Biochemical and Biophysical Research Communications 447 (2014) 437–440

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Protein phosphatase 1 dephosphorylates Orc2

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ARTICLE INFO

*Article history:*
Received 21 March 2014
Available online 13 April 2014

*Keywords:*
ORC
Protein phosphatase 1
Dephosphorylation
DNA replication
Cell cycle

ABSTRACT

Phosphorylation of $Thr^{116}$ and $Thr^{226}$ on 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. The phosphorylated Orc2 becomes dephosphorylated in the late M phase of the cell cycle. Here we show that protein phosphatase 1 (PP1) dephosphorylates Orc2. Dephosphorylation of Orc2 was accompanied by associating the dissociated Orc subunits with chromatin. Inhibitors of PP1 preferentially inhibited the dephosphorylation of Orc2. The overexpression of the α, β and γ PP1 isoforms decreased the amount of phosphorylated Orc2, and the depletion of these isoforms by RNA interference increased the amount of phosphorylated Orc2. These results suggest that PP1 dephosphorylates Orc2 to promote the binding of ORC to chromatin.



## 1. Introduction

In eukaryotes, the origin recognition complex (ORC) binds to the replication origin to assemble the pre-replicative complex (pre-RC), which is composed of the ORC, Cdc6, Cdt1 and MCM2-7, and thereby enables the subsequent processes of chromosome replication [1–4]. DNA synthesis in S phase can only occur at the origins where the pre-RC has formed during the G1 phase of the cell cycle. The ORC consists of six different subunits and contains ATP-binding and ATP-hydrolysis activities [5,6]. ATP binding is involved in ORC complex formation, DNA binding, and pre-replicative complex formation. In mammalian cells, the ORC dissociates from chromatin and from replication origins during S phase [7–9]. In addition to its involvement in the formation of the pre-replicative complex, the ORC participates in heterochromatin formation, gene silencing, sister chromatid cohesion, and other functions [10].

Families of Protein Serine/Threonine phosphatases (PSPs) are composed of phosphoprotein phosphatases (PPPs), metal-dependent protein phosphatases (PPMs), and aspartate-based phosphatases represented by FCP/SCP (TFIIF-associating component of RNA polymerase II CTD phosphatase/small CTD phosphatase) [11]. Among these, protein phosphatase 1 and 2A are included in representative members of the PPP family and can be inhibited by several toxins such as okadaic acid (OA), calyculin A, microcystin-LR (MCLR), nodularin, tautomycin and cantharidin [12].

Recently, we reported that cyclin A/CDK2 phosphorylates the $Thr^{116}$ and $Thr^{226}$ of the Orc2 subunit of the human ORC during S phase [9]. The phosphorylated state of Orc2 is maintained until M phase. The phosphorylation of Orc2 dissociates the ORC from chromatin and from replication origins. The binding of this dissociated ORC to chromatin and replication origins is required for the initiation of chromosome replication during the subsequent round of the cell cycle. In this report, we demonstrated that PP1 dephosphorylates phosphorylated Orc2, thereby allowing the ORC to bind to chromatin.

## 2. Materials and methods

### 2.1. Antibodies, inhibitors, DNA and siRNAs

The anti-phospho-$Thr^{116}$ and anti-phospho-$Thr^{226}$ Orc2 antibodies were described in previously published studies [9]. The antibodies against Orc1, PP1α, PP1γ, and Lamin A/C were purchased from Santa Cruz Biotechnology, Inc.; the antibody against PP1β was purchased from Abcam; the antibody against Orc2 was purchased from BD Pharmingen, Inc. and Calbiochem; the antibody against Orc4 was purchased from US Biological; the antibodies against β-actin and α-tubulin were purchased from Sigma; the antibody against His was purchased from Clontech; and the antibody against Cdc25C was purchased from Cell Signalling

*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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http://dx.doi.org/10.1016/j.bbrc.2014.04.029
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Technologies, Inc. The antibodies against Orc3 and Orc5 were gifts from Dr. Anindya Dutta (the University of Virginia).

