Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 356 (2007) 668–673
BBRC
www.elsevier.com/locate/ybbrc

Phosphorylation of threonine 204 of DEAD-box RNA helicase DDX3 by cyclin B/cdc2 in vitro

Takeshi Sekiguchi *, Yoshiko Kurihara, Junko Fukumura

Department of Molecular Biology, Graduate School of Medical Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

Received 15 February 2007
Available online 15 March 2007

Abstract

DDX3 is a DEAD-box RNA helicase involved in human immunodeficiency virus mRNA export and translation. Previously, we reported that DDX3 is required for cyclin A expression. To examine whether DDX3 is regulated at the post-transcriptional level, we determined the phosphorylation sites of hamster DDX3 in vitro. Threonine 204 (Thr204) is a conserved amino acid residue of DDX3 homologues in yeast, frog, hamster, and human that is located within motif Q of DEAD-box RNA helicases. A Thr204 to Glu204 DDX3 mutant protein lost its function, suggesting that phosphorylation at Thr204 affects DDX3 function. Thr204 was phosphorylated by cyclin B/cdc2. Thr323 in motif Ib was also phosphorylated by cyclin B/cdc2 kinase. We propose a novel function of cyclin B/cdc2 kinase in mitosis, which is to cause a loss of DDX3 function to repress cyclin A expression and to decrease ribosome biogenesis and translation during mitosis.

Keywords: Cyclin B/cdc2; DDX3; Temperature-sensitive mutant; RNA helicase

DDX3/DBX is a DEAD-box RNA helicase involved in mRNA export and translation. DDX3 has an essential role in human immunodeficiency virus (HIV) mRNA export [1]. DDX3 interacts with the hepatitis C virus core protein, which induces liver cancer in transgenic mice [2]. Thus, DDX3 might have multiple roles in the production of various viruses and in the development of cancer. *Saccharomyces cerevisiae* Ded1p, which is one of the DEAD-box proteins required for general translation together with the eIF4A DEAD-box RNA helicase [3–5]. In *Schizosaccharomyces pombe*, a mutation of the RNA helicase Ded1p translation initiation factor inhibits B-type cyclins Cdc13 and Cig2 translation and cell-cycle progression [6]. Hamster tsET24 cells are temperature-sensitive (ts) mutant cells with a DDX3 gene mutation that exhibit normal cell growth at the permissive temperature, but G1 arrest at the nonpermissive temperature of 39.5 °C [7]. Cyclin A expression is decreased in tsET24 cells at 39.5 °C, suggesting that DDX3 is required for cyclin A expression.

Cyclin B/cdc2 kinases are key regulators in cell cycle progression in and out of mitosis. Cyclin B/cdc2 activation is required for the onset of mitosis. Cyclin/cdc2 kinases phosphorylate numerous substrates, such as nuclear lamins, kinesin-related motors and other microtubule-binding proteins, condensins, Golgi matrix components, and Golgi fragmentation, and regulate the anaphase-promoting complex/cyclosome; thereby regulating substrate activity (for review, see [8]). During mitosis, cdc2 kinases are believed to repress anabolic processes in the cells. Cdc2 kinases inhibit RNA polymerase II, TFIID, TFIIIB [9], poly(A) polymerase [10], elongation factor 1γ [11], and ribosomal S6 kinase 1 [12,13] to undergo mitotic silencing to spare energy for various mitotic events.

We report here that DDX3 might be a novel cyclin B/cdc2 kinase substrate protein. We determined the cyclin B/cdc2 kinase phosphorylation sites of hamster DDX3 at Thr204 and Thr323 in vitro, both of which were localized in conserved motifs (Q and Ib) of DDX proteins. Either Glu204

* Corresponding author. Fax: +81 92 642 6183.
E-mail address: sekigu@molbiol.med.kyushu-u.ac.jp (T. Sekiguchi).

