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The cyclin-dependent kinase 11 interacts with NOT2

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Abstract

The caspase-processed cyclin-dependent kinase 11 (formerly known as PITSLRE) is implicated in apoptotic signaling. However, the mechanism of apoptotic signal transduction through CDK11^{p46} is still unclear. We used a yeast two-hybrid screening strategy and identified NOT2 as an interacting partner of caspase-processed C-terminal kinase domain of CDK11 (CDK11^{p46}). We demonstrate that CDK11^{p46} directly interacts with NOT2 in vitro and in human cells. The NOT domain in the C-terminal part of NOT2 is responsible for the association between CDK11^{p46} and NOT2. Both NOT2 and CDK11^{p46} predominantly co-localized in the nucleus. Furthermore, we show that overexpression of NOT2 reduces luciferase mRNA and induces apoptosis. However, NOT2 is not phosphorylated by CDK11^{p46}. These findings suggest that CDK11 may contribute to apoptosis by regulating the activity of NOT2 independent of its kinase activity.

Keywords: Cyclin-dependent kinase 11; CDK11^{p46}; PITSLRE; NOT2; Apoptosis; CCR4–NOT complex; Caspase; RNA processing; Yeast two-hybrid; Phosphorylation

It is becoming increasingly clear that in addition to controlling the cell cycle, cyclin-dependent kinases may have other functions within the cell. The CDK11 (also known as PITSLRE) protein kinases are members of the cyclin-dependent kinase superfamily. Two distinct but closely related human CDK11 genes (Cdc2L 1 and Cdc2L 2) express several CDK11 isoforms [1,2]. CDK11 homologues exist in several species including human, mouse, chicken, *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Xenopus* [3–5]. The highly conserved nature of the CDK11 suggests important cellular functions.

Although CDK11 is a member of the cyclin-dependent kinase superfamily, their function within the cell is not totally clear. However, recent studies indicate that the p110 isoform of CDK11 (CDK11^{p110}) may be involved in some aspect of RNA processing or transcription by virtue of the fact that CDK11 co-immunoprecipitate and/or co-purify with multiple transcriptional elongation factors [3,4]. Furthermore, CDK11^{p110} associates with Cyclin L [5]. Cyclin L is an RS domain protein that may function in pre-mRNA splicing [5]. These observations suggest that CDK11^{p110} kinases play some role in the production of translatable RNA transcripts in proliferating cells. The CDK11^{p110} isoforms contain an internal ribosome entry site (IRES) which leads to the generation of a CDK11^{p58} isoform during the G_2/M phase of the cell cycle [6]. Elevated expression of CDK11^{p58} in eukaryotic cells alters normal cytokinesis and can delay cells in late telophase [7]. CDK11^{p58} appears to interact with cyclin D3 [8]. This suggests that CDK11^{p58}/cyclin D3 may play a role in mitosis.

With regard to apoptosis, increased expression of CDK11^{p58} reduces cell growth due to apoptosis [1]. In addition, our group and others have shown that the CDK11^{p110} isoform and the CDK11^{p58} isoform are cleaved by caspases to generate a smaller 46–50 kDa protein that contains the catalytic portion of the protein [2,9,10]. This smaller CDK11^{p46} isoform can be triggered

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by Fas, tumor necrosis factor A, or staurosporine and phosphorylate histone H1. Collectively, these observations suggest that CDK11 may play a role in apoptotic signaling. However, little is known about the substrates potentially regulated by CDK11 during apoptosis. Recently, studies by our group indicate that CDK11^{p46} interacts and phosphorylates the eukaryotic initiation factor 3f (eIF3f) which leads to inhibition of translation [11]. Another study showed that CDK11^{p46} interacts with p21-activated kinase (PAK1) and inhibits its activity [12]. These observations suggest that the caspase-processed CDK11^{p46} isoform may be a downstream effector protein kinase in apoptotic signaling pathway.

To identify cellular factors involved in apoptotic signaling function of CDK11^{p46}, we performed a yeast two-hybrid screen using CDK11^{p46} as bait. We identify NOT2 as a protein associated with the caspase-processed isoform of CDK11 (CDK11^{p46}). The interaction between CDK11^{p46} and NOT2 occurs in vitro and in vivo. In addition, we show that NOT2 reduces luciferase mRNA and induces apoptosis in melanoma cells.

Materials and methods

Cell culture and transfection. A375 human melanoma cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured at 37 °C with 5% CO_2 in RPMI 1640 medium (Mediatech, Herndon, VA), supplemented with 5% fetal bovine serum (Omega Scientific, Tarzana, CA), 1% L-glutamine, and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA). HEK293 (human embryonic kidney 293) cell line was cultured in Dulbecco's modification of Eagle's medium (Mediatech) supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin at 37 °C with 5% CO_2. All transfections were carried out using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instruction.

Antibodies. Rabbit polyclonal HA antibody and monoclonal c-Myc antibody were purchased from Sigma. GN1 is an affinity-purified rabbit polyclonal antibody raised by injection of rabbit with purified recombinant glutathione *S*-transferase (GST) fused with the sequence coding for amino acids 341–413 of CDK11^{p110} (Rockland, Gilbertsville, PA). CDK11 monoclonal antibody P1C recognizes the last 75 amino acids of CDK11^{p110} and was a gift from Drs. Vincent Kidd and Jill Lahti, St. Jude Children's Research Hospital, Memphis, Tennessee [4]. NOT2 antibody was a gift from Dr. H.Th. Marc Timmers (University Medical Center Utrecht, Universiteitsweg 100, 3584 CG Utrecht, The Netherlands). Xpress antibody was purchased from Invitrogen.

