624E) in 141–155. Amino acids 129–159 of AlF-C2 were rich in basic residues (32%). Moreover, 46% (6/13) were basic in 147–159. Thus, basic amino acids in 147–159 might be important for binding to DNA.

To clarify the role of the N-terminal region, we constructed N-terminal truncation mutants from AlF-C2(1-159) (Fig. 5A). AlF-C2(59-159) and AlF-C2(67-159) bound to site C (lanes 19 and 22) and their sequence-specific binding was confirmed by competitive assays (lanes 20–21 and 23–24). In contrast, AlF-C2(76-159) did not bind to site C (lane 25). Therefore, the amino acids 67–75 in AlF-C2 were also important for binding to site C. Protein motif search with Blocks identified a 7 kDa DNA-binding protein motif (Block name: IPB003212B) in amino acids 71–96. The 7 kDa DNA-binding proteins were small abundant DNA-binding proteins in thermoacidophilic archaeon (13).

Taking these results together, a stretch of amino acids 67–159 in AlF-C proteins is responsible for the interaction with site C DNA, and amino acids 67–75 and 147–159 are indispensable. In the region from position 76 to 146, the Pfam protein families’ database search (14) identified the RNA recognition motifs (RRM). AlF-C proteins contain two RRM motifs at 76–146 and 160–230 (Fig. 5A). However, deletion of the latter RRM did not affect the DNA-binding activity.

**DISCUSSION**

In this work, we found that Orc1 directly bound to novel growth-regulated DNA-binding proteins, termed AlF-C1 and AlF-C2, which represses the AldB gene. AlF-C1 and AlF-C2 are members of a hnRNP family that have two RNA-binding domains (RRM and also RBD) (7). Although hnRNPs were first described as a major group of chromatin-associated RNA-binding proteins (15), recent studies revealed that they are involved in various biological functions, including repression...
of viral DNA replication (16), repression of estrogen- (17) and vitamin D-induced transcription (18) and transcriptional regulation of various genes (19–24). In addition, hnRNP, which binds telomeric DNA and is involved in mammalian telomere biogenesis, has been reported (25). The scaffold attachment factor A (SAF-A) and P130, components of the nuclear scaffold, are also members of hnRNP (26,27). Another hnRNP, MSSP, which binds to an origin of replication in the e-myc gene, interacts with DNA polymerase α and stimulates its activity (28). Usually, hnRNP proteins contain two to three RRMs, and amino acids outside of RRM are variable (29,30). The variable region seems to be important to confer different functions of hnRNP proteins.

AIF-C1 and AIF-C2 are novel members of hnRNP family, in that they have separate domains to bind DNA sequence-specifically, to bind Orc1, and to repress transcription. These features of hnRNP have not been reported to date. We demonstrated that a stretch of amino acids 67–159 in AIF-C2 (and AIF-C1) was required for the interaction with site C DNA. Although this region contains RRM (amino acids 76–146), our study clearly indicated that RRM alone was not sufficient for DNA binding, but required flanked variable regions. However, both of the two RRMs in AIF-C2 are not required. This is contrasting to the MSSP protein, which requires two RRMs for all of its functions (31).

To the binding of Orc1, the amino acid sequence 249–263 of AIF-C proteins was shown to be responsible, which was not found in other hnRNPs except for closely related AIF-C proteins found in rat, mouse and human. This sequence is rich in Gly and Arg. Interestingly, a similar sequence was also found in the Epstein–Barr (EB) virus EBNA1 protein that binds to the replication origin of the viral DNA and activates DNA replication. Deletion of Gly- and Arg-rich sequence in EBNA1 protein resulted in decreased replication activity (32). In addition, it has recently been shown by immunoprecipitation experiments, that EBNA1 and Orc1 are co-precipitated as a complex (33). Although direct Orc1-EBNA1 interaction was not strictly proven, it might be possible that Gly- and Arg-rich sequences in EBNA1 and AIF-C proteins are important for interaction with Orc1.

