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The cyclin-dependent kinase 11^{p46} isoform interacts with RanBPM

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Abstract

We identified Ran-binding protein (RanBPM) as an interacting partner of the caspase-processed C-terminal domain of cyclin-dependent kinase 11 (CDK11^{p46}) by using the yeast two-hybrid system. CDK11^{p110} protein kinases are members of the cyclin-dependent kinase superfamily. During staurosporine-, Fas-, and tumor necrosis factor α-induced apoptosis caspase-processed activated CDK11^{p46} is generated from larger CDK11^{p110} isoforms. CDK11^{p46} promotes apoptosis when it is ectopically expressed in human cells. However, the mechanism of signal transduction through CDK11^{p46} is still unclear. In this study, we demonstrate that CDK11^{p46} directly interacts with RanBPM in vitro and in human cells. RanBPM contains a conserved SPRY (repeats in splA and Ryr) domain and is localized both in the nucleus and cytoplasm. The SPRY domain of RanBPM is responsible for the association between CDK11^{p46} and RanBPM. Furthermore, we show that CDK11^{46} phosphorylates RanBPM.

Keywords: Cyclin-dependent kinase 11; CDK11^{p46}; RanBPM; Apoptosis; Phosphorylation; PITSLRE

Cyclin-dependent kinase 11 isoforms (CDK11; also named PITSLRE) are members of the cyclin-dependent kinase superfamily. Two closely related human CDK11 kinase genes (Cdc2L 1 and Cdc2L 2) express several CDK11 isoforms [1] whose functions appear somewhat diverse. The larger CDK11^{p110} isoforms associate with cyclin L and are involved in pre-mRNA splicing [2,3] and in the regulation of transcription [4]. During the G2/M phase of the cell cycle, an internal ribosome entry site in the CDK11^{p110} cDNA produces a smaller CDK11^{p58} isoform. The cyclin partner of CDK11^{p58} appears to be cyclin D3 [5]. The CDK11^{p58} isoform may play a role in mitosis [5–7]. During anoikis, tumor necrosis factor α- and Fas-induced apoptosis, CDK11^{p110} and CDK11^{p58} are cleaved by caspases to generate smaller 46–50-kDa proteins containing the catalytic kinase domain [8–12]. It was shown that ectopic expression of a CDK11^{p46} isoform that resembles the final caspase-modified product induces apoptosis [5,12,13]. Although the CDK11^{p46} isoform clearly participates in regulating some aspect of apoptotic signaling, little is known about its substrates. Recent studies indicate that CDK11^{p46} interacts and phosphorylates eukaryotic initiation factor 3f (eIF3f) which leads to inhibition of translation [13]. Another study showed that CDK11^{p46} interacts with p21-activated kinase (PAK1) and inhibits its activity [12]. Collectively, these observations suggest that the caspase-processed CDK11^{p46} isoform may be a downstream effector protein kinase in apoptotic signaling cascades.

To identify cellular factors involved in apoptotic signaling functions of CDK11^{p46}, we performed a yeast two-hybrid screen of a human fetal brain cDNA library, using CDK11^{p46} as a bait. Here, we report that CDK11^{p46} interacts with RanBPM, in vitro and in vivo. We determined that SPRY domain of RanBPM is responsible for this interaction. In addition, we show that CDK11^{p46} phosphorylates RanBPM.

Materials and methods

Cell culture and transfection. A375 human melanoma cell line was obtained from the American Type Culture Collection. The cells were

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cultured at 37 °C with 5% CO_2 in RPMI 1640 medium (Mediatech), supplemented with 5% fetal bovine serum (Omega Scientific), 1% L-glutamine, and 1% penicillin/streptomycin (Invitrogen). All transfections were carried out using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instruction.

Antibodies. RanBPM antibody is a polyclonal antibody raised by injection of goat with purified recombinant GST-RanBPM (Rockland). GN1 is an affinity-purified rabbit polyclonal antibody obtained by injection of GST-CDK11 containing amino acids 341–413 of CDK11 (Rockland). CDK11 monoclonal antibody P1C recognizes the C-terminal 75 amino acids of $CDK11^{p110}$ and was a gift from Drs. Vincent Kidd and Jill Lahti (St. Jude Children's Research Hospital, Memphis, TN) [4]. Rabbit polyclonal HA antibody and monoclonal c-Myc antibody were purchased from Sigma.

