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Dephosphorylation of Orc2 by protein phosphatase 1 promotes the binding of the origin recognition complex to chromatin

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ABSTRACT

Phosphorylation of Orc2, one of the six subunits of the origin recognition complex (ORC), by cyclin A/CDK2 during S phase leads to the dissociation of Orc2, Orc3, Orc4, and Orc5 subunits (Orc2–5) from human chromatin and replication origins. Dephosphorylation of the phosphorylated Orc2 by protein phosphatase 1 (PP1) is accompanied by the binding of the dissociated subunits to chromatin. Here we show that PP1 physically interacts with Orc2. The binding of PP1 to Orc2 and the dephosphorylation of Orc2 by PP1 occurred in a cell cycle-dependent manner through an interaction with 119-KSVSF-123, which is the consensus motif for the binding of PP1, of Orc2. The dephosphorylation of Orc2 by PP1 is required for the binding of Orc2 to chromatin. These results support that PP1 dephosphorylates Orc2 to promote the binding of ORC to chromatin and replication origins for the subsequent round of the cell cycle.

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1. Introduction

Protein functions are often controlled by phosphorylation and dephosphorylation. The phosphorylation of the proteins that are involved in the formation of the pre-replicative and pre-initiation complexes for the cell cycle-dependent initiation of chromosome replication [1–4] suggests that protein phosphatases may be necessary to reverse these phosphorylation effects. Both protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) are serine/threonine phosphatases that belong to the family of phosphoprotein phosphatases [5–7]. The association of the PP1 catalytic subunit with diverse regulatory subunits and/or interacting proteins confers distinct substrate specificities that control various physiological processes, including mitotic exit. The PP1 catalytic subunit is conserved among eukaryotic cells. Over 100 regulatory subunits or interacting proteins of PP1 have been identified. The PP1 catalytic subunit binds proteins possessing an RVXF motif, $[R/K][X]_{0-1}[V/I]X[F/W]$ [8,9]. The valine (V) and phenylalanine/tryptophan (F/W) residues of this binding motif are crucial for anchoring the interacting proteins. Several regulatory subunits, such as Aurora-A, Aurora-B and Nek2, are also substrates for dephosphorylation and contain an RVXF motif [6,10–12]. Mammalian PP1 comprises three isoforms: PP1α, PP1β/δ (which is known as PP1β throughout the remainder of this manuscript) and PP1γ. PP1α localizes in the nuclear matrix and at the centrosome, whereas PP1β is found on the chromatin [13]. PP1γ exists in the nucleoli and mitotic spindle of the cell [13,14].

The origin recognition complex (ORC), which is composed of six different subunits, binds to the replication origins during the late M to early G1 phase of cell cycle for subsequent processes of chromosomal replication initiation [1,2]. The bound ORC becomes dissociated from replication origin from the S phase [15]. The Orc2 subunit of ORC contains consensus sequences for phosphorylation by CDK and its mutation of the CDK phosphorylation site results in re-replication [16] and delayed cell cycle progression in yeast [17]. In human cells, Orc2 is phosphorylated by cyclin A/CDK2 at Thr^{116} and Thr^{226} during S phase, which results in dissociation of Orc2–5 subunits from the origins [15]. Dephosphorylation of Orc2 accompanies the association of ORC with chromatin [18]. The overexpression and depletion of PP1 isoforms along with specific inhibitor studies demonstrated that PP1 dephosphorylates Orc2. In this study, we show that PP1 interacts with Orc2 through the 119-KSVSF-123 motif. This interaction is essential for the dephosphorylation of Orc2 and binding to chromatin for the next round of cell cycle.

Abbreviations: α-pT116, anti-phospho-Thr-116 Orc2 antibody; α-pT226, anti-phospho-Thr-226 Orc2 antibody; CDK, cyclin-dependent kinase; ORC, origin recognition complex; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; pre-RC, pre-replicative complex.

