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Novel phosphorelay-dependent control of ZFP36L1 protein during the cell cycle

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ABSTRACT

The ZFP36 family is a prototypical member of a highly conserved group of proteins with CCCH-type RNA-binding domains, whose functional role and regulatory mechanism in mitotic cells remain obscure. In this study, we provide the first evidence that ZFP36L1 phosphorylation is modulated in a cell cycle-dependent manner. The C-terminal region of ZFP36L1 is critical for its cell cycle-dependent phosphorylation of this protein. We also suggest that the phosphorelay-dependent regulation of ZFP36L1 influences mitotic spindle organization. Thus, our data demonstrate a new class of regulatory mechanism for CCCH-type zinc-finger proteins in cell cycle control.

1. Introduction

The timely and accurate regulation of gene expressions is critical for coordinating many biological processes, including cell cycle control. Recently, the tristetraprolin family of RNA-binding protein, ZFP36, has been recognized as an important regulatory target for ensuring precise gene expression, though its importance in cell cycle control has only recently been receiving increased attention [1–3]. ZFP36 family proteins are critical for controlling mRNA stability [4,5]. They possess highly conserved CCCH-type zinc-finger domains, which are critical for high binding affinity with AU-rich elements (AREs) in the 3′-untranslated region of target mRNAs [6]. The specific binding of ZFP36 family proteins to AREs results in the destabilization of the corresponding mRNAs, such as tumor necrosis factor α [7]. ZFP36L2 has also been implicated in the ARE-dependent mRNA decay of G1/S cyclin family transcripts [1,2]. The expression and activity of ZFP36 family members is modulated in response to several mitogens, including serum, insulin, PDGF and FGF. The N-terminal region of ZFP36 proteins is phosphorylated in mitogen-treated cells, for example, by p38 mitogen-activated protein (MAP) kinase and its downstream kinase MK2 at Ser^{52} and Ser^{178} [8–10], leading to inhibition of their ARE-binding activity [11,12]. Previous LC/MS/MS analyses identified a number of potential phosphorylation sites in mammalian ZFP36 proteins [10,13–15], although the cell cycle-related regulation of these posttranslational modifications has not been elucidated.

In the nematode *Caenorhabditis elegans*, we and others have shown that a ZFP36-related CCCH protein, OMA-1, is expressed in oocytes, and is essential for female meiotic cell cycle progression [16,17]. After fertilization, OMA-1 proteins are specifically phosphorylated by several cell cycle regulatory kinases at the C-terminus during the first mitosis of one-cell stage embryos [18,19], although the C-terminal sequence of OMA-1 is unique in nematodes. The OMA-1 phosphorylation is critical for its rapid elimination in zygotes, which is absolutely required for initiating appropriate embryogenesis [18–20]. These results indicate that the cell cycle-dependent phosphorylation of OMA-1 is functionally important, at least in nematode zygotes. However, no vertebrate CCCH-type zinc-finger proteins have as yet been identified that associate with cell cycle-dependent phosphorylation. In the present study, we provide the first evidence that C-terminal phosphorylation of the vertebrate CCCH protein ZFP36L1 is cell cycle-dependent, and that phosphorelay-dependent regulation of ZFP36L1 *in vivo* influences chromosome segregation in *Xenopus* embryos. Thus, our approach has revealed a new class of regulatory mechanism for CCCH-type zinc-finger RNA binding proteins,

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which are collectively required for the coordinated progression of mitosis.

2. Materials and methods

2.1. Expression of proteins in Xenopus embryos

The full-length cDNAs of *Xenopus zfp36l1* was amplified by PCR from cDNA libraries prepared from *Xenopus* oocytes, and the *in vitro* synthesized mRNAs were microinjected into a blastomer of *Xenopus* embryos as described previously [21,22]. At the indicated stage, each embryo was individually harvested, crushed in PBS, and centrifuged to collect the cytoplasmic fraction for subsequent analysis.

2.2. Immunological analysis

The anti-ZFP36L1 antibody was prepared for this study as follows. Two hundreds μg of bacterially-produced *Xenopus* ZFP36L1 was mixed and emulsified with an equal amount of TiterMaxGold™ (TiterMax USA, Inc., Norcross, GA) and was then inoculated into a mouse. The antibody was obtained after four rounds of immunization at two-week intervals and used after affinity purification. Immunoprecipitation (IP), western blotting and immunocytochemical analysis were performed as described previously [1,17,23].

2.3. Cell-free phosphorylation assay of ZFP36L1

The expression and purification of GST-fusion proteins were performed as described previously [21,22]. For *in vitro* phosphorylation reactions with purified kinases, 1 μg of GST-fused recombinant ZFP36L1 protein and its mutant derivatives were mixed with 5 units of commercially available kinases, CK1, CK2, GSK3, p42 MAPK (New England BioLabs, Ipswich, MA), in the presence of 5 μCi γ-$^{[32]}$P-ATP in reaction buffer (25 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 10 μM ATP) and incubated at 25 °C for 1 h. The reaction was stopped by adding SDS, followed by SDS-PAGE and autoradiography to measure the incorporation of $^{[32]}$P into the substrates.

