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Two nuclear export signals of Cdc6 are differentially associated with CDK-mediated phosphorylation residues for cytoplasmic translocation

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ABSTRACT

Cdc6 is cleaved at residues 442 and 290 by caspase-3 during apoptosis producing p49-tCdc6 and p32-tCdc6, respectively. While p32-tCdc6 is unable to translocate into the cytoplasm, p49-tCdc6 retains cytoplasmic translocation activity, but it has a lower efficiency than wild-type Cdc6. We hypothesized that a novel nuclear export signal (NES) sequence exists between amino acids 290 and 442. Cdc6 contains a novel NES in the region of amino acids 300–315 (NES2) that shares sequence similarity with NES1 at residues 462–476. In mutant versions of Cdc6, we replaced leucine with alanine in NES1 and NES2 and co-expressed the mutant constructs with cyclin A. We observed that the cytoplasmic translocation of these mutants was reduced in comparison to wild-type Cdc6. Moreover, the cytoplasmic translocation of a mutant in which all four leucine residues were mutated to alanine was significantly inhibited in comparison to the translocation of wild-type Cdc6. The Crm1 binding activities of Cdc6 NES mutants were consistent with the efficiency of its cytoplasmic translocation. Further studies have revealed that L468 and L470 of NES1 are required for cytoplasmic translocation of Cdc6 phosphorylated at S74, while L311 and L313 of NES2 accelerate the cytoplasmic translocation of Cdc6 phosphorylated at S54. These results suggest that the two NESs of Cdc6 work cooperatively and distinctly for the cytoplasmic translocation of Cdc6 phosphorylated at S74 and S54 by cyclin A/Cdk2.

1. Introduction

Cdc6, a component of the pre-replication complex, is required for loading the MCM complex onto chromosomes to commence DNA replication initiation with ORC and Cdt1 during S phase of the cell cycle [1,2]. In yeast, as cells pass into S phase, Cdc6 is targeted for degradation by SCF^{CDC4}-dependent ubiquitination and is subsequently degraded by the proteasome. Several mutations in consensus sites for Cdk phosphorylation at the N-terminus of Cdc6 in *Sacchromyces pombe* and *Sacchromyces cerevisiae* inhibit degradation, indicating that phosphorylation triggers this proteolysis [2]. In mammalian cells, phosphorylation of Cdc6 by cyclin A/Cdk2 at the N-terminal consensus CDK phosphorylation sites facilitates translocation of Cdc6 from the nucleus to the cytoplasm, thus preventing re-initiation of replication during S and G2 phases [3,4]. The conformational changes induced by phosphorylation of Cdc6 may unmask the C-terminal nuclear export signals (NES) required for nuclear export [5–7]. Overexpression of a mutant form of Cdc6/Cdc18 lacking CDK phosphorylation sites leads to the nuclear accumulation of the protein [8,9]. Thus, chromosomal re-replication can be induced in the absence of cell division by a mechanism that involves preventing cytoplasmic translocation of Cdc6 [10–12].

Cellular proteins containing both nuclear import and export signals can shuttle between the nucleus and the cytoplasm. Cells can regulate this dynamic movement and subsequently control the localization and activity of individual proteins and protein complexes [13,14]. Nuclear export signal (NES) sequences have been identified in many cellular proteins. In the HIV-1 Rev protein, the NES motif comprises a minimal 11-amino acid peptide (LQLPPLERLTL), and mutations of critical leucines abolish its export activity [15,16]. Functional NESs consist of a core of closely spaced leucines or other large hydrophobic amino acids. NESs have been detected in several very diverse cellular proteins and function by directly binding to a nuclear export receptor called the chromosomal region maintenance 1 (Crm1/exportin1) [17,18]. Cdc6 is reported to have a NES in residues 462–488, which contains a core of 15 highly hydrophobic amino acid residues, ILVCSLMLLIRQLKI [5].