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.03.038

or Glu323 mutation abolished the complementation activity in tsET24 cells at a nonpermissive temperature, thus suggesting that loss of DDX3 function is caused by phosphorylation at residue threonine 204 by the cdc2 protein kinase.

Materials and methods

Cell culture and transfection. tsET24, BHK21, and HeLa cells were grown as described previously [14]. tsET24 cells were maintained at the permissive temperature of 33.5 °C, and ts^+ cells were selected in the presence of hygromycin at 39.5 °C, the nonpermissive temperature. tsET24 cells (2×10^5 cells) were transfected with DNA-lipid complex using LipofectAMINE™ Reagent (Invitrogen, Carlsbad, CA) as described previously [15].

Purification of GST-fusion or His-tagged proteins. *Escherichia coli* BL21 harboring a GST or a pET28 plasmid was grown in 750 ml of Luria Bertani medium, treated with isopropyl β-D-thiogalactoside (final conc., 0.2 mM) for 4 h at 30 °C as described previously [16]. Cells were dispersed in lysis solution at a ratio of 1:5 [cell volume: lysis solution (1× PBS, 2 mM EDTA, 0.1% β-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin)] and sonicated three times for 5 min on ice (Sonicator™, Heat System-Ultrasonics Inc., Farmingdale, NY; with a microtip, 40% cycle, and output control 4). After centrifugation at 10,000*g* for 30 min at 4 °C, 10 ml of the supernatant was mixed with 1 ml of a 50% (v/v) slurry of glutathione Sepharose-4B beads (GE healthcare Piscataway, NJ) or nickel agarose beads (Qiagen, Chatworth, CA) and rotated for 30 min at 4 °C. The beads were washed four times with the lysis buffer and processed as described previously [17].

Immunoblotting and antibodies. Protein samples were electrophoresed on a 5–20% gradient gel (PAGEL, ATTO, Japan) and analyzed by immunoblotting. The anti-DDX3 antibody was described previously [14]. The rabbit anti-phospho-Thr204 antibody was raised against the synthetic peptide containing phospho-Thr204 (TRYTRP-phospho-T-PVQKHAI). The anti-cdc2 and cyclin B antibodies were described previously [18].

In vitro kinase assay. GST-fusion proteins were mixed with reaction buffer [50 mM Hepes (pH 7.5), 1 mM $MgCl_2$, 1 mM dithiothreitol, 2.5 mM EGTA, 10 mM β-glycerophosphate, 0.1 mM Na–orthovanadate, 1 mM NaF, 20 μM ATP] and [γ-^{32}P]ATP (10 μCi) (GE Healthcare) in 40 μl reaction solution as recommended by the supplier. The reaction mixtures were then mixed with either cyclin B/cdc2 (Upstate Biotechnology Waltham, MA; Catalog No. 14-450) or cyclin A/cdk2 (Cell Signaling Technology Beverly, MA) and incubated at 30 °C for 30 min.

In vitro mutagenesis. pCDEBΔ-DDX3 was mutagenized in vitro by PCR with KOD polymerase (Toyobo, Japan) as described previously [19]. The following oligonucleotides were used: Thr323 to Glu323, 323-glu-d; 5′-GATCTTTTAGTCGCTGAACCAGGTCGTTTGAATG-3′, 323-glu-r; 5′-CATTCAAACGACCTGGTTCAGCGACTAAAAGATC-3′, Thr323 to Ala323, 323-ala-d; 5′-GATCTTTTAGTCGCTGCTCCAGGTCGTT TGAATG-3′, 323-ala-r; 5′-CATTCAAACGACCTGGAGCAGCGACT AAAAGATC-3′. Thr204 to Glu204, 204-glu-d; 5′-GTTATACTCGC CCAGAACCAGTACAGAAGCA-3′, 204-glu-r; 5′-TGCTTCTGTACT GGTTCTGGGCGAGTATAAC-3′. Thr204 to Ala204, 204-ala-d; 5′-GT TATACTCGCCCAGCACCAGTACAGAAGCA-3′, 204-ala-r; TGCTT CTGTACTGGTGCTGGGCGAGTATAAC-3′. Thr204 to Leu204, 204-leu-d; 5′-GTTATACTCGCCCACTTCCAGTACAGAAGCA-3′, 204-le u-r; TGCTTCTGTACTGGAAGTGGGCGAGTATAAC-3′.