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2. Materials and methods

2.1. Cell culture and site-directed mutagenesis

U2OS, HeLa and HEK293T were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and antibiotics (penicilline/streptomycin). Inducible HeLa or U2OS Tet-On cell lines expressing the Orc2 protein were constructed and manipulated in accordance with previously described procedures [15]. To generate PP1-binding motif mutants of human ORC2, we used the following primers: For V121A/F123A, forward 5′-CACCACAAAAAAGTGCTTCAGCCA GTTTGAAGAATGATCCTGAG-3′ and reverse 5′-CTCAGGATCATTCTTCAAACTGGCTGAAGCACTTT TTTGTGGTG-3′; For Δ119–123, forward 5′-CAGAACTAGCAAAAACA CCACAAAGTTTGAAGAATGATCCTGAG-3′ and reverse 5′-CTCAG-GATCATTCTTCAAACTTTGTGGTGTTTTTGCTAGTT CTG-3′. Mutagenesis was performed using *pfu* turbo polymerase (Stratagene) by manufacturer's methods.

2.2. Yeast two hybrid assay

Human ORC subunits were inserted into pGBKT7-rec and then each construct was transformed into *Saccharomyces cerevisiae* AH109 which contains distinct ADE2, HIS3, lacZ and MEL1 reporter constructs. Transformed AH109 cells were mixed with Y187 cells that contains HeLa cDNA libraries (Clontech). After gently swirling at 30 °C for 24 h, mated strains were plated on high stringent growth medium (SD/-Trp/-Leu/-His/-Ade). Plasmids were obtained from selectively growing strains and transformed into Top10F′ competent cells. Finally, library plasmid was selected on ampicillin-supplemented LB media and sequenced. For the interaction of ORC subunits with PP1 and PP2A, PP1 isoform or PP2A was inserted into pGADT7-rec. This construct was transformed into AH109 that already contained ORC construct. Co-transformed AH109 were plated on high stringent growth medium.

3. Results and discussion

3.1. The 119-KSVSF-123 motif of Orc2 is required for the binding of PP1 to Orc2

Orc2 is dephosphorylated by PP1 isoforms α, β and γ [18]. To examine the physical interaction of Orc2 with PP1 isoforms, a yeast two-hybrid assay was performed (Fig. 1(A)). The interaction between each of the PP1 isoforms and Orc2 enabled growth on the selective medium, whereas no growth on this medium was observed if PP1 isoforms were absent, and PP2A did not interact sufficiently with Orc2 to enable growth. However, no other subunit of the ORC exhibited an interaction with the PP1 isoforms (Fig. 1(B)). These results suggest that of the six ORC subunits, PP1 specifically interacts with Orc2.

The interaction between Orc2 and the PP1 isoforms was verified in human cells. The immunoprecipitation of Orc2 using a monoclonal anti-Orc2 antibody co-precipitated the PP1 isoforms α, β and γ, whereas no significant co-precipitation was observed with a control antibody (Fig. 1(C)). The reciprocal immunoprecipitation with an anti-PP1 antibody contained Orc1, Orc3, Orc4 and Orc5 in addition to Orc2, implying that PP1 is able to bind to Orc2 within the ORC.

The PP1 catalytic subunit recognizes a binding motif, $[R/K][X]_{0-1}[V/I]X[F/W]$, on its regulatory subunit or interacting protein [9]. Using a data-based motif search, we found that this PP1-binding motif, 119-KSVSF-123, exists in the N-terminus of human Orc2 (Fig. 1(D)). The 119-KSVSF-123 motif and its flanking amino acids

