Available online at www.sciencedirect.com
SCIENCE DIRECT
Cellular Signalling 18 (2006) 1318–1326
CELLULAR SIGNALLING
www.elsevier.com/locate/cellsig

Phosphorylation of the Pro-X-Thr-Pro site in phosphatase inhibitor-2 by cyclin-dependent protein kinase during M-phase of the cell cycle☆

Mingguang Li [a], Bjarki Stefansson [a], Weiping Wang [a], Erik M. Schaefer [b], David L. Brautigan [a,*]

[a] *Center for Cell Signaling and Department of Microbiology University of Virginia School of Medicine Charlottesville, VA 22908, USA*
[b] *BioSource International 94 South Street Hopkinton, MA 01748, USA*

Received 28 September 2005; accepted 7 October 2005
Available online 27 December 2005

Abstract

Protein phosphorylation serves as a primary mechanism for triggering events during mitosis and depends on coordinated regulation of kinases and phosphatases. Protein Ser-Thr phosphatase-1 (PP1) activity is essential for the metaphase to anaphase transition and the most ancient regulator of PP1 conserved from yeast to human is inhibitor-2 (I-2), an unstructured heat-stable protein. A unique sequence motif in I-2 from various species surrounds a phosphorylation site PXTP that can be phosphorylated in biochemical assays by GSK3, MAPK and CDK kinases. Here we used a phosphosite specific antibody to investigate the phosphorylation of I-2. We fractioned extracts from HeLa cells arrested with nocodazole and assayed for PXTP kinases using recombinant I-2. One major and two minor peaks of kinase activity were identified and the major peak contained both active MAPK and cdk1∶cyclinB1, confirmed by immunoblotting. Cells released from a double thymidine block synchronously progressed through mitosis and immunoblotting revealed transient phosphorylation of endogenous I-2 in cells only during mitosis, and corresponding phosphorylation of histone H3 (Ser10) and PP1 (Thr320). Activation of cdk1∶cyclinB1 was coincident with I-2 phosphorylation, but neither MAPK nor GSK3 were phosphorylated at this time, so we concluded that in living cells only cdk1∶cyclinB1 phosphorylated the PXTP site in I-2. Immunofluorescent staining of cells with the PXTP phosphosite antibody revealed highly specific staining of mitotic cells prior to anaphase, at which point the staining disappeared. Thus, phosphorylation of I-2 is catalyzed by cdk1∶cyclinB1 and staining with a specific antibody should prove useful as a selective marker of cells in the early stages of mitosis.

Keywords: CDC2; Cyclin B1; GSK3; ERK1/2; MAPK; MEK; Histone H3; Nocodazole; Roscovitine

1. Introduction

Phosphorylation of thousands of cellular proteins predominantly occurs on Ser and Thr residues and the level of phosphorylation is under stringent control by protein Ser/Thr phosphatases, the major phosphatase is known as protein phosphatase-1 (PP1) [1]. In turn PP1 is regulated by a variety of subunits as well as inhibitor phosphoproteins [2–6]. The most ancient of PP1 regulators is the protein inhibitor-2 (I-2) that is conserved among all eukaryotes, from the GLC8 gene in yeast [7] to the abundant I-2 protein widely distributed in mammalian tissues [8,9]. Research about 20 years ago established that phosphorylation of the conserved PXTP site in Inh2 (Thr72 in the rabbit protein) causes changes in the conformation and activity of PP1 when the proteins are bound together in a heterodimer [10]. This PXTP site in I-2 is phosphorylated in biochemical assays by multiple protein kinases, in particular GSK3, ERK and CDC2 [11–14]. These assays used purified I-2 and/or the heterodimer of I-2∶PP1 reacting with purified protein kinases. This set of kinases is known to recognize substrates with a Pro residue following the phosphoacceptor Ser or Thr residue. Even after more than 20 years, whether any or all of these kinases phosphorylate the PXTP site in I-2 in living cells or tissues has not been examined and is unknown.