Results

DDX3 is phosphorylated by cyclin B/cdc2 kinase in vitro

The DDX3 sequence has three possible cyclin B/cdc2 kinase phosphorylation sites (TP and SP); Thr204, Thr323, and Ser492, in which Thr204 and Thr323 are well conserved among yeast, frog (*Xenopus laevis*), hamster, and human (Fig. 1 and [14]), suggesting that these two sites are important for its function. Human DDX3 has 98.5% homology with hamster DDX3 [14]. At first, we focused on the Thr204 phosphorylation site. Thr204 (TPVQ) locates within the motif Q [20,21] (Fig. 1). Kinase(s) pulled down from a HeLa cell extract with p13 beads and anti-cyclin B beads efficiently phosphorylated GST-DDX3 (Fig. 2A). We raised an antibody that specifically recognized phosphorylated-Thr204 and found that cyclin B/cdc2 phosphorylated wild-type DDX3 (Fig. 2B, lane 1), but failed to phosphorylate mutant DDX3 protein (204E, 323E) (Fig. 2B, lane 3). To further demonstrate that Thr204 was phosphorylated by cyclin B/cdc2, GST-fusion protein containing residues Gly157 to Leu239 was used for an in vitro phosphorylation assay (Fig. 2C). The GST fusion protein was efficiently phosphorylated by cyclin B/cdc2 kinase (Fig. 2C, lane 1). In contrast, the Glu204 GST fusion protein was not phosphorylated (Fig. 2C, lane 2), demonstrating that Thr204 is phosphorylated by cyclin B/cdc2 kinase. Because the substrate motifs for cyclin B/cdc2 are the same for cyclin A/cdk2, we examined whether cyclin A/cdk2 kinase phosphorylates DDX3. Unexpectedly, cyclin A/cdk2 failed to phosphorylate Thr204 (Fig. 2D).

Another possible cyclin B/cdc2 kinase phosphorylation site is Thr323 (TPGR), which locates within a motif Ib consensus site for DEAD-box RNA helicase activity (Fig. 1). GST-fusion proteins containing residues Arg294 to Asp368 were used for an in vitro phosphorylation assay (Fig. 2E). The GST fusion protein was efficiently phosphorylated by cyclin B/cdc2 kinase (Fig. 2E, lane 1). In contrast, when we introduced a mutation in which Thr323 was substituted with glutamic acid (Glu323), the GST-fusion protein was not phosphorylated by the kinase (Fig. 2E, lane 2), demonstrating that Thr323 is phosphorylated by cyclin B/cdc2 kinase. GST protein was used as a control to measure the background level of cyclin B/cdc2 kinase to GST protein.

DDX3 is phosphorylated in mitosis

Cyclin B/cdc2 is activated during mitosis. Therefore, we examined whether DDX3 was phosphorylated in mitosis. We treated mitotic HeLa cells with nocodazole to produce mitosis-arrested cells. For the G1 phase sample, HeLa cells were arrested at the G1 to S transition with thymidine and released to progress into the next G1 phase, which was examined by FACScan analysis. A phospho-protein-specific column was used to purify phosphorylated proteins at mitosis or at the G1 phase, as described in Materials and methods. In mitosis, the majority of DDX3 was bound to the column, while in the G1 phase only a small of DDX3 was bound to the column (Fig. 3, top panel, lane 3), suggesting that DDX3 was phosphorylated mainly in mitosis. Cdc2 was phosphorylated and enriched in the elution fraction and used as a positive (phosphorylated protein)