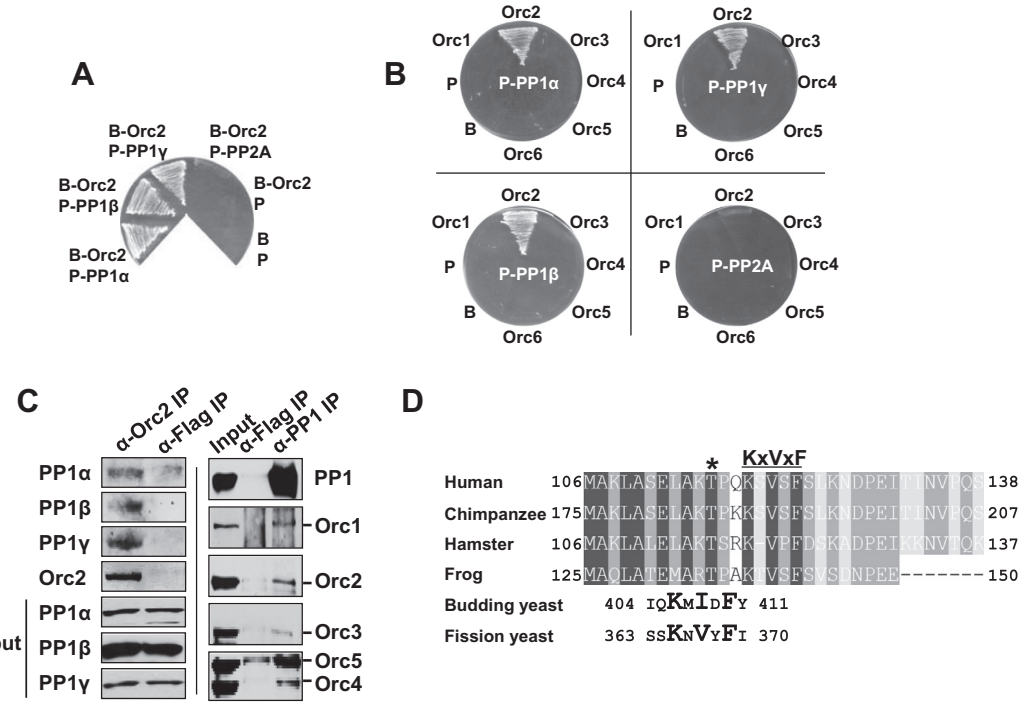

Fig. 1. PP1 binds to Orc2. (A) The open reading frame of Orc2 was inserted into the bait vector pGBKT7-rec, and the indicated protein phosphatase open reading frame was inserted into the prey vector pGADT7-rec. Yeast two-hybrid assays were performed on QDO selection plates as described in Section 2. B, the bait vector pGBKT7-rec; P, the prey vector pGADT7-rec. (B) The open reading frame of each Orc subunit was inserted into the bait vector pGBKT7-rec and yeast two-hybrid assays were performed with the prey vector containing the open reading frame of the indicated protein phosphatase. (C) Orc2 was immunoprecipitated from HEK293 lysate with either a monoclonal anti-Orc2 antibody or a monoclonal anti-Flag antibody (Left). PP1 was immunoprecipitated with an anti-PP1β antibody (Right). The co-precipitating proteins were detected by immunoblotting. (D) The sequence alignment of Orc2 was performed using the ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2). The PP1-binding consensus motif, $[R/K][X]_{0-1}[V/I]X[F/W]$, corresponds to the 119-KSVSF-123 sequence of human Orc2. The asterisk indicates Thr^{116}.