Selection of replication origins in Saccharomyces cerevisiae is mediated by the direct binding of ORC (34). In contrast, studies of metazoan ORCs have been less clear as to specific ORC-binding sequences (2). Recently, chromatin immunoprecipitation assays with antibodies against human Orc1 and Orc2 demonstrated that an Orc1- and Orc2-binding region on the chromatin coincided with an origin of DNA replication (35). However, direct binding of any metazoan ORC subunits to origin DNA has yet to be reported.

The way in which ORC interacts with chromatin differs between yeast and metazoan cells. Yeast ORC is stably associated with the chromatin throughout the cell cycle (36,37), whereas the interaction of metazoan ORC with chromatin changes during the cell cycle. Metazoan Orc1 is selectively released from chromatin during M phase (38) and S phase (39). Ubiquitination of the released mammalian Orc1 is sequestered from re-association (40,41). ORC activity is restored during M to G1 phase, which is concomitant with the reappearance of Orc1 tightly bound to chromatin, and with formation of pre-RCs at specific genomic sites (38,42,43). Therefore, mammalian Orc1 is thought to play a key role in such a cell cycle-regulated ORC activity for replication initiation.

Comparison of Orc1 among 11 different species, including single cell to Metazoa, revealed 52% similarity in the C-terminal regions that contain homologies with Cdc6 protein and the AAA+ family of ATPases, while exhibited 35% overall similarity (44). Presumably, the highly conserved C-terminal region of Orc1 is that involved in its functions conserved in all those species. The BAH domain which is identified in eukaryotic DNA methyltransferases and several proteins involved in transcription regulation, locates in the N-terminal region of Orc1 (11). In contrast, the middle part of the Orc1 proteins is highly variable among species, and thus it is conceivable that the middle part functions in species-specific mode of origin recognition. Accordingly, we found an Orc1 domain responsible for the interaction with AIF-C2 in the highly variable region. The amino acid sequence of this domain is conserved among human, mouse and Chinese hamster, but not in Drosophila, X. laevis and yeast (Fig. 3). This sequence is also conserved in rat (data not shown).

Likewise, the amino acid sequence required for binding to Orc1 was perfectly conserved among rat, human and mouse AIF-C homologs (7). Therefore, it is considered that the interaction of Orc1 and AIF-C or AIF-C homologs occurs only in mammals.

It has been reported that human Orc2, Orc3, Orc4 and Orc5 form a core complex and that the interaction of Orc1 with the core complex is labile or cell-cycle dependent (45,46). Although we showed the direct interaction of Orc1 with AIF-C proteins (Fig. 1), we do not know at present whether AIF-C proteins can bind to Orc1 and subsequently associate with Orc2–5 complex, as such described above. Further experiments in vitro and in vivo are needed to answer the question to this point.

Concerning the origin selection by ORC, the ARS consensus sequence plays a large part in S. cerevisiae (34). In Schizosaccharomyces pombe, Orc4 is solely responsible for selection of specific ORC-binding sites; it binds to AT-rich sequences in the origin through its nine AT-hook motifs (47,48). In this view, the presence of an AT-rich sequence juxtaposed to the AIF-C-binding site at the rat AldB gene origin is of great interest; the AT-rich sequence has been shown to be required for ARS activity of the origin sequence (8). It is also interesting to consider that an unknown AT-hook protein might also be involved in recruiting ORC to the origin region of the rat AldB gene.

Unlike yeast S. cerevisiae, origins of DNA replication do not share common specific sequences in mammalian cells. If mammalian ORC does not have the ability to select origins based solely on its own affinity for specific consensus DNA sequence, how does it become localized to origins of replication? As mentioned above, a role for sequence-specific DNA-binding factors in the recruitment of ORC to specific genomic loci is currently suggested. It has been reported that the viral transcription factor EBNA1 binds to the EB virus origin of replication and recruits ORC to specific DNA-binding factors would be involved in recruiting ORC to their selected origins. It is interesting to note here that the AIF-C1 and AIF-C2 proteins bound to site C in the AldB origin/
promoter (7), which was one necessarily required element for autonomous replication in transfected cells (8). The association of AIF-C proteins with Orc1 implied that AIF-C proteins play a role in the recruitment of ORC to the replication origins in mammalian cells, similar to EBNA1 protein.

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REFERENCES