Previous work from this laboratory studied the phosphorylation of I-2 in living cells [15,16]. The I-2 purified from HeLa cells was analyzed by mass spectrometry, showing constitutive stoichiometric phosphorylation of multiple Ser sites reactive

☆ This work was supported in part by grants from the United States Public Health Service, National Institutes of Health GM-56362 and CA-77584 to D.L.B.
* Corresponding author. P.O. Box 800577, West Complex, MSB-7225, 1400 Jefferson Park Avenue, USA. Tel.: +1 434 924 5892; fax: +1 434 243 2829.
E-mail address: db8g@virginia.edu (D.L. Brautigan).

0898-6568/$ - see front matter © 2005 Published by Elsevier Inc.
doi:10.1016/j.cellsig.2005.10.020

with CK2, but no significant phosphorylation of the Thr72 site in the PXTP motif. However, we discovered that phosphorylation of the PXTP site in I-2 increased >25-fold in HeLa cells arrested in mitosis by nocodazole. Phosphorylation of PXTP was determined by retarded migration of I-2 in SDS–PAGE and use of a phosphosite antibody. Lysates of HeLa cells contained kinases that could phosphorylate the PXTP site in I-2, but only about half that activity was inhibited by the selective chemical inhibitor roscovitine, suggesting that the reaction was due to both cyclin-dependent kinases and other kinases [16]. Other work using yeast strains deleted for each of the 108 protein kinases showed that the I-2 homologue GLC8 containing the conserved PXTP motif was phosphorylated by just one cyclin-dependent kinase, Pcl6/7::Pho 85 [17]. Thus, the question remained: which kinases phosphorylate the PXTP site in I-2 in mammalian cells? This study set out to address this question. We found that I-2 in cells is only a substrate for cdk1::cyclinB1. The result is consistent with dedicated phosphorylation of the I-2 homologue GLC8 in yeast. We found ERK is activated in cells arrested by nocodazole, but not in cells proceeding through mitosis from a block at G_1/S, reconciling current and previous observations. Our experiments employed and characterized a new and better PXTP phosphosite-specific I-2 antibody that shows highly specific staining of cells in early stages of mitosis.

2. Materials and methods

2.1. Materials

Phosphosite-specific (PXTP) I-2 (Thr72) antibody was produced by BioSource International using a peptide DYTSpTP for immunization and affinity purification. The following antibodies were purchased from the indicated suppliers: mouse monoclonal IgG for His_6 tag (His-probe H-3) and anti-cyclin B1 antibody (Santa Cruz Biotechnology), anti-phospho-CDC2(Thr161) and CDC2 (Tyr15), anti-GSK-3β and phospho-GSK-3α/β (Ser21/9), anti-phospho-MEK1/2 (Ser217/221) (Cell Signaling Technology), anti-phospho-ERK1/2 and anti-phospho-histone H3(Ser10) (Upstate), anti-actin antibody (Sigma). Roscovitine and GSK3 inhibitor (3-(3-carboxy-4-chloroanilino)-4-(3-nitrophenyl)maleimide) were purchased from Calbiochem. Recombinant His_6-*S*-tagged I-2 was prepared as previously described by Eto et al. [5].

2.2. Column chromatography

HeLa cells were blocked in mitosis by incubating with 200 ng/ml nocodazole for 16 h and mitotic cells were shaken off from four T75 cell culture bottles. Mitotic HeLa cells were collected by centrifugation at 1000×*g* for 5 min, washed once by resuspension in PBS, and repelleted. Cells were lysed in a buffer containing 50 mM MOPS buffer, pH 8.0, 30 mM β-mercaptoethanol, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM PefaBloc, 1 mM vanadate, 1 μM microcystin-LR, 1% (v/v) Igepal CA-630, in a final volume of 2 ml. Insoluble material was removed by centrifugation at 13,000×*g* for 10 min in a microfuge. The supernatants were fractionated by fast protein liquid chromatography (Pharmacia) on a Mono Q HR5.5 column (Pharmacia) equilibrated with buffer A containing 50 mM MOPS, pH 8.0, 30 mM β-mercaptoethanol, 5 mM $MgCl_2$, 1 mM EGTA. A total of 8 mg of protein was applied. Elution was carried out with a linear gradient of NaCl from 0 to 1.0 M in buffer A and 0.5 ml fractions were collected.