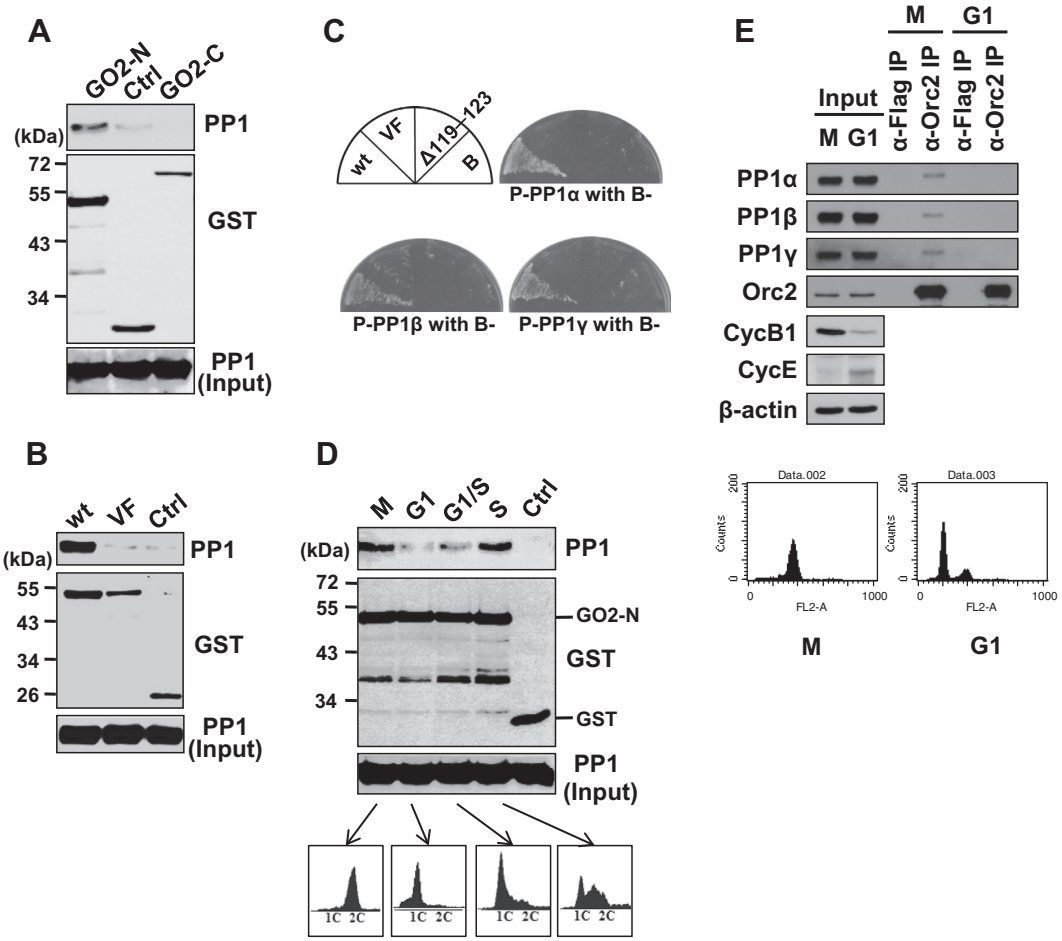

Fig. 2. PP1 binds to Orc2 via its interaction with the 119-KSVSF-123 motif of Orc2 in a cell cycle-dependent manner. (A) DNA constructs expressing the GST-tagged N-terminal 230 (GST-Orc2-N) or C-terminal 347 amino acid residues (GST-Orc2-C) of Orc2 were transfected into HEK293T cells; the lysates were then used in glutathione pull-down assays. GO2-N, GST-Orc2-N; GO2-C, GST-Orc2-C; Ctrl, empty vector. (B) The indicated proteins expressed in HEK293T cells were obtained by glutathione pull-down followed by analysis by immunoblotting. wt, GST-Orc2-N; VF, GST-Orc2-N-Val121A/Phe123A; Ctrl, empty vector. (C) A yeast two-hybrid assay was performed. wt, Orc2 wild type; VF, Orc2-Val121A/Phe123A; Δ119–123, Orc2-(Δ119–123). (D) HEK293T cells expressing GST-Orc2-N were arrested by treatment with nocodazole followed by mitotic shake-off (M) and released into fresh media for 4 h (G1) or treated with a double thymidine block (G1/S) and released into fresh media for 4 h (S). The FACS profiles of cells were shown in the below. A glutathione pull-down was performed, and the indicated protein was detected by immunoblotting. (E) HeLa cells were synchronized by nocodazole treatment and mitotic shake-off (M) and released into fresh media for 4 h (G1); immunoprecipitations were then performed using an anti-Orc2 or an anti-Flag antibody (Upper). FACS profiles of the M and G1 phase cells (Lower).

are preserved within higher eukaryotes ranging from frogs to humans. Yeasts also contain this motif.