The cDNAs of human PP1β, PP1γ and PP2A were purchased from Korean UniGene Information (KUGI), and PP1α cDNA was cloned from a HeLa cDNA library (Clontech). The following siRNA oligonucleotides were purchased from Samchully Pharm. Co.: control siRNA (GL3), CUUACGCUGAGUACUUCGA; PP1α, GAGACGCTACAACATCAAA; PP1β, AGAAGUUCGAGGCUUAUGU; PP1γ, CUAUCCUACUAGAACUUGA.

### 2.2. Chromatin fractionation assay

The cells were resuspended in buffer C (10 mM PIPES pH 7.9, 0.2 M NaCl, 0.3 M sucrose, 0.1% Triton X-100, 1 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EGTA, 200 μM $Na_3VO_4$, 10 mM NaF, 20 nM calyculin A and a protease inhibitor cocktail) and lysed on ice for 10 min. The lysis was followed by centrifugation at 1300×*g* for 5 min. The supernatant was then further centrifuged at 16,000×*g* for 15 min to obtain a soluble fraction, and the precipitate was washed once with two volumes of buffer C to obtain the precipitate, which is a chromatin-enriched fraction.

### 2.3. The in vitro dephosphorylation assay using mitotic HeLa extracts

The *in vitro* dephosphorylation assay was performed in accordance with previously published procedures [13], with minor modifications. HeLa cells arrested in mitosis with nocodazole (100 ng/ml) treatment for 16 h were collected by shake-off and then released in fresh media for 1 h. The cells were collected, and the collected cells were washed twice with PBS. The cells were lysed in hypotonic buffer (20 mM HEPES, pH 7.5, 5 mM KCl, 1.5 mM $MgCl_2$ and 1 mM dithiothreitol); this wash was followed by Dounce homogenization, and the supernatants were then obtained by centrifugation for 15 min at 16,000×*g*. DMSO or inhibitor-2 protein (4 μM; New England Biolabs) was added to the cell lysate, and the lysate was incubated at room temperature.

## 3. Results and discussion

### 3.1. The dephosphorylation of phosphorylated Orc2 associates the ORC with chromatin

Of the six subunits of the ORC, the Orc2, Orc3, Orc4, and Orc5 subunits (Orc2-5) form a stable complex [14]. The phosphorylation of Orc2 at its $Thr^{116}$ and $Thr^{226}$ by cyclin A/CDK during the S phase of the cell cycle dissociates Orc2-5 from chromatin and replication origins, although this phosphorylation does not affect the assembly of the ORC [9]. We examined whether the dephosphorylation of the phosphorylated Orc2 allows Orc2-5 to bind to chromatin. HeLa cells that had been arrested at the G1/S transition of the cell cycle with a double thymidine block were released for 6 h; at this time, the cells were in late S phase (Fig. 1(A)). Although the incubation of the harvested S-phase cells in phosphate-buffered saline did not significantly alter the cell cycle phase or the overall quantity of Orc2 in the cells, the incubation reduced the levels of Orc2 with phosphorylated $Thr^{116}$ and $Thr^{226}$. The S-phase cells contained higher levels of Orc2 with phospho-$Thr^{116}$ and phospho-$Thr^{226}$ in the soluble fraction and lesser amounts of chromatin-bound Orc2-5 than the G1/S cells (Fig. 1(B)). The incubation of the harvested S-phase cells in phosphate-buffered saline decreased the levels of Orc2 with phospho-$Thr^{116}$ and phospho-$Thr^{226}$ in the soluble fraction but increased the chromatin-bound fraction of each of Orc2-5. These decreases and increases were proportional to the duration of the incubation. These results suggest that the dephosphorylation of Orc2 enables the dissociated Orc2-5 to bind to chromatin.

