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The cyclin-dependent kinase 11 interacts with 14-3-3 proteins

Yongmei Feng [a,b,*], Wenqing Qi [b], Jesse Martinez [b], Mark A. Nelson [a,b,*]

[a] Department of Pathology, University of Arizona, 1501 N Campbell Avenue, Tucson, AZ 85724, USA
[b] Arizona Cancer Center, 1515 N. Campbell Avenue, Tucson, AZ 85724, USA

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Abstract

Cyclin-dependent kinase 11 isoforms (CDK11) are members of the $p34^{cdc2}$ superfamily. They have been shown to play a role in RNA processing and apoptosis. In the present study, we investigate whether CDK11 interacts with 14-3-3 proteins. Our study shows that the putative 14-3-3 binding site (113-RHRSHS-118) within the N-terminal domain of $CDK11^{p110}$ is functional. Endogenous $CDK11^{p110}$ binds directly to 14-3-3 proteins and phosphorylation of the serine 118 within the RHRSHS motif seems to be required for the binding. Besides, $CDK11^{p110}$ is capable of interacting with several different isoforms of 14-3-3 proteins both in vitro and in vivo. The interaction of 14-3-3 γ with $CDK11^{p110}$ occurs throughout the entire cell cycle and reaches maximum at the G2/M phase. Interestingly, 14-3-3 γ shows strong interaction with N-terminal portion of caspase-cleaved $CDK11^{p110}$ ($CDK11^{p60}$) product at 48 h after Fas treatment, which correlates with the maximal cleavage level of $CDK11^{p110}$ and the maximum activation level of CDK11 kinase activity during apoptosis. Collectively, these results suggest that CDK11 kinases could be regulated by interaction with 14-3-3 proteins during cell cycle and apoptosis.

Keywords: CDK11; 14-3-3 proteins; Cell cycle; Apoptosis

Cyclin-dependent kinase 11 isoforms (CDK11; formerly named PITSLRE) are members of the cyclin-dependent kinase superfamily. The larger $CDK11^{p110}$ isoforms associate with cyclin L isoforms and appear to regulate different cellular functions, including RNA processing and apoptosis [1–6]. One specific isoform $CDK11^{p58}$, which is generated from an internal ribosome entry site of $CDK11^{p110}$, is found to be expressed during the G2/M phase of the cell cycle and implicated in mitosis [6–9]. Studies have also indicated that proteolytic processing of CDK11 by caspase 3 might serve as effectors in apoptotic signaling pathways [10–12]. During apoptosis induced by Fas, tumor necrosis factor, or staurosporine, $CDK11^{p110}$ isoforms are cleaved into two fragments—one fragment is 60 kDa in size and contains the regulatory domains of CDK11; whereas the other fragment is 46–50 kDa in size and contains the catalytic domain [10–12]. Overexpression of $CDK11^{p46}$ inhibits protein synthesis and induces apoptosis, suggesting that $CDK11^{p46}$ might play a role in the execution phase of apoptosis [13].

In addition to cyclin L family members, proteins that associate with CDK11 are only now becoming clear. Previous studies by our group demonstrate that heat shock protein (Hsp90) interacts with $CDK11^{p110}$ and $CDK11^{p46}$, which can stabilize CDK11 kinase and therefore is crucial for its pro-apoptotic function [14]. In the N-terminal region of $CDK11^{p110}$, a putative 14-3-3 binding site (R-H-R-S-H-S, codon 113–118) was identified by sequence analysis using several molecular tools online (http://elm.eu.org; http://scansite.mit.edu). The 14-3-3 proteins are a family of widely expressed phosphoserine/phosphothreonine-binding proteins [15]. They are highly conserved in higher eukaryotes, invertebrates,

* Corresponding authors. Fax: +1 520 626 8864 (M.A. Nelson).
E-mail addresses: yfeng@email.arizona.edu (Y. Feng), mnelson@azcc.arizona.edu (M.A. Nelson).

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and plants. Several 14-3-3 isoforms (β, γ, ε, ζ, η, τ, and θ) have been identified, which bind to many cellular proteins. Depending on the target proteins, 14-3-3 binding may inhibit or activate the catalytic activity of its associated enzymes, alter the interaction of its protein targets with other proteins, or induce relocalization of its binding partners [15,16].

Two duplicate genes *Cdc2L1* and *Cdc2L2* express several different CDK11 protein kinase isoforms, many arising by alternative splicing [17]. The most common alternatively spliced transcripts generate proteins with or without the putative 14-3-3 consensus binding site, as well as proteins with or without the caspase cleavage sites that are used during TNF- and Fas-mediated apoptosis [17]. To confirm the interaction of $CDK11^{p110}$ and 14-3-3 proteins, and to further investigate the functional significance of the interaction, we performed immunoprecipitation and GST pull-down assay. Here, we report that $CDK11^{p110}$ interacts with several isoforms of 14-3-3 proteins both in vitro and in vivo, and the interactions could play a role in cell cycle and apoptosis.

Materials and methods

Cell culture and treatment. Human melanoma cell lines were obtained from ATCC (Manassas, VA). A375 cells were grown as monolayers in RPMI 1640 (Mediatech, Herndon, VA) supplemented with 5%(v/v) fetal bovine serum (Omega Scientific, Tarzana, CA), 1% L-glutamine, and 1% penicillin–streptomycin. A375 cells (1×10^6) were seeded and incubated overnight at 37 °C in a humidified 5% CO_2 environment and treated with 0.5 μg/ml anti-Fas monoclonal antibody CH-11 (Upstate Biotechnology, Lake Placid, NY). After 48 and 60 h at 37 °C, control and treated cells were harvested by low-speed centrifugation, washed twice with PBS, and lysed as previously described [10].