In earlier reports, we suggested that caspase-3-mediated cleavage of Cdc6 disturbs the cytoplasmic localization of truncated Cdc6 and induces apoptosis [6,19]. In addition, caspase-3-mediated cleavage of Cdc6 generates two N-terminal Cdc6 truncation fragments, p49-tCdc6 and p32-tCdc6, which have lost the putative carboxy-terminal NES, but are differentially retained in the nucleus [6]. Our recent study showed that the phosphorylation site serine 74 is critical for the

Abbreviations: NES, nuclear export signal; NMT-Cdc6, a mutant version of Cdc6 with Lys to Ala in NES sequences; ASA-Cdc6, a mutant version of Cdc6 with S54A and S106A; SAA-Cdc6, a mutant version of Cdc6 with S74A and S106A; p49-tCdc6, a truncated Cdc6 at D442, producing a 49 kDa fragment; p32-tCdc6, a truncated Cdc6 at D290, producing a 32 kDa fragment

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transport of Cdc6 into the cytoplasm [7]. Interestingly, phosphorylation at S54 also contributes to its localization in the cytoplasm, but phosphorylation at S106 does not [7].

Based on these previous studies, we hypothesized that Cdc6 contains another NES sequence between amino acid residues 290 and 442, since p49-tCdc6 can still translocate into the cytoplasm by phosphorylation, albeit to a lesser extent than intact Cdc6 [6]. Furthermore, nuclear export of p32-tCdc6 is more strongly inhibited, even when it is phosphorylated at both S74 and S54 by cyclin A/Cdk2 [6,7]. Here, we show that Cdc6 interacts with Crm1 and that Cdc6 contains two distinct NES motifs. The cytoplasmic translocation of Cdc6 is mediated by interaction with Crm1 and the two NES motifs in the regions of amino acids 300–315 and 462–476. We further demonstrate that the cytoplasmic translocation driven by phosphorylation of S54 or S74 of Cdc6 is differentially regulated by two NES sequences.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) and calf serum were purchased from Life Technologies, Inc. (Carlsbad, CA, USA). The transfection reagent Polyfect® was purchased from Qiagen (Valencia, CA, USA), and other chemical reagents were purchased from Sigma (St. Louis, MO, USA).

2.2. Plasmids

Human Cdc6 cDNA was kindly provided by Dr. R. S. Williams. For mutation of the NES sequences, Leu 306, Leu 311, Leu 313, Leu 468, and Leu 470 residues were converted to Ala by altering the Leu codons GAT or GAC to Ala codons AAT or AAC, respectively. To generate chimeric E2F1, NES2 cDNA was generated by PCR using 5′-GTTGAATTCAATGG CCTTGGCCGGG-3′ (with added EcoRI site) and 5′-GAGGCCTGAAATCC AGGGGGGTG-3′ (with added StuI site) and inserted into pCMV-HA-E2F1 cDNA.

2.3. Cell culture, transfection and imaging

Human cervical carcinoma (HeLa) cells were maintained at 37 °C and 5% CO_2 as a monolayer culture in DMEM supplemented with 10% (v/v) heat-inactivated calf serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. HeLa cells were seeded on 35-mm culture plates or slides at a density of 5×10^4 cells/mL for 24 h before transfection with 3 μg of the appropriate plasmids using Polyfect (Qiagen) according to the manufacturer's instructions. For DAPI staining, cells were fixed in 4% paraformaldehyde solution (Sigma) for 10 min at room temperature after transfection, treated with DAPI solution for 10 min at 37 °C, and washed twice with PBS. Anti-HA polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Images were made with a camera connected to a fluorescence microscope (Nikon, Eclipse Ti, Tokyo, Japan).

2.4. Co-transfection

HeLa cells were transfected in 35-mm dishes with 1.5 μg of the appropriate plasmids using 12 μL Polyfect according to the manufacturer's instructions and harvested 24 h later in lysis buffer (0.5% Triton X-100, 20 mM Tris [pH 7.5], 2 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, 50 mM β-glycerophosphate, 25 mM NaF, 1 mM Na_3VO_4, 2 μg/mL leupeptin, 2 μg/mL pepstatin A, 100 μg/mL PMSF, 1 μg/mL antipain). Translocation analysis of HeLa cells was performed 24 h after transfection using a propidium iodide staining solution as described above.