2.3. Cell synchronization and flow cytometric analysis

During double thymidine block the cells become arrested at the G1/S phase border. HeLa cells (2×10^5) were seeded in a series of 6-cm plates and cultured in complete medium. At 12 h after replating, 2 mM thymidine was added and 16 h later, the thymidine was washed away with PBS and the cultures were grown in complete medium for 8 h. The entire cycle of treatment was repeated once. After release from the second thymidine block, individual plates of cells were harvested at various times by aspiration of the media and direct addition of 300 μl of 2× SDS sample buffer. The cells were scraped and transferred to tubes that were heated for 5 min at 95 °C, then stored at −20 °C. The cells for flow cytometry were washed with PBS, released from the plates with trypsin and fixed with 70% ethanol overnight at 4 °C. The ethanol was removed by centrifugation for 5 min at 300×*g*. Cells were washed with PBS and stained for 30 min at room temperature with 40 μg/ml propidium iodide in PBS (containing 200 μg/ml RNase A and 0.1% Triton X-100). Analysis was done at the University of Virginia Flow Cytometry Facility and cell cycle distribution was analyzed using ModFit LT version 3.1 software (Verity Software House, Inc.). For each time at least 1×10^6 cells were analyzed. The entire analysis was repeated with identical results.

2.4. Cell culture, western blotting, and kinase assay

HeLa cells were cultured in Dulbecco's modified Eagle's Medium supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. MonoQ fractions and samples from thymidine block/release were immunoblotted with specific antibodies using 12% SDS–PAGE. For kinase assay of MonoQ fractions reaction mixtures contained, in a final volume of 50 μl, 25 mM MOPS, pH 7.4, 0.1% β-mercaptoethanol, 5 mM $MgCl_2$, 1 mM EGTA, 0.1 mM ATP and 25 μl of MonoQ fraction plus 5 μg of recombinant I-2 as substrate. After incubation at 25 °C overnight, the reactions were stopped by adding 50 μl of 2× SDS sample buffer. The phosphorylated Thr72 in I-2 was detected by immunoblotting with anti-phospho-I2 (Thr72) antibody (anti-PXTP) and development using Super-Signal West Pico system from Pierce (Rockford, IL). The relative intensity of staining was quantitated by densitometry (Molecular Dynamics) and ImageQuant software. HeLa cells 9 h after release from double thymidine block were collected by centrifugation and lysed in 50 mM MOPS, pH 8.0, 0.1% β-mercaptoethanol, 5 mM $MgCl_2$, 150 mM NaCl, 1 mM EGTA, 1 mM PefaBloc, 1 μM microcystin-LR, 1 mM vanadate, 20 mM β-glycerophosphate, 1% (v/v) Nonidet P-40 (Igepal CA-630). The cell extracts obtained after centrifugation at 13,000×*g* for 10 min were incubated with recombinant His_6-*S*-tag-I-2 in the same kinase buffer as described above. Kinase reactions were stopped by 2× SDS sample buffer and the samples were immunoblotted with anti-phospho-PXTP (Thr72) I-2 antibody.

2.5. Immunofluorescence microscopy

Microscopic images of HeLa cells were obtained as described previously by Leach et al. [18] and Eto et al. [19]. HeLa cells were seeded onto 22 mm cover slips, grown overnight, then rinsed with PBS and fixed with 2% paraformaldehyde in PBS at 37 °C for 20 min. Cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min, washed three times with PBS and blocked with 3% bovine serum albumin in PBS for 1 h at 37 °C. Primary antibody (anti-phospho-PXTP (Thr72) I-2 at 1:1000 and the anti-phospho(Ser10) histone H3 at 1:200 dilution) was incubated overnight at 4 °C and following three washes with PBS the secondary antibodies plus Hoechst (1:20,000 dilution) were incubated 1 h at 37 °C. The specimens were washed three times with PBS then mounted with Vectashield (Vector Labs, Burlingham, CA) and microscopic images acquired on a Nikon Microphot SA with a Hamamatsu C4742 digital camera operated by OpenLab software (Improvision) and processed in Adobe PhotoShop.

3. Results

We prepared soluble extracts from HeLa cells shaken off the plates after 16 h of treatment with nocodazole and separated the proteins by gradient elution in MonoQ anion exchange chromatography. The individual fractions were used as a source of kinase in an assay with recombinant tagged I-2 plus MgATP. Phosphorylation of the PXTP site in I-2 was analyzed by