To verify the interaction of Orc2 with PP1 through the PP1-binding motif of 119-KSVSF-123, glutathione pull-down experiments were performed in human cells. The N-terminal 230-amino-acid fragment containing the PP1-binding motif of Orc2 (GST-Orc2-N) successfully bound to PP1, whereas the C-terminal 347-amino-acid fragment (GST-Orc2-C) did not bind to PP1 (Fig. 2(A)). We generated a substitution mutant in the binding motif, Orc2-(Val121A/Phe123A), which contained substitutions of Val^{121} and Phe^{123} with Ala. PP1 did not significantly bind to GST-Orc2-(Val121A/Phe123A) (Fig. 2(B)). In yeast two-hybrid assays, PP1α, PP1β and PP1γ did not interact with either the Orc2-(Val121A/Phe123A) mutant or an Orc2-(Δ119–123) mutant that contained a deletion of the binding motif (Fig. 2(C)). These results indicate that PP1 is bound to Orc2 through the interaction of PP1 with the PP1-binding motif, 119-KSVSF-123.

We examined the interaction of Orc2 with PP1 during cell cycle progression. GST-Orc2-N-expressing cells were synchronized using either the nocodazole or double thymidine approaches (Fig. 2(D)). The glutathione pull-down of GST-Orc2-N co-precipitated PP1 in S and M phase; during these phases, Orc2 existed in a phosphorylated state [15]. By contrast, no significant co-precipitation of PP1 was observed for the glutathione pull-downs that were conducted in the G1 phase. Moreover, endogenous Orc2 bound to endogenous PP1α, PP1β and PP1γ to a greater extent in the M-phase cells than in the G1-phase cells (Fig. 2(E)).

3.2. The dephosphorylation of Orc2 by PP1 is required for the binding of ORC to chromatin

To verify the dependence of the dephosphorylation of Orc2 by PP1 upon the PP1-binding motif of Orc2, wild-type GST-Orc2, phospho-defective GST-Orc2-(Thr116A/Thr226A), and GST-Orc2-(Val121A/Phe123A) containing mutations in the PP1-binding motif were separately expressed in human cells and isolated by glutathione pull-down (Fig. 3(A)). Because GST-Orc2-(Thr116A/Thr226A) contained substitutions of both Thr^{116} and Thr^{226} with Ala, this protein could not be phosphorylated. The PP1-binding-motif mutant contained 3.3- and 4.4-fold more phospho-Thr^{116} and phospho-Thr^{226}, respectively, than the wild-type protein. There was no detectable recognition of the mutant protein by either antibody.