Yeast two-hybrid assay. Matchmaker GAL4 two-hybrid system 2 (Clontech) was used to perform yeast two-hybrid screening according to the manufacturer's instruction. The wild type or 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 [24]. A total of 5×10^6 transformants from a human fetal brain matchmaker cDNA library (Clontech) were screened in the yeast strain AH109 (Clontech), 28 colonies were identified as His^+, Ade^+, and 13 clones were positive for β-galactosidase activity. 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 2 of these clones (clones 37 and 50) encode the partial sequences of RanBPM. To confirm the interaction between $CDK11^{p46}$ and RanBPM interaction in yeast, 0.1 μg of clone 37 or 50 was co-transfected with wild-type $CDK11^{p46}$ into yeast strain AH109 using the lithium acetate transformation method. Growth selection was performed according to the manufacturer's protocol (Clontech).

Construction of vectors. pCMV-Myc-$CDK11^{p46}$ vector containing the $CDK11^{p46}$ coding sequence (GenBank Accession No.: U04824) with a c-Myc tag at the N terminus was constructed by PCR using $p110KS^+$ (a gift from Dr. Vincent Kidd) as template. pCMV-HA-RanBPM and pGEX-4T-2-RanBPM were constructed by inserting the RanBPM sequence isolated from the original fetal brain cDNA clone that we obtained by a yeast two-hybrid assay into vectors pCMV-HA (Clontech) or pGEX-4T-2 (Amersham–Pharmacia Biotech) using *Sma*I/*Xho*I restriction enzyme sites. Fragments encoding truncated RanBPM, containing amino acids 207–339 and 334–729, were obtained by PCR amplification and cloned into pGEX-4T-2 vector to generate GST-RanBPM deletion proteins.

Purification of recombinant protein from Escherichia coli. GST, GST-RanBPM, and GST deletion mutants of RanBPM were induced with 0.6 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 using Centricon 30 (Amicon) to an appropriate concentration and stored as aliquots at −70 °C.

GST pull-down assay. Equal amounts of GST, GST-RanBMP, or GST deletion mutants of RanBPM bound to glutathione–Sepharose were 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 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). Total cell lysates (250 μg) were pre-cleared with protein A or G–agarose beads (Oncogene) and mouse or goat IgG (Santa Cruz Biotech) at 4 °C for 1 h. c-Myc-$CDK11^{p46}$ fusion protein or RanBPM was then immunoprecipitated using c-Myc monoclonal antibody (Sigma) or goat RanBPM polyclonal antibody for 4 h at 4 °C. Approximately 25 μl of packed protein A or G–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 (BioRad) and the blots were probed with different antibodies. A secondary probe with horseradish peroxidase conjugated antibodies (Sigma) was detected by enhanced chemiluminescence (ECL) (Amersham–Pharmacia Biotech).

Immunofluorescence confocal microscopy. pCMV-HA-RanBPM 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 removed. The coverslips were then incubated with CDK11-specific antibody P1C (1:500 dilution) and rabbit anti-HA antibody (1:50 dilution) for 1 h at RT. Coverslips were washed three times with PBS for 5 min each and then incubated with fluorescence labeled secondary antibodies FITC-conjugated anti-mouse IgG + IgM (1:100 dilution) and Cy5-conjugated anti-rabbit IgG (1:100 dilution) in the dark for 1 h at RT. Following incubation, coverslips were washed three times with PBS, mounted with DAKO mounting media, and stored at 4 °C overnight for immunofluorescence confocal microscopy analysis.

Protein kinase assay. Immunoprecipitations were performed as described above. For the kinase reaction, protein A–agarose beads were resuspended in 10 μl of kinase buffer (25 mM glycerophosphate, pH 7.5, 1.25 mM EGTA, 10 mM $MgCl_2$, 1 mM DTT, and 40 μM ATP). Substrates (3 μg in 10 μl) were added, followed by 10 μl of 2 × kinase buffer supplemented with 2.5 μCi [γ-^{32}P]ATP. The kinase reactions were incubated for 15 min at 30 °C. Phosphorylation was analyzed by 12% SDS–PAGE and autoradiography. Molecular Dynamic ImageQuant software was used to quantitate the relative phosphorylation level of the substrates.