Antibodies. GN1 is an affinity-purified rabbit polyclonal antibody obtained by injection of GST-CDK11 containing amino acids 341–413 of CDK11 (Rockland, Gilbertsville, PA). Antibodies against 14-3-3 proteins were purchased from Santa Cruz Biotechnology. The antibody used for detecting all the 14-3-3 proteins is 14-3-3 β (H-8). The antibody used specific for 14-3-3 β, 14-3-3 γ, 14-3-3 ε, 14-3-3 θ, 14-3-3 ζ, and 14-3-3 σ is A-6, C-16, T-16, C-17, C-16, and N-14, respectively. Monoclonal c-myc antibody was obtained from Sigma.

Construction of vectors and site-directed mutagenesis. Plasmids GST-14-3-3 β, 14-3-3 γ, 14-3-3 ε, 14-3-3 θ, and14-3-3 ζ were constructed by RT-PCR and inserted into pGEX vector (Amersham Biosciences). pCMV-Myc-$CDK11^{p60}$ construct containing the N-terminal portion of $CDK11^{p110}$ coding sequence (GenBank Accession no: U04824) was constructed by RT-PCR. The primers used were: 5′-GGAATTCAAATGGGTGATGAAAAGGACTCTT-3′ and 5′-GG GGTACCTAGGTACTTGGGCAGCTCCTG-3′, in which *Eco*RI and *Kpn*I restriction sites were added to the forward and reverse primers, respectively. The 1.2-kb fragment was then cloned in-frame into a mammalian expression vector pCMV-Myc (Clontech, Palo Alto, CA) with myc tag at the N-terminal of fusion pCMV-Myc-$CDK11^{p60}$. Substitution mutation pCMV-Myc-$CDK11^{p60}$SA (with serine substituted by alanine at position 118) was generated with the QuickChange site-directed mutagenesis kit (Stratagene, Cedar Creek, TX) according to the manufacturer's instructions and confirmed by DNA sequencing.

Transient transfection. Transient transfections of A375 cells were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Briefly, A375 cells were seeded in six-well plates at a density of 1×10^6 cells/well. After 24 h, cells were transfected with complexes containing 10 μl LF2000 and 4 μg of myc-tagged $CDK11^{p60}$ or $CDK11^{p60}$SA.

Purification of GST-fused 14-3-3 proteins and GST pull-down assay. GST and GST-fused different isoforms of 14-3-3 proteins were induced with 0.2 mM IPTG and expressed in BL21 bacteria for 5 h at 30 °C. The recombinant proteins were purified using the Bulk GST Purification Module according to the manufacturer's instruction (Amersham Pharmacia Biotech). Purified proteins were concentrated to an appropriate concentration and stored as aliquots at −80 °C. Equal amounts of GST and GST-fused 14-3-3 isoforms bound to glutathione–Sepharose were incubated with 250 μg A375 cell lysates overnight at 4 °C. The beads were then washed five times with binding buffer and boiled in SDS sample buffer. The bound CDK11 protein was analyzed by Western blot analysis after separation by SDS–polyacrylamide gel electrophoresis (PAGE).

Immunoprecipitation and Western blotting. Cells were harvested, washed twice with cold PBS, and lysed in lysis buffer (25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EGTA, 50 mM glycerophosphate, and 0.1% NP 40) containing 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 1% protease inhibitor cocktail (Sigma) for 30 min on ice. Following lysis, cells were centrifuged at 13,000*g* for 10 min at 4 °C and the protein content was determined using the bicinchoninic acid assay (Pierce, Rockford, IL). Total cell lysates (250 μg) were pre-cleared with protein A–agarose beads (Oncogene, La Jolla, CA) and rabbit IgG at 4 °C for 1 h. 14-3-3 proteins were then immunoprecipitated using anti-14-3-3 antibodies overnight at 4 °C. Approximately 20μl of packed protein A–agarose beads was added, and the incubation was continued for another 2 h. The immunocomplexes were then washed three times with lysis buffer and subjected to SDS–PAGE. The proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad) and the blots were probed with anti-CDK11 antibody. A secondary probe with horseradish peroxidase conjugated antibodies (Sigma) was detected by enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech).

Cell synchronization and cell cycle analysis. A375 cells were grown until confluent in regular media in a T-175 flask. RPMI containing 0.5% FBS was added for 72 h to arrest the cells. Cells were then released from arrest by feeding with regular medium and plated in a 100-mm tissue culture plate at a density of 1.5×10^6 cells/plate. Cells were collected for analysis at 6, 9, 12, 18, 21, 24, and 30 h after release.

Synchronized cells were harvested at the indicated time points and stained with propidium iodide as described before [18]. One million cells were pelleted and washed with cold PBS. Pellets were then resuspended in 1 ml PBS. Three milliliters of 100% ethanol was added drop by drop while the sample was vortexed and then stored at −20 °C. After all samples were collected, they were pelleted, and washed again before resuspended in 1 ml of Krishan's buffer. The samples were incubated in dark for 30 min at room temperature and sent for FACS analysis.

Results

Some isoforms of CDK11 contain a putative 14-3-3 binding site

14-3-3 proteins bind to two types of consensus sites: R-(S/Ar)-X-pS-X-P and R-X-(S/Ar)-(+)-pS-X-P, where Ar is an aromatic amino acid, pS is phosphoserine, + is a basic amino acid, and X is any amino acid [19,20].