### 3.2. Protein phosphatase 1 dephosphorylates Orc2

The dephosphorylation of Orc2 during the incubations that are indicated in Fig. 1 prompted us to identify the protein phosphatase that was responsible for this dephosphorylation. HeLa cells treated with various protein phosphatase inhibitors, solubilized in dimethyl sulfoxide, were analyzed by immunoblotting to detect the phosphorylation state of the $Thr^{116}$ and $Thr^{226}$ of Orc2 (Fig. 2). Calyculin A inhibits both PP1 and protein phosphatase 2A (PP2A) [12], whereas tautomycin preferentially inhibits PP1 [15,16]. The inhibition of dephosphorylation by either calyculin A or tautomycin increased the amounts of the phospho-$Thr^{116}$ and phospho-$Thr^{226}$ of Orc2 in a dose-dependent manner (Fig. 2(A) and (B)). Okadaic acid preferentially inhibits PP2A [15]. PP2A dephosphorylates hyperphosphorylated Cdc25C and downshifts the migration of this protein to 60 kDa [17]. Okadaic acid inhibited the dephosphorylation of hyperphosphorylated Cdc25C, resulting in the accumulation of hyperphosphorylated forms of Cdc25C (Fig. 2(C)). However, up to 1 μM of okadaic acid did not significantly affect the quantities of phosphorylated Orc2 that were detected.

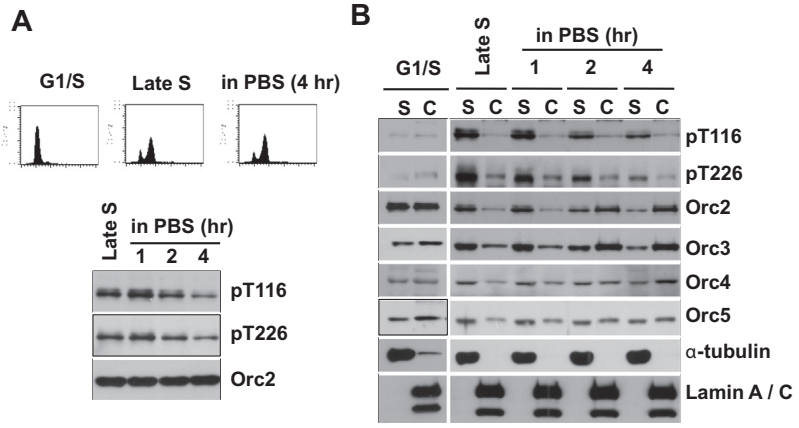


**Fig. 1.** The dephosphorylation of the phospho-$Thr^{116}$ and phospho-$Thr^{226}$ of Orc2 allows the ORC to bind to chromatin. (A) HeLa cells arrested with a double thymidine block (G1/S phase) were released for 6 h (late S phase). The cells were washed twice with phosphate-buffered saline and were then incubation in the same buffer at 37 °C for the indicated lengths of time. The cells were analyzed by FACS (Upper) and immunoblotting, using the appropriate corresponding antibody (Lower). (B) Chromatin fractionation was performed as described in Section 2. The indicated proteins were detected by immunoblotting. The phospho-$Thr^{116}$ and phospho-$Thr^{226}$ of Orc2 were detected using anti-phospho-$Thr^{116}$ (α-pThr116) and anti-phospho-$Thr^{226}$ (α-pThr226) antibodies, respectively [9]. PBS, phosphate-buffered saline; pThr116, phospho-$Thr^{226}$ of Orc2; pThr226, phospho-$Thr^{226}$ of Orc2; S, soluble fraction; C, chromatin-bound fraction. These and following experiments were performed at least three times.