Results and discussion

Identification of RanBPM as $CDK11^{p46}$-interacting protein

To identify novel proteins interacting with $CDK11^{p46}$, yeast two-hybrid analysis was completed with the ATP-binding site mutant (K451M) caspase-processed C-terminal portion containing the kinase catalytic domain of $CDK11^{p110}$ or wild-type as bait. Thirteen positive cDNA clones were isolated with K451M $CDK11^{p46}$, whereas one clone was isolated with wild-type $CDK11^{p46}$ as bait. DNA sequencing revealed that 2 of the positive clones encoded partial sequences of RanBPM. The specificity of interaction was confirmed by co-transformation of K451M $CDK11^{p46}$ or wild-type $CDK11^{p46}$ with the 2 RanBPM cDNA clones into yeast followed by growth selection and a β-galactosidase assay (data not shown).

Binding of RanBPM with CDK11^{p46} in vitro and in vivo

To examine the interaction between RanBPM and CDK11^{p46} in vitro, GST pull-down assays were performed. In vitro translated ^{35}S-labeled CDK11^{p46} was tested for binding to purified GST-RanBPM. RanBPM contains a conserved SPRY domain that has been shown to mediate protein–protein interactions [14]. RanBPM also contains a LisHCTLH motif (Lis1-homologous and C-terminal to LisH) which is possessed by proteins that are involved in microtubule dynamics, cell migration, nucleokinesis, and chromosome segregation [15,16]. To determine the region in RanBPM responsible for the interaction with CDK11^{p46} we constructed 2 RanBPM deletion constucts (Fig. 1A), one containing only the SPRY domain (GST-RanBPM 207–339) and the other lacking the SPRY domain and the N-terminal region (GST-RanBPM 334–729). The GST pull-down assays demonstrated that CDK11^{p46} binds to GST-RanBPM and GST-RanBPM 207-339, but not to GST-RanBPM 334–729 (Fig. 1B). This indicates that the SPRY domain of RanBPM is responsible for direct interaction with CDK11^{p46}.

To determine whether RanBPM and CDK11^{p46} interact in mammalian cells co-immunoprecipitation assays were performed. The CDK11^{p46} was tagged at its amino terminus with a c-Myc epitope and transiently expressed in the human melanoma cell line, A375. The expression of CDK11^{p46} was confirmed by Western blotting with GN1 antibody recognizing CDK11 (Fig. 2A). Whole cell extracts were immunoprecipitated with anti-RanBPM antibody followed by immunoblot analysis with anti-c-Myc antibody. As shown in Fig. 2B, CDK11^{p46} was co-immunoprecipitated with RanBPM. This suggests that RanBPM and CDK11^{p46} associate in human cells.

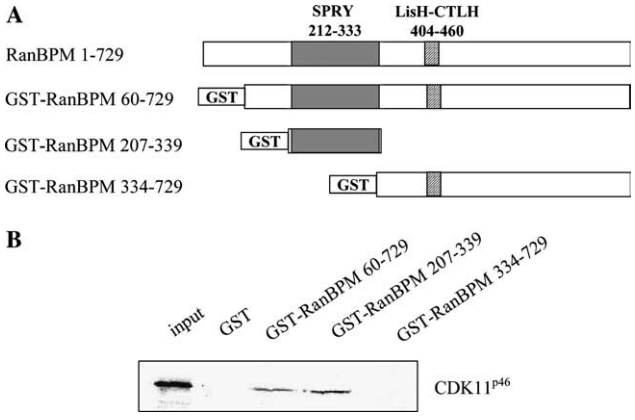

Fig. 1. RanBPM interacts with CDK11^{p46} directly in vitro. (A) Deletion constructs of GST-RanBPM were engineered as described in "Materials and methods." The SPRY domain and LisH-CTLH motif are indicated. (B) Analysis of the interaction between RanBPM and CDK11^{p46} by GST pull-down assay. In vitro translated ^{35}S-labeled CDK11^{p46} was incubated with GST-RanBPM 60–729, GST-RanBPM 207–339, GST-RanBPM 334–729, or GST. Proteins that interact with GST or the GST-RanBPM permutations along with 50% of the ^{35}S-CDK11^{p46} supplemented to the pull-down reactions (input) were resolved on a SDS–PAGE followed by autoradiography.