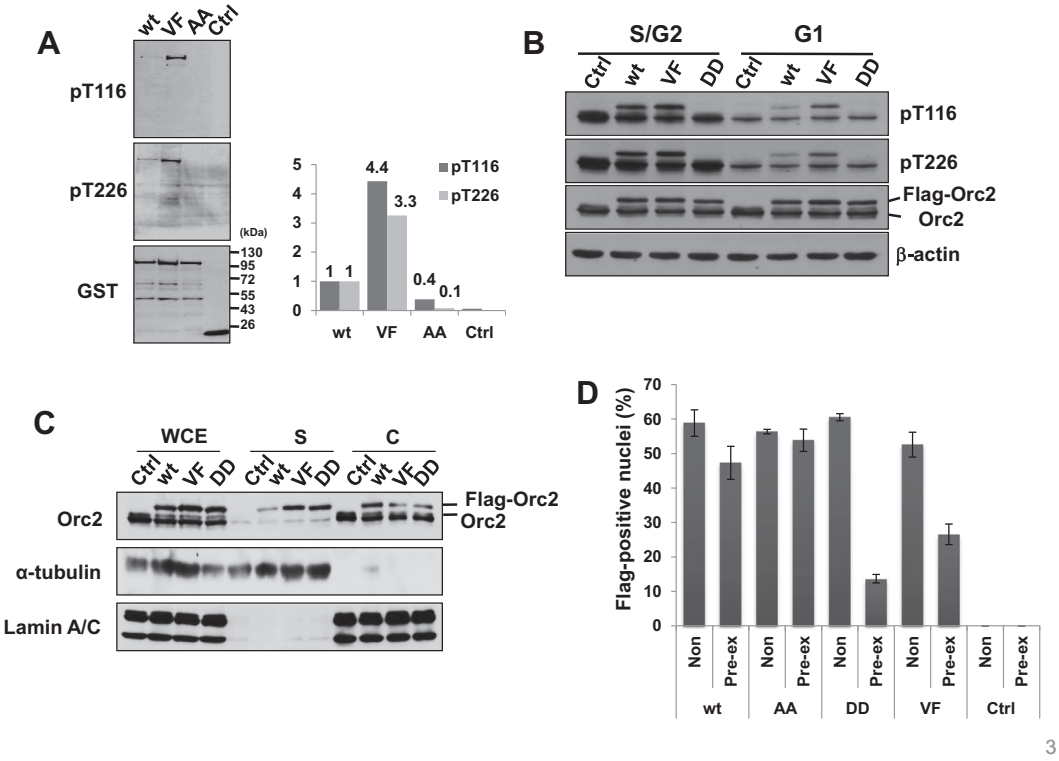

Fig. 3. The dephosphorylation of Orc2 by PP1 allows the Orc2 to bind to chromatin. (A) HEK293T cells were transfected with DNA constructs expressing GST-Orc2 (wt), GST-Orc2-Val121A/Phe123A (VF), GST-Orc2-Thr116A/Thr226A (AA), or GST (Ctrl). The phospho-Thr^{116} and phospho-Thr^{226} of the indicated proteins that were isolated by glutathione pull-downs were detected by immunoblotting (Left). The relative intensity of each band over the Orc2 band was described (Right). (B) HeLa Tet-On cell lines grown in the presence of 2 μg/mL of doxycycline for 48 h were treated with a double thymidine block and released for 8 h (S/G2) or 12 h (G1). The indicated proteins were detected by immunoblotting. Ctrl, HeLa Tet-On cells; wt, HeLa Tet-On cells expressing Flag-Orc2 wild-type protein; VF, HeLa Tet-On cells expressing Flag-Orc2-(Val121A/Phe123A); DD, HeLa Tet-On cells expressing Flag-Orc2-(Thr116D/Thr226D). (C) The indicated proteins were induced from the corresponding U2OS Tet-On cell lines by the addition of 2 μg/mL of doxycycline for 48 h. Chromatin fractionation was performed as described in Section 2, except that buffer C containing 0.1 M NaCl was used. WCE, whole-cell extract; S, soluble fraction; C, chromatin-bound fraction. (D) U2OS Tet-On cells were pre-extracted using 0.5% Triton X-100 in phosphate-buffered saline for 1 min before fixation followed by immunostaining. The nuclei exhibiting a Flag-positive signal were quantified using the ImageJ software package and described as the ratio of positive nuclei over the total number of nuclei. Non, non-extracted; Pre-ex, pre-extracted.