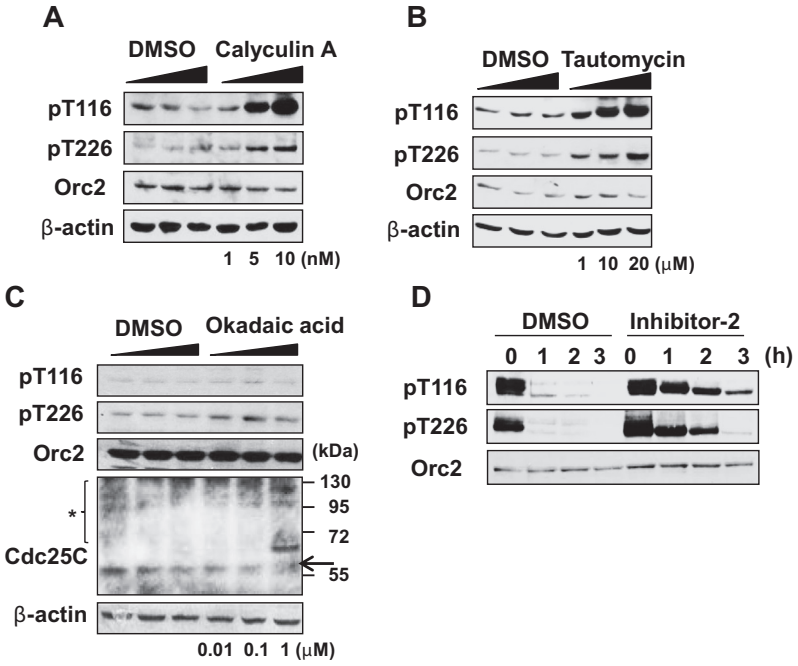


**Fig. 2.** PP1 dephosphorylates Orc2. The indicated concentration of (A) calyculin A for 2 h, (B) tautomycin for 4 h, or (C) okadaic acid for 4 h was added to HeLa cell cultures before cell harvest. The hyperphosphorylated and dephosphorylated forms of Cdc25C are denoted by the asterisk and the arrow, respectively. (D) Mitotic HeLa extracts prepared as described in Section 2 were incubated with 4 μM of inhibitor-2 protein (New England Biolabs) at room temperature for the indicated time. The protein phosphatase inhibitors were resuspended in dimethyl sulfoxide (DMSO).

Because mitotic cells contain abundant phosphorylated Orc2 [9], a cell extract was prepared from nocodazole-arrested HeLa cells (Fig. 2(D)). Inhibitor-2 protein binds to PP1 and specifically inhibits the catalysis of PP1 [13]. The addition of inhibitor-2 delayed the dephosphorylation of the phospho-Thr$^{116}$ and phospho-Thr$^{226}$ of Orc2. The inhibition of Orc2 dephosphorylation by tautomycin and inhibitor-2, as well as the lack of inhibition of this dephosphorylation by okadaic acid, suggests that PP1 dephosphorylates the phospho-Thr$^{116}$ and phospho-Thr$^{226}$ of Orc2.

### 3.3. PP1α, PP1β and PP1γ dephosphorylate Orc2

Three isoforms of the PP1 catalytic subunit exist: PP1α, PP1β and PP1γ [18]. To identify the PP1 isoform that is responsible for dephosphorylating Orc2, each isoform was expressed (Fig. 3(A)). The expression of each isoform decreased the levels of both the phospho-Thr$^{116}$ and the phospho-Thr$^{226}$ of Orc2. By contrast, the expression of PP2A did not significantly affect the phosphorylated Orc2. Individual and combinatorial depletions of PP1α, PP1β and PP1γ were performed with the corresponding PP1 isoform-specific siRNA (Fig. 3(B)). The depletion of one of the three isoforms did not show significant increase of the phospho-Thr$^{116}$ and phospho-Thr$^{226}$ of Orc2. However, the co-depletion of any two or three of the PP1 isoforms resulted in an increase in the phospho-Thr$^{116}$ and phospho-Thr$^{226}$ of Orc2. The ectopic expression and depletion results suggest that all three of the PP1 isoforms, rather than a certain isoform, participate in the dephosphorylation of Orc2.