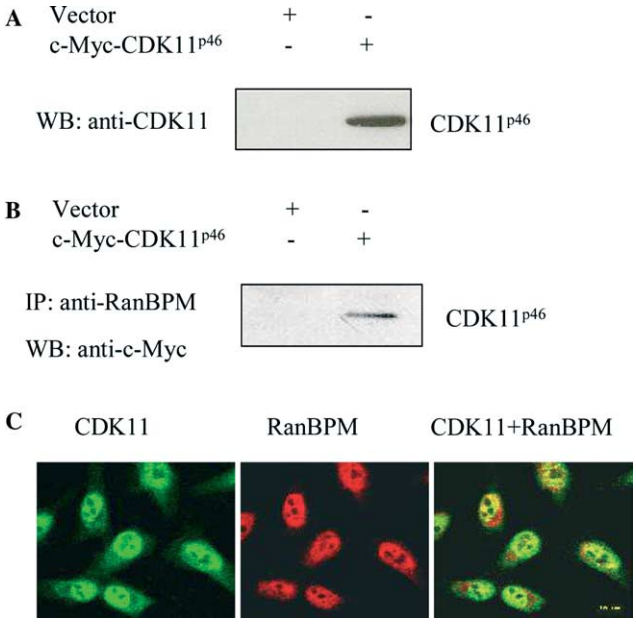

Fig. 2. RanBPM associates with CDK11^{p46} in vivo. (A) Immunoblot analysis with CDK11 specific GN1 antibody of cell extracts from cells transfected with empty vector or c-Myc-CDK11^{p46}. (B) In vivo co-immunoprecipitation of RanBPM with c-Myc-CDK11^{p46}. Extracts from cells transfected with empty vector or c-Myc-CDK11^{p46} were subjected to immunoprecipitation with anti-RanBPM antibody followed by anti-c-Myc immunoblotting. (C) Co-localization of CDK11^{p110} and RanBPM in human cells. A375 cells were transfected with HA-RanBPM and a confocal immunofluorescence microscopy assay was performed using CDK11 specific P1C antibody and anti-HA antibody. The subcellular localization of CDK11^{p110} (green) and RanBPM (red) and their co-localization (yellow) are shown.

To further demonstrate this interaction, the subcellular localization of RanBPM and CDK11 was determined by confocal microscopy analysis. A375 cells were transiently transfected with HA epitope-tagged RanBPM. Immunofluorescence analysis revealed that RanBPM (red image) is localized both in the cytoplasm and the nucleus. CDK11 (green image) is localized predominantly in the nucleus. Merging of the two images demonstrates that RanBPM co-localizes with CDK11 in the nucleus (Fig. 2C, yellow image).

CDK11^{p46} phosphorylates RanBPM in vitro

CDK11^{p110} is processed by caspases during Fas-induced apoptosis. Caspases cleave CDK11^{p110} into a 60-kDa N-terminal fragment and a 46–50-kDa C-terminal fragment containing the kinase catalytic domain. Cleavage is correlated with an increase in histone H1 kinase activity [9–11]. We tested whether CDK11^{p46} could phosphorylate RanBPM. A375 cells were tran-