2.5. Preparation of nuclear and cytoplasmic cell extracts and immunoblot analysis

Nuclear and cytoplasmic cell extracts were prepared from cells in lysis buffer (10 mM HEPES [pH 7.4], 10 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA, 25 μg/mL leupeptin, 5 μg/mL pepstatin A, 1 mM phenyl methyl sulfonyl fluoride [PMSF], 40 mM β-glycerophosphate, 1 mM dithiothreitol [DTT]). Nuclei were collected by centrifugation at 800 ×*g* for 10 min through 30% sucrose (800 ×*g*, 10 min at 4 °C) and suspended in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris [pH 8.0], 20 mM β-glycerophosphate, 50 mM NaF, 1 μg/mL leupeptin, 1 μg/mL antipain, 1 μg/mL pepstatin A, 0.1 mM PMSF). The cytosol was centrifuged at 100,000 ×*g* for 1 h, and the supernatant was collected. For Western blot studies, nuclear lysates were centrifuged at 12,000 rpm for 15 min at 4 °C, and the supernatants were collected. After adjusting the protein concentration (BCA assay, Pierce, Chester, UK), cell lysates were boiled and resolved by 12% SDS-PAGE before Western blot analysis with appropriate antibodies. An anti-Cdc6 monoclonal antibody raised against full-length Cdc6 of human origin (sc-9964), anti-p-S54-Cdc6 polyclonal (sc-12920-R), anti-pS74-Cdc6 polyclonal (sc-12921-R), anti-Cdk2 polyclonal and anti-cyclin A monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-flag monoclonal antibody was from Upstate (New York, NY, USA). Immune complexes were revealed using ECL™ Western blotting detection reagents (GE Healthcare Life Sciences, Piscataway, NJ, USA) and an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA).

2.6. Immunoprecipitation assay

Crm1 was immunoprecipitated from tCdc6-overexpressing HeLa cells using an anti-Crm1 polyclonal antibody (Santa Cruz) in buffer (0.5% Triton X-100, 20 mM Tris pH 7.5, 2 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, 50 mM β-glycerophosphate, 25 mM NaF, 1 mM Na_3VO_4, 2 μg/mL leupeptin, 2 μg/mL pepstatin A, 100 μg/mL PMSF, 1 μg/mL antipain), followed by incubation with protein A agarose at 4 °C for 2 h. The samples were combined with protein A agarose, suspended in SDS loading buffer, and resolved by SDS-PAGE.

2.7. GST pull-down assay

Glutathione S-transferase (GST) fusion polypeptides were expressed in *Escherichia coli* strain BL17 and purified using immobilized glutathione-sepharose 4B beads (GE Healthcare Life Sciences) according to the manufacturer's instructions. *E. coli* cells were lysed in lysis buffer (25 mM HEPES [pH 7.5], 5 mM EDTA, 2 mM DTT, 0.1% CHAPS 1 mM PMSF protease inhibitor cocktail). The GST fusion protein was purified and confirmed on SDS-polyacrylamide gels stained with Coomassie blue. For preparation of Cdc6, GFP-tagged wild type Cdc6 and NES mutants were expressed in HeLa cells. For affinity chromatography of transfectant GFP-tagged Cdc6 bound to GST-fusion polypeptides, 200 μg of the cell lysates was incubated with 40 μL GST-Crm1 resin (50% slurry) in PBS buffer for 4 h at 4 °C. The resins were washed four times with binding buffer, eluted with SDS-PAGE sample buffer, and subjected to SDS-PAGE and Western blotting.

2.8. FACS analysis

To observe cell cycle phase after treatment with hydroxyurea, cells were collected by trypsinization, fixed in 75% ethanol, stained with 500 μL of a 50 μg/mL propidium iodide solution, and subjected to fluorescence-activated cell sorting (FACS) analysis. Cells were sorted and analyzed using Guava Easy Cyte™ 8 and Guava Soft 2.2.3 (Millipore, Billerica, MA, USA).

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