The phosphorylated Orc2 that is dissociated from chromatin is distributed throughout the interphase nucleus. In M phase, the disruption of the nuclear envelope diffuses the phosphorylated Orc2 throughout the cell. Therefore, PP1α, PP1β and PP1γ, which localize to different compartments and organelles of cells may not be discriminated during the binding and dephosphorylation of Orc2. The re-association of the dissociated Orc2-5 with chromatin through the *in vitro* dephosphorylation of Orc2 (Fig. 1) suggests that the binding to and dissociation from chromatin of Orc2-5 could be dynamic; these transitions are regulated by the dephosphorylated and phosphorylated states of Orc2, even in the absence of cell cycle progression.

Chromosome initiation and replication, including the formation of the pre-RC and pre-initiation complex, require the cell cycle-dependent kinases CDK and Cdc7/Dbf7 [4,14,19–21]. For the cell cycle-dependent control of chromosome initiation, these kinases inhibit and activate various replicative proteins, such as Cdc6,

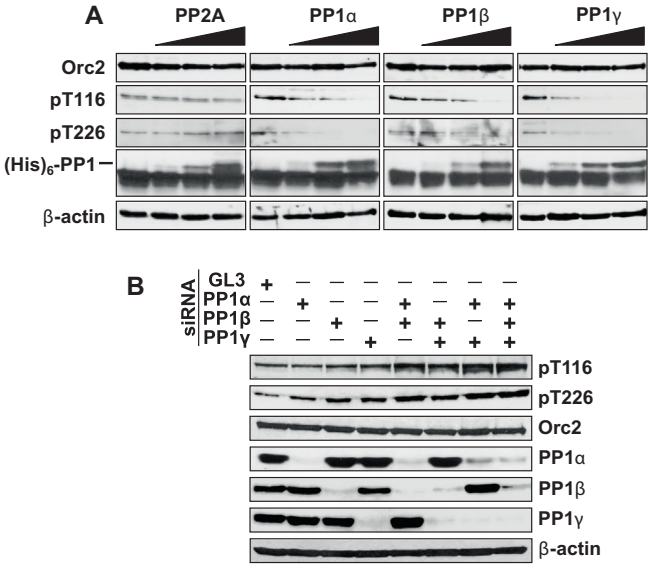


**Fig. 3.** The PP1 isoforms α, β and γ dephosphorylate the phospho-Thr$^{116}$ and phospho-Thr$^{226}$ of Orc2. (A) HeLa cells were transfected with 0, 0.5, 1 and 2 μg of DNA constructs expressing the indicated $(His)_6$-protein phosphatase; this transfection was followed by a 48-h incubation. (B) Each PP1 isoform of HeLa cells was depleted using individual or various combinations of PP1α-, PP1β- and PP1γ-specific siRNA oligonucleotides, and GL3 was used as a control siRNA (Section 2). The proteins were detected by immunoblotting.

Cdt1, MCM2-7 and Slds [21–23]. This phosphorylation-mediated activation or inhibition suggests that dephosphorylation must be required to counteract the effects of phosphorylation. However, few investigations have reported findings regarding the function of protein phosphatases in the regulation of chromosome replication. It has been suggested that the interaction of Cdc6 with protein phosphatase 2A and its regulatory subunit PR48 participates in G1 phase progression [24]. The depletion of protein phosphatase 2A from Xenopus egg extract has also been shown to diminish the loading of Cdc45 to the pre-replicative complex [25]. Rif1 (Rap1-interacting factor 1) was suggested as a novel PP1 substrate targeting subunit to prevent premature phosphorylation of MCM4 [26]. In this report, we addressed that Orc2 is dephosphorylated by PP1 to activate the ability of the ORC to bind to chromatin. Protein phosphatase function must be further elucidated with respect to the regulation of chromosome replication in coordination with the cell cycle.

## Acknowledgments

We thank Dr. Anindya Dutta for providing us with the anti-Orc3 and anti-Orc5 antibodies. This work was supported by a grant from the Basic Science Research Program through the National Research Foundation of Korea (NRF-2013R1A1A2012109). J.S.B and G.S.K were supported by the BK21 Research Fellowship from the Ministry of Education, Science and Technology, Republic of Korea.

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