Yeast two-hybrid assay. Matchmaker GAL4 two-hybrid system 2 (Clontech, Palo Alto, CA) was used to perform yeast two-hybrid screening according to the manufacturer's instruction. The wild-type and K451M substituted CDK11 C-terminal portion (CDK11^{p46}) were used as baits. In the K451M altered CDK11^{p46}, one lysine residue that is associated with phosphate transfer was substituted with a methionine. This substitution was performed to stabilize the interaction between the kinase and its substrate [16]. A total of 5×10^6 transformants from a human fetal brain matchmaker cDNA library (Clontech) were screened in the yeast strain AH109 (Clontech) and 28 colonies were identified as His^+Ade^+, out of which 13 were positive for β-galactosidase. Among the 13 positive clones, 12 were isolated when the K451M mutant CDK11^{p46} was used as bait, whereas one clone was isolated using wild-type CDK11^{p46} as bait. Sequence analysis revealed that one of these clones (clone 22) encodes the partial sequence of NOT2. To confirm the CDK11^{p46}/NOT2 interaction in yeast, 0.1 μg of clone 22 and wild-type bait plasmid was co-transfected into yeast strain AH109 using the lithium acetate transformation method. Growth selection was performed according to the manufacturer's protocol (Clontech).

Construction of vectors. Two DNA fragments encoding truncated NOT2, amino acids 1–291 and 290–540 were obtained by PCR amplification using B42-NOT2 (a gift from Dr. H.Th. Marc Timmers) as template and cloned into pGEX-4T-2 vector (Amersham Pharmacia Biotech, Piscataway, NJ) to generate deletion GST-NOT2 fusion proteins. pCMV-Myc-CDK11^{p46} containing the CDK11^{p46} coding sequence (GenBank Accession No. U04824, nt 1282–2465) with a c-Myc tag at the N-terminus was constructed by PCR using p110KS$^+$ (a gift from Dr. Vincent Kidd) as template. pcDNA3-CDK11^{p46} was constructed by cloning the PCR amplified CDK11^{p46} with an in-frame start codon ATG (nt 1121–2465) into the *Eco*RI site of an eukaryotic expression vector pcDNA3 (Invitrogen) for in vitro transcription and translation. pCMV-HA-NOT2 was constructed by inserting the NOT2 sequence isolated from the original fetal brain cDNA clone that we obtained by yeast two-hybrid assay into pCMV-HA vector (Clontech) using *Eco*RI/*Xho*I restriction enzymes. The full-length NOT2 was PCR amplified using B42-NOT2 as template and cloned into pcDNA4/HisMax-TOPO (Invitrogen).

Purification of recombinant protein from Escherichia coli. GST and GST fused NOT2 fragments were induced by 0.2 mM IPTG and expressed in BL21 bacteria for 4 h at 30 °C. The recombinant proteins were purified using Bulk GST Purification Module according to the manufacturer's instruction (Amersham Pharmacia Biotech). Purified proteins were concentrated using Centricon 30 (Amicon) to an appropriate concentration and stored as aliquots at −70 °C.

GST pull-down assay. The assay was performed as described previously [11]. Briefly, GST or GST fusion proteins were expressed in BL21 cells and equal amount of bacterial lysates was incubated with 25 μl of glutathione–Sepharose beads for 30 min. The beads were then washed three times with PBS and incubated with 5 μl of in vitro transcribed and translated [^{35}S]methionine-labeled CDK11^{p46} overnight at 4 °C. [^{35}S]Methionine-labeled CDK11^{p46} was produced using a TNT coupled reticulocyte lysate system (Promega). The beads were then washed five times with binding buffer and boiled in SDS sample buffer. The bound CDK11^{p46} protein was analyzed by autoradiography after resolved by SDS–PAGE.

Immunoprecipitation and Western blotting. Cells were harvested, washed twice with cold PBS, and lysed in lysis buffer (10 mM Hepes, pH 7.2, 142.5 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, and 0.2% 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 lysate (500 μg) was pre-cleared with protein A– or G–agarose beads (Oncogene, La Jolla, CA) and rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C for 1 h. HA-NOT2 or endogenous NOT2 was then immunoprecipitated using HA antibody or NOT2 antibody and protein A–agarose overnight at 4 °C. The immune complex was then washed three times with lysis buffer and subjected to SDS–polyacrylamide gel electrophoresis (PAGE). The proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA) and the blots were probed with different antibodies. A secondary probe with horseradish peroxidase-labeled antibodies (Sigma) was detected by enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech).

Immunofluorescence confocal microscopy. pCMV-HA-NOT2 transiently transfected A375 cells were grown on coverslips, washed twice with cold PBS, and fixed in 4% paraformaldehyde in PBS for 20 min at room temperature (RT). Cells were rinsed three times with PBS and permeabilized with 100% methanol at −20 °C for 6 min. Cells on coverslips were washed with PBS again and incubated with 5% bovine serum albumin (BSA) in PBS for 10 min at RT and then the BSA was