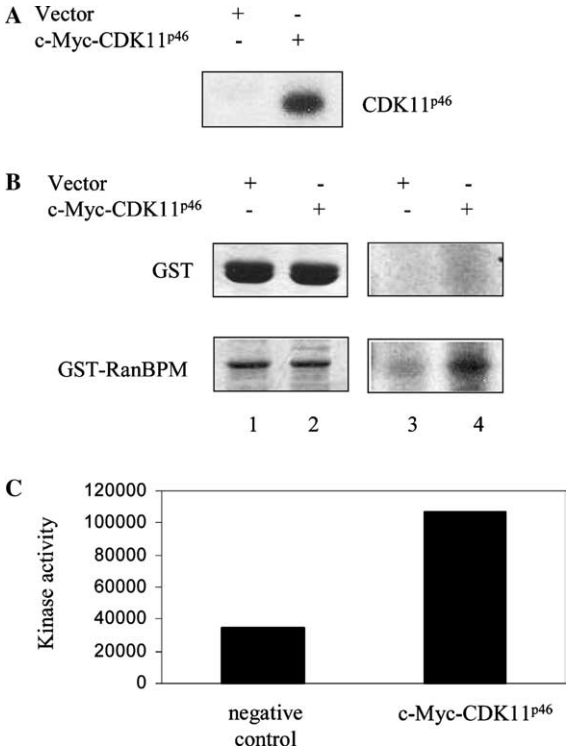

Fig. 3. CDK11^{p46} possesses autophosphorylation activity and phosphorylates RanBPM in vitro. Extracts from A375 cells transfected with empty vector or c-Myc-CDK11^{p46} were immunoprecipitated with anti-c-Myc antibody, followed by in vitro kinase analysis without added substrate (A) or using GST or GST-RanBPM as substrates (B, lanes 3 and 4). Coomassie blue staining of GST and GST-RanBPM used for the kinase reaction is shown in lanes 1 and 2. (C) Relative kinase activity of CDK11^{p46} was determined by quantification of the labeled GST-RanBPM bands with ImageQuant software. Extracts from cells transfected with empty vector served as negative control. Results are representative of three independent experiments.

siently transfected with vector or c-Myc epitope tagged CDK11^{p46}. CDK11^{p46} was immunoprecipitated from cell extracts using anti-c-Myc monoclonal antibody and kinase assays were performed without substrate or with GST or GST-RanBPM as substrates. The results shown in Fig. 3A demonstrate that CDK11^{p46} is an active kinase because it is capable of autophosphorylation. In addition, it phosphorylates GST-RanBPM, but not GST alone (Fig. 3B). The relative kinase activities are shown in Fig. 3C.

It is postulated that CDK11^{p46} plays a role in apoptotic signal transduction. It has been previously shown that CDK11^{p110} isoforms are cleaved by caspases during Fas- and tumor necrosis factor α-induced cell death [8–11,13]. Furthermore, ectopic expression of CDK11^{p46} in A375 cells induces apoptosis and inhibits overall protein synthesis [13]. Here, we demonstrated that CDK11^{p46} interacts with RanBPM and phosphorylates RanBPM. RanBPM was originally cloned as a centrosomal 55-kDa Ran-binding protein that is highly conserved between human, mouse, and hamster [17]. However, the original study was completed with N-terminally truncated cDNA. More recently, a full-length RanBPM cDNA was discovered that encodes a larger 90-kDa protein which localizes in both the nucleus and the cytoplasm [18]. The function of RanBPM is still unclear, but it has recently been linked to activation of the Ras/Erk signaling pathway through recruiting Sos to MET (protein–tyrosine kinase receptor for hepatocyte growth factor) [14]. Additionally, it was shown that RanBPM regulates androgen and glucocorticoid receptor activity [19]. Furthermore, RanBPM was identified as an interacting partner with psoriasin, a protein highly expressed in breast cancer cells [20], and homeodomain-interacting protein kinase-2 (HIPK2), a nuclear serine/threonine kinase [21]. HIPK2 is activated by ultraviolet radiation and regulates p53 activity through phosphorylation at Ser 46. It promotes p53-dependent gene expression, which results in growth arrest and the enhancement of ultraviolet-induced apoptosis [22,23]. In addition to HIPK2, we determined here that RanBPM interacts with a second kinase involved in apoptotic signaling, CDK11^{p46}. We also showed that CDK11^{p46} phosphorylates RanBPM. It is too early to postulate a functional role for this activity, but we speculate that both CDK11^{p46} and RanBPM are components of the same signal transduction pathway. The arising question is whether CDK11^{p46}, RanBPM, and HIPK2 act cooperatively during apoptosis. Further analysis of the interaction between CDK11^{p46} and RanBPM may result in better understanding of the regulation of apoptotic signaling through CDK11^{p46}.

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