For *in vitro* phosphorylation in *Xenopus* extracts, purified Flag-tagged ZFP36L1 and its mutant derivative proteins were incubated in *Xenopus* meiotic CSF extracts [24]. For this assay, 500 ng of a series of Flag-ZFP36L1 proteins and 20 μL of extracts were mixed and then incubated at 20 °C for indicated times with protease inhibitor MG-132. The reactions was stopped by adding 1% SDS and the modification of ZFP36L1 was analyzed by Western blot analysis with anti-Flag antibody.

2.4. Mammalian cell culture, synchronization and transfection

The cDNA cloning for human ZFP36L1, HeLa cell culture, transfections and cell synchronization experiments were preformed as described previously [1].

3. Results

3.1. ZFP36L1 phosphorylation is modulated in a cell cycle-dependent manner in Xenopus dividing embryos

Xenopus ZFP36L1 is a maternally expressed protein that belongs to a large family of CCCH-type zinc-finger proteins [25]. Since the phosphorylation and degradation of the nematode CCCH protein OMA-1 is essential for the completion of meiosis [18,19], we investigated whether ZFP36L1 is also subjected to cell cycle-dependent modification in *Xenopus* dividing embryos. Using a specific antibody, we found that endogenous ZFP36L1 protein can be detected in *Xenopus* embryos as multiple bands of Mr 48,000-58,000 (Fig. 1A), suggesting that some covalent modifications of ZFP36L1 might occur. These signals can be absorbed by the corresponding antigen and were not detected in pre-immune serum (Fig. S1A). We subsequently noticed that the modifications of endogenous ZFP36L1 protein fluctuated during the cell cleavage cycle (Fig. 1A).

Similar to the case of endogenous ZFP36L1 protein, Flag-tagged ZFP36L1 protein was more severely modified in the dividing embryo (indicated as I) than in interphase embryo (indicated as II) (Fig. 1B and C). At the time of cell cleavage, the majority of Flag-ZFP36L1 was detected in the slowest moving band (Fig. 1C). This diversity in SDS-PAGE migration can mainly be attributed to phosphorylation, since the slower migrating bands were transformed into faster bands upon *in vitro* dephosphorylation with alkaline phosphatase (CIAP) (Fig. 1D). These results indicate that ZFP36L1 is expressed as a number of differentially hyper-phosphorylated forms. Time course analysis of ZFP36L1 modification further supported its cell cycle-dependency; the amount of slowly migrating ZFP36L1 species gradually increased, and then disappeared and/or were converted to faster migrating forms after egg cleavages (Fig. 1E and Fig. S1B). As a result, the faster and slower migrating forms could be detected in different proportions, depending on cell cycle stage. These observations imply that ZFP36L1 phosphorylation is linked to the mitotic events.

3.2. C-terminus of ZFP36L1 is essential for its phosphorylation

Most of previously identified phosphorylated sites in vertebrate ZFP36 family proteins exist in either the N-terminal or central domains, except for the case of nematode OMA-1 protein [18,19]. Therefore, we investigated whether removal of the ZFP36L1 N-terminal and C-terminal regions modified its susceptibility to mitosis-specific phosphorylation. Five fragments, namely, N (amino acids 1-126), Z (amino acids 99-196), C (amino acids 176-345), and their combinations (NZ and ZC), were tested as substrates for *in vivo* phosphorylation (Fig. 1F). Only full-length ZFP36L1 and fragments containing the C-terminal region were found to be efficiently phosphorylated (Fig. 1G, indicated by arrowheads). These results imply that the C-terminal half of ZFP36L1 plays an essential role in the cell cycle-dependent phosphorylation of this protein in *Xenopus* embryonic cells.

To further investigate the region required for this cell cycle-dependent phosphorylation, we prepared a series of C-terminally-truncated mutants of ZFP36L1 (Fig. 1H). The results clearly showed that deletion of the C-terminal 30 (C30) amino acids from full-length ZFP36L1 was sufficient to abolish its CIAP sensitivity completely (Fig. 1I; 1-315). This suggests that the C30 residues of ZFP36L1 (designated C30-ZFP36L1, indicated by gray color in Fig. 1H) contain critical phosphorylation sites that might stimulate the hyper-phosphorylation of ZFP36L1 protein.

3.3. The 317-325 Ser cluster of ZFP36L1 is essential for its phosphorylation in vivo

Inspection of the C30-ZFP36L1 amino acid sequence revealed the presence of Ser/Thr clusters (Fig. 2A, WT), including potential consensus phosphorylation motifs for MAP kinases (Ser^{325}), caseine kinase 2 (CK2) (Ser^{320}/Ser^{323}/Ser^{341}), protein kinase A (PKA) (Ser^{341}) and glycogen syntase kinase-3 β (GSK-3β) (Ser^{317}/Ser^{321}/Ser^{325}). To determine the vital phosphorylation site(s) in C30-ZFP36L1, we systematically substituted the Ser/Thr clusters to Ala as shown in Fig. 2A. We found that substitution of 4 Ser residues in the 317 to 320 cluster to Ala (designated the SA1 mutant) abolished