The levels of phospho-Thr^{116} and phospho-Thr^{226} of Flag-Orc2 proteins, which were induced by the addition of doxycycline to HeLa Tet-On cell lines, were compared in the S/G2- and G1-phase cells (Fig. 3(B)). The Thr^{116} and Thr^{226} of Orc2 became phosphorylated during S phase and dephosphorylated during late M phase [15]. Therefore, higher levels of phospho-Thr^{116} and phospho-Thr^{226} were contained in the endogenous Orc2 and the wild-type Flag-Orc2 of the S/G2-phase cells than in the G1-phase cells. However, the reduction in the levels of phospho-Thr^{116} and phospho-Thr^{226} was less efficient for the PP1-binding-motif mutant protein, Flag-Orc2-(Val121A/Phe123A) than for the wild-type protein. Flag-Orc2-(Thr116D/Thr226D) was not phosphorylated because of its substitution of both Thr^{116} and Thr^{226} with Asp. The inefficient reduction of the phospho-Thr^{116} and phospho-Thr^{226} of Flag-Orc2-(Val121A/Phe123A) in G1 phase, together with the increased phosphorylation at GST-Orc2-(Val121A/Phe123A) in Fig. 2(A), indicates that PP1 dephosphorylates Orc2 through its interaction with the PP1-binding-motif of Orc2, 119-KSVSF-123.

The effect of the dephosphorylation of Orc2 was examined by chromatin fractionation (Fig. 3(C)). Compared with the wild-type protein, the defect in the ability of the phospho-mimetic Flag-Orc2-(Thr116D/Thr226D) to bind to chromatin led to a reduction in the amount of protein in the chromatin-bound fraction and an increase in the amount of protein in the soluble fraction. Similarly, the PP1-binding-motif mutant Flag-Orc2-(Val121A/Phe123A) decreased in the chromatin-bound fraction but increased in the soluble fraction. The pre-extraction of the cells with detergent removes soluble non-chromatin-associated protein from the nucleus [15,19]. The pre-extraction of the cells expressing various Flag-Orc2 proteins also revealed that both the PP1-binding-motif mutant protein and the phospho-mimetic Flag-Orc2 were defective in chromatin association (Fig. 3(D)). Whereas the phospho-defective Orc2 persists in binding to chromatin and replication origins throughout the cell cycle, the phospho-mimetic protein is defective with respect to this binding [15]. These results indicate that the dephosphorylation of Orc2 by PP1 is necessary for the binding of the ORC to chromatin and replication origins.

The Thr^{116} and Thr^{226} of Orc2 are phosphorylated by cyclin A/CDK during S phase, thereby dissociating the ORC from chromatin and replication origins. This phosphorylation is maintained until M phase and contributes to inhibiting the ORC from binding to newly replicated DNA. To activate the ORC to bind to chromatin and replication origins for the subsequent round of the cell cycle, PP1 dephosphorylates the Orc2 during the late M phase. Because the function of the ORC precedes the functions of other proteins in the initiation of chromosome replication, the control of the ORC through the phosphorylation and dephosphorylation of Orc2 may contribute to the cell cycle-regulated initiation of chromosome replication.

PP1 binds to Orc2 during the S to M phases of the cell cycle (Fig. 2(D) and (E)); during these phases, Orc2 exists in its phosphorylated form [15]. However, dephosphorylated Orc2 apparently accumulates in the cell from the late M to the G1 phase of the cell cycle. It is possible that the phosphorylation of Orc2 by CDK dom-

inates the dephosphorylation of the Orc2 by PP1 to maintain the phosphorylated state of Orc2 from the S phase to the G2 phase of the cell cycle. Alternatively, the dependence of PP1 dephosphorylation activity upon a regulatory subunit or inhibitor would also allow for PP1 dephosphorylation during late M phase.

PP1 participates in both M phase progression and the M/G1 phase transition [6,20]. PP1 antagonizes Aurora kinases at the spindle assembly checkpoint and during chromosome congression [21]. In combination with AKAP149, PP1 dephosphorylates lamin B, which is phosphorylated by protein kinase C (PKC), enabling the reassembly of the nuclear envelope [22]. Moreover, hyperphosphorylated Rb is dephosphorylated by PP1, favoring the formation of the Rb–E2F complex [23]. Together with these functions, PP1 might contribute to the M/G1 transition by dephosphorylating Orc2 to enable the ORC to bind